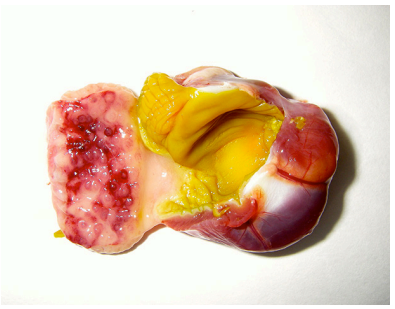





Diseases of Poultry

14th Edition

Editor-in-Chief
David E. Swayne

Associate Editors
Martine Boulianne
Catherine M. Logue
Larry R. McDougald
Venugopal Nair
David L. Suarez



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Diseases of Poultry

VOLUME I

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Dedicated to



Dr. J.R. Glisson, Associate Editor,
Diseases of Poultry, 11th, 12th and 13th editions



Dr. Lisa K. Nolan, Associate Editor,
Diseases of Poultry, 12th and 13th editions

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Preface: Historical Review of *Diseases of Poultry*

Forewords for *Diseases of Poultry*, beginning with the first one written by John R. Mohler in 1943, have briefly described the nature and contents of the edition, along with substantive reasons for its publication and distribution to potential users. Mohler pointed out that for a profitable poultry industry, "Knowledge of the characteristics of each disease is necessary…as the first step in building up an effective barrier against it." He further noted that "This unusually comprehensive book is intended for students, veterinarians, pathologists, and workers in specialized fields." These words are as applicable today as they were 70 years ago and the importance of the text is supported by publication of 14 editions.

For the 6th edition in 1972, Dr. P.P. Levine offered an accounting of some of the changes in the poultry industry that moved it from a small-scale farm activity to "a highly sophisticated industry marketing products worth over $6 billion per year in the United States alone." He correctly attributed some of the many advances in disease control through eradication, genetic selection, immunization practices, management improvements, and so on, to major advances founded in research. Such new knowledge strongly dictates a need for revised texts. Levine further predicted that "infectious diseases will decline in importance; toxicologic, nutritional, genetic, and husbandry problems will demand increasing attention. Change is the order of life, and avian diseases are no exception." In the 7th edition (1978), he pointed out many of the important new advances in identifying the etiology of several conditions, and the need for *Diseases of Poultry* to "keep up with the rapid developments in avian diseases."

Ben Pomeroy, in the 8th (1984) and 9th (1991) editions, reiterated the need for new editions to keep up with the "explosion of knowledge on the prevention and control of avian diseases." The inclusion of contributions from experts from many countries of the world, and the importance of such in the face of global issues of disease control, was emphasized by Charles Beard in the Foreword of the 10th (1997) edition. He pointed out that understanding the molecular genetics of causative agents is also important and the use of molecular methods is necessary for poultry disease researchers to understand and control infectious diseases; yet another reason for timely updates.

The message is clear: a changing and global poultry industry and its many allied industries need the most recent information available to keep pace with the challenges of providing adequate health care and disease prevention. It is important not only to poultry flocks, but also to the consumers who expect safe, as well as nutritious, poultry products. This, the 14th edition, upholds the long-standing reputation of this book for keeping scientists, breeders, poultry producers, and poultry health professionals supplied with the latest and most comprehensive information available.

Seventy-five years have passed since the first edition was printed. Before all details are lost forever, it is fitting to look back at how this "Bible" in the field of poultry diseases came to be and how it has evolved into what it is today. It all began in the 1930s. In a memorandum addressed to the American Association of Avian Pathologists (AAAP) dated December 22, 1965, H.E. Biester related the events that preceded the decision by the Iowa State College (now University) Press (ISU Press) to undertake the publication of *Diseases of Poultry*. During the 1930s, Louis DeVries, a member of the Department of Modern Languages at the college, translated a 1929 German book entitled *Handbuch der Geflügelkrankheiten und der Geflügerzucht*, published by Ferdinand Enke, Stuttgart. The translation laid dormant for several years until Dr. D.M. Campbell, the Chicago publisher of *Veterinary Medicine*, saw the translation and expressed some interest in it. Dr. Biester, who described himself as an "innocent bystander, having no special interest in the project," told Dr. Campbell that the manuscript was unacceptable for a variety of reasons and he suggested that if he were serious about publication, then selected specialists should edit or rewrite the material. Dr. Biester later was pulled into the project and he ultimately concluded that the German book was obsolete. Apparently, a number of men had accepted invitations to cooperate in developing an American book, and according to Biester, they agreed that "it would be better to prepare a totally new book based on American conditions."

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Thus, the die was cast. Dr. Campbell gave up his plans, and the ISU Press decided to publish an original text. Drs. Biester and DeVries served as editors, and 34 American investigators were engaged in the project. There were chapters on general subjects such as anatomy, digestion, genetics, hematology, hygiene and sanitation, nutrition, and surgery, as well as those dealing with specific infectious and noninfectious diseases and conditions. A separate chapter dealt with diseases of turkeys. In 1943, the 1st edition was ready. The publication costs were considerable for a book that was thought to have limited distribution, so it was decided to omit royalties and accept a subsidy for illustrations from the Dean of the College. Fifteen hundred copies were printed and placed on sale for $7.50; to everyone's surprise, a second printing of 2,500 copies was needed after less than nine months and there was yet another printing of 2,500 copies two years later. Royalties were then instituted! The ISU Press was concerned that without some remuneration, the authors might be reluctant to remain "dedicated."

Edition	Year	Editors	Pages	Chapters	No. USA authors	No. non-USA authors (no. countries)
1st	1943	H.E. Biester	1,005	40	34	0
		Louis Devries				
2nd	1948	H.E. Biester	1,154	40	33	0
		L.H. Schwarte				
3rd	1952	H.E. Biester	1,245	41	35	0
		L.H. Schwarte				
4th	1959	H.E. Biester	1,103	41	33	0
		L.H. Schwarte				
5th	1965	H.E. Biester	1,382	41	37	0
		L.H. Schwarte				
6th	1972	M.S. Hofstad (EC)	1,176	33	40	0
		B.W. Calnek				
		C.F. Helmboldt				
		W.M. Reid				
		H.W. Yoder, Jr.				
7th	1978	M.S. Hofstad (EC)	949	33	45	1 (Czechoslovakia)
		B.W. Calnek				
		C.F. Helmboldt				
		W.M. Reid				
		H.W. Yoder, Jr.				
8th	1984	M.S. Hofstad (EC)	831	34	51	4 (United Kingdom)
		H. John Barnes				
		B.W. Calnek				
		W.M. Reid				
		H.W. Yoder, Jr.				
9th	1991	B.W. Calnek (EC)	929	35	61	17 (Australia, Belgium, Canada, Germany, Hungary, Israel, Japan, United Kingdom)
		H. John Barnes				
		C.W. Beard				
		W.M. Reid				
		H.W. Yoder, Jr.				

(*Continued*)

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Edition	Year	Editors	Pages	Chapters	No. USA authors	No. non-USA authors (no. countries)
10th	1997	B.W. Calnek (EC) H. John Barnes C.W. Beard L. R. McDougald Y.M. Saif	1,081	37	78	18 (Australia, Belgium, Canada, Germany, Japan, Pakistan, United Kingdom)
11th	2003	Y.M. Saif (EC) H. John Barnes A.M. Fadly J.R. Glisson L.R. McDougald D.E. Swayne	1,231	34	63	25 (Australia, Belgium, Canada, Denmark, France, Germany, Japan, Israel, Netherlands, Saudi Arabia, United Kingdom)
12th	2008	Y.M. Saif (EC) A.M. Fadly J.R. Glisson L.R. McDougald L.K. Nolan D.E. Swayne	1,324	33	71	25 (Australia, Belgium, Canada, Denmark, France, Germany, Japan, Israel, Mexico, Netherlands, United Kingdom)
13th	2013	D.E. Swayne (EC) J.R. Glisson L.R. McDougald V. Nair* L.K. Nolan D.L. Suarez	1,420	33	72	30 (Australia, Austria, Belgium, Canada, Chinese Taipei, Denmark, France, Germany, Hungary, Italy, Japan, Mexico, The Netherlands, Sweden, United Kingdom)
14th	2020	D.E. Swayne (EC) M. Boulianne[a] C. Logue L.R. McDougald V. Nair[a] D.L. Suarez	1,477	33	79	31 (Australia, Austria, Belgium, Canada, China, Chinese Taipei, France, Germany, Hungary, Indonesia, Israel, Italy, Mexico, The Netherlands, Sweden, United Kingdom)

EC = Editor-in-Chief
[a] Non-USA Associate Editors (Canada, United Kingdom)

The inclusion of Dr. DeVries as an editor is a bit puzzling since he had no medical background; perhaps it was in recognition of his effort with the translation of the German text. In any case, he was replaced in subsequent editions by Dr. L.H. Schwarte, a member of the Veterinary Research Institute in Ames who had written four chapters in the 1st edition. The book was thereafter referred to by many as "Biester and Schwarte," even for a period after they were no longer associated with it. They continued at the helm through the 5th edition, published in 1965. Although Dr. Schwarte contributed several chapters in each of the first five editions, Dr. Biester apparently confined his efforts to editorial tasks. Their memo to the AAAP stated that they both were responsible for making the index, and they personally checked practically all of the references because they felt that they "owed to the reader accuracy." A total of 61 persons served as authors under their editorial supervision; 12 of them contributed to all five editions.

Ultimately, the passage of time dictated that Drs. Biester and Schwarte should relinquish their roles as editors and they decided that the 5th edition (1965) would be their last. As noted in the Preface to the 6th edition, it was their wish "that future editions of the book become the responsibility of the AAAP…" which had become a strong and representative organization to which many of the

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users of *Diseases of Poultry* belonged. Also the AAAP was already in the business of publishing the journal *Avian Diseases* and so it was considered a logical move. The AAAP appointed a committee, chaired by Dr. M.S. Hofstad who had been one of the book's authors and who was on the faculty at Iowa State University (ISU). Drs. Biester, J.E. Williams, B.S. Pomeroy, and C.F. Helmboldt filled out the committee and, in June 1966, they recommended that the AAAP sponsor future editions of *Diseases of Poultry* which would continue to be published by ISU Press in Ames. They asked the Board of Directors to appoint an editorial committee consisting of an editorial chairman and four associate editors by January 1, 1967. A letter from Dr. G.H. Snoeyenbos (AAAP secretary-treasurer) to Dr. C.A. Bottorff (AAAP president) dated November 23, 1966, suggested that Dr. P.P. Levine had declined a proposal that he assume the editorship for the book. Dr. Hofstad was subsequently named editor, and he personally requested that Drs. Helmboldt, B.W. Calnek, W.M Reid, and H.W. Yoder, Jr., be invited to be the associate editors. Each was given responsibility for a group of chapters that largely represented their individual interests and strengths. An agreement between the AAAP and the ISU Press was executed on May 8, 1967, and it was agreed that manuscripts would be delivered to the publisher by September 1, 1969. So the transfer was complete and official.

The 6th edition, under totally new editorial support, underwent some significant changes. The length of the book was beginning to be of concern and there was some discussion about perhaps needing to split it into two volumes. To avoid this, several chapters (anatomy, nutrition, genetics, and hematology) were eliminated based on good coverage in other publications. Also, there was a consolidation of other material; for instance, all neoplastic diseases were placed in a single chapter and turkey diseases were incorporated in other chapters based on etiology. There were sweeping changes in authorship. Only 14 of the 40 contributors to the 6th edition had participated in the 5th. Clearly, a new era had arrived!

Also, following concern for the book's length, Dr. Hofstad asked that the number of listed references be reduced by *selective* citation. He agreed that the reader should find, *or be directed to*, all pertinent literature on each of the covered topics, the latter through citation of review papers, and so on. Space allocated to references became an issue in subsequent editions as well. In the 7th and 8th editions, titles of all references were removed. This was controversial and not all editors agreed, including B.W. Calnek—senior author of this review—and reference titles appeared again beginning with his tenure as editor of the 9th edition. Interestingly, based on the number of pages, the 3rd edition (1,245 pages) was actually longer than the 11th (1,231 pages), but by increasing page size, decreasing type size, and splitting into two columns/page, it was possible to include more than twice the amount of written material in the latter.

Unlike the situation with Drs. Biester and Schwarte, citations and their accuracy became the responsibility of the individual authors. When it was observed that many errors existed, authors of the 9th edition were asked by Dr. Calnek to check every single reference against the original work to assure accuracy. This met with an enormous number of groans and considerable resistance until each author (with perhaps an exception or two) followed this strict instruction. The subsequent turnaround in their attitude was truly amazing when nearly all of them found errors, including the citation of references that did not even exist. It was not uncommon to detect mistakes in as many as 10% of citations in some chapters, probably due in large part to a common practice of copying reference citations from other lists.

Beginning with the 9th edition, the book entered the electronic age. All material was submitted or copied into a word processing program that allowed spellchecking and reformatting. Initially, it was a tedious job, particularly because personal computers at that time were slow and the skill of the individual authors in mastering a new approach varied considerably. However, improvements in software and computers and the possibility of rapid transfer of texts between authors, editors, and the publisher made the preparation of a new edition pleasurable compared to the old "hard-copy" approach.

There has been a continuum of changes that have improved *Diseases of Poultry* and kept it relevant over the years. For the 10th edition, the editors carefully reviewed and upgraded illustrations and for the first time included a number of color plates. Another major improvement that was gradually incorporated was the inclusion of molecular biology in many of the chapters. This was particularly important with regard to new applications of molecular techniques in diagnostic procedures, descriptions of etiological agents and significant elements of their molecular makeup, understanding the significance of selected genes in the pathogenesis of the diseases, and the development of genetically engineered vaccines. Our understanding of the fundamental nature of many diseases is now founded on the use of molecular approaches in the research laboratory.

Another of the more significant evolutionary changes was the addition of foreign authors to make the book truly international in flavor. One of the original AAAP-appointed editors argued strongly that *Diseases of Poultry* should be an "American" book, and the authorship was so aligned. The 6th edition had the first "foreign" contributor, although she (Bela Tumova,

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from Prague, Czechoslovakia) was actually a visiting professor at the University of Wisconsin working with B.C. Easterday on avian influenza at the time. It wasn't until the 8th edition that invitations to contribute to the book were extended to workers outside of the United States. Drs. P.M. Biggs and L.N. Payne from England and Drs. J.B. McFerran and M.S. McNulty from Northern Ireland thus paved the way by providing parts of the chapters on neoplastic diseases, adenoviruses, and miscellaneous viral infections. The next edition (9th) was truly international with 17 contributors from 8 countries outside of the United States, and by the 14th edition, there had been a total of 31 different contributors from 16 countries. Beginning with the 13th edition, under David E Swayne, the first non-USA associate editor was selected, Venugopal Nair, and with the 14th edition, the second non-USA associate editor, Martine Boulianne. The worldwide reputation of the book was certainly enhanced by the selection of authors and associate editors based on their knowledge and contributions to our understanding of individual diseases and conditions without regard to their geographic location.

The importance of *Diseases of Poultry* as a text for the world is also reflected in its translation into foreign languages or publication in a "copied" form in other countries. There have been several authorized translations into Spanish, Chinese, and Russian, and an agreement between the publisher and India has allowed what is essentially a photocopied version of the original to be made.

An ongoing review of the relative importance of individual diseases or conditions has led to a good deal of reshuffling over the years. Chapters have been added, combined, split, or eliminated to meet the changing picture of what is important to the field of avian diseases and disorders. Periodically, and especially with a change in authorship, major rewriting of some sections takes place. New chapters such as one dealing with new and emerging diseases appear when needed.

Beginning with the 12th edition emphasis was placed on the significance of each disease to public health considering the ever increasing interest in food safety. Emphasis was also placed on standardizing the format for all the subchapters by using the same headings thus making the book more reader friendly.

Despite our increased understanding of disease processes there remains much to learn. Only in very few cases do we know the molecular markers of virulence, pathogenicity, or immunogenicity of pathogens and, needless to say, the molecular basis for disease resistance is a wide open field. In many cases, our understanding of disease-triggering mechanisms is poor and diseases of multiple etiologies remain problematic. There has been a major research undertaking to address these areas and since the 12th edition, such new information has been incorporated in newer editions.

As indicated, the introduction of molecular techniques has greatly enhanced our understanding of disease but it has also created some confusion as we strive to understand the underlying genetics of important biologic characteristics of pathogens. The early euphoria from thinking that one gene is responsible for a given biologic characteristic is being replaced by the realization that more than one gene is usually involved. This makes it difficult to decipher our observations. Another point of confusion resulted from the definition of the term genotype and early attempts to relate it to serotype or protective type. Again, it became clear that such a relationship is lacking in most cases and genotyping is useful mainly for epidemiologic studies. Considering all these gaps in our knowledge, and the unprecedented speed of knowledge generation, it is understandable that we need a new edition every 5–6 years to keep our text continually updated.

The first 10 editions were available in hard-bound books only but the arrival of the computers and personal readers saw expansion into electronic books with a CD-ROM version for use on personal computers with the 11th edition, replaced by Adobe Digitals downloadable electronic versions with the 12th, 13th, and 14th editions, Kindle version with 13th and 14th editions, and online institutional Oxford book with the 13th and 14th editions. However, through the 13th edition, the hard-copy was still the main seller for *Diseases of Poultry*.

Finally, the euphoria of the last century, suggesting that we will conquer infectious diseases as stated to the US Congress in 1969, by then Surgeon General of the United States William H. Stewart ("We can close the books on infectious diseases…"), and our own P.P. Levine's prediction in 1973 stated earlier in this section ("Infectious diseases will decline in importance…") proved terribly wrong. Microbes are a tough and nimble foe capable of changing and adjusting to new environments mostly created by man. Thus, we think that infectious diseases will continue to be a top health priority for poultry, another reason why we continue to need new editions of this book.

Changes in editors occurred over the years so that by the 11th edition, none of the 1968 group appointed by the AAAP remained. After riding herd on three editions (6th–8th), Dr. Hofstad retired and was replaced by Dr. Calnek (9th and 10th) and he, in turn, passed the baton to Dr. Y.M. Saif (11th and 12th), and most recently to Dr. D.E. Swayne (13th and 14th). Likewise, associate editors that have replaced or been added to the original group appointed by the AAAP in 1968 include Drs. H.J. Barnes (8th–11th), C.W. Beard (9th and 10th), L.R. McDougald (10th–14th),

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Y.M. Saif (10th), J.R. Glisson (11th–13th), A.M. Fadly (11th and 12th), D.E. Swayne (11th and 12th), L.K. Nolan (12th and 13th), D.L. Suarez, and V. Nair (13th and 14th), and Catherine Logue and Martine Boulianne (14th).

In summary, it is obvious that the "Bible" in the field of avian diseases is an evolving, vibrant, and ever-current source of information relevant to all practitioners in the field of poultry medicine. It continues to be a reference source of significance to a vast number of persons with many different relationships to the poultry industry.

Bruce W. Calnek (9th and 10th editions)
Y.M. Saif (11th and 12th editions)
David E. Swayne (13th and 14th editions)

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Introduction

This edition is appropriately dedicated to two colleagues who have devoted their careers to creating new knowledge and passing on existing wisdom on poultry health through successive editions of *Diseases of Poultry*: Dr. J.R. Glisson, who served as Associate Editor for the 11th–13th editions, and Dr. Lisa K. Nolan, who served as an Associate Editor for the 12th and 13th editions. Both have been instrumental in maintaining the high quality of this book, and, indeed, we are highly grateful for their efforts. With this edition, Drs. Catherine M. Logue and Martine Boulianne joined the existing Associate Editors, Drs. Larry R. McDougald, Venugopal Nair, and David L. Suarez. We are very appreciative of their services.

This edition expands on a major emphasis, initiated in 13th edition, on electronic versions with expansion of color figures and availability of three specific electronic formats to accommodate a variety of media from traditional computers to standalone electronic books to smartphones. The new generation of poultry veterinarians and scientists are living and working in the electronic age and rely upon instantaneous access to crucial information to do their jobs, and *Diseases of Poultry* will be at their fingertips or in their pockets for daily and mobile use. In addition, the widely used high quality, hard copy is also preserved as a reference text, but with fewer color figures than in the electronic formats.

This 14th edition represents a continuation of the tradition established earlier of providing the latest information on poultry diseases. Earlier trends of expansion of authorship to include authors from around the globe were continued in this edition, as was the appointment of our second non-USA Associate Editor, Martine Boulianne. The Preface was updated and expanded to a more comprehensive review of the history of *Diseases of Poultry*, including valuable new metric data.

All of the book chapters were updated with the most current and accurate knowledge and many with new figures, especially color figures of gross lesions. Much of the historical information and antiquated or historical diagnostic tests were removed and readers are referred to prior editions for in-depth coverage. A new subchapter on Disease Prevention and Control in Antibiotic-Free Production was added to Principles of Disease Prevention, Diagnosis, and Control, (Chapter 1). New subchapters were added to Chapter 33, Emerging Diseases and Diseases of Complex or Unknown Etiology: (1) White Chick Syndrome, (2) Focal Duodenal Necrosis in Table Egg Layers, (3) Wooden Breast and Other Muscle Abnormalities, and (4) Idiopathic Egg Production Drops in Brown Layers. Major revisions were accomplished for Chicken Infectious Anemia and Circovirus Infections in Commercial Flocks, Avian Reovirus Infections, Marek's Disease, Salmonella Infections, Mycoplasmosis, and Coccidiosis. With the 14th edition, we collected clinical input from seven poultry veterinarians throughout the world (Drs. Ian Rubinoff, Sjaak de Wit, Tom Grimes, Deirdre Johnson, Michele Kromm, Guillermo Zavala, and Teguh Yodiantara Prajitno) and incorporated their insights in prevention and treatments of specific poultry diseases into individual chapters. This has improved the field relevancy of the book.

Subchapters on *Mycoplasma meleagridis*, Hypoglycemia-Spiking Mortality Syndrome of Broiler Chickens, Proventriculitis and Proventricular Dilatation of Broiler were merged into other subchapters. These changes were dictated by the increasing or decreasing significance of some diseases or the increasing knowledge on a given disease. Some subchapters have been moved to different chapters because of recent findings indicating that they fit within different areas.

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A sincere thank you goes to the authors who contributed to the current and earlier editions of the book. It has been a wonderful experience working with all of you.

Over the past five years, the personnel at Wiley that worked on this edition have been most helpful and accommodating, and we sincerely appreciate their support especially Erica Judisch, Purvi Patel, Susan Engelken, and Catriona Cooper.

This is my second time to serve as Editor-in-Chief of *Diseases of Poultry*, and I (DES) am indebted to my colleagues, the Associate Editors, and the past Editor-in-Chief Y.M. Saif, for their tireless efforts in the review process and their support and advice.

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Section I

General Concepts of Poultry Diseases

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1

Principles of Disease Prevention, Diagnosis, and Control

Introduction

Stephen R. Collett

In an effort to cost-effectively meet demand, producers have increased the size and throughput of their production systems. These large, close-confinement rearing systems, designed to improve economies of scale and maximize productivity by optimizing bird comfort, also increase the risk and impact of disease challenge. The close proximity of susceptible hosts increases the chance and rate of infectious disease spread. Replication of mutable viruses (including live virus vaccines) in large populations mathematically increases the probability of the emergence of variants, and reliance on immunization to control these diseases selects for antigenically dissimilar mutants that escape adaptive immunity. Diseases previously recognized as unimportant, because they have been adequately controlled, have now re-emerged as significant concerns. Many of today's disease challenges are not new problems, they have merely expanded their geographic distribution or re-emerged primarily because of management techniques and production system design constraints.

Disease control priorities have evolved with intensification of the industry. While initially focused on diseases of catastrophic nature, attention has shifted from defined, clinical disease at the individual house or farm level, to less well-defined sub-clinical disease, performance shortfalls, and bird welfare. Disease prevention, diagnosis, and control strategies have changed to prevent physiological, nutritional, and agent-induced pathologies from affecting performance.

Since the production system is profit driven, decisions regarding management of disease challenge can no longer be made based solely on biological grounds. Unless a disease poses a specific risk to human health, animal welfare, productive efficiency, or product quality, its mere presence in a flock may not be significant from a business perspective. It is often difficult for the veterinarian, trained in disease prevention, diagnosis, and control to appreciate that the presence of a disease in a flock could be considered superfluous. Unless it is economically advantageous to take action against a disease challenge, its presence in a flock is tolerated. Intervention strategies are consequently chosen based on both their economic and biological efficiency. This process requires a dynamic, integrated combination of first, an epidemiologic and economic analysis to determine and quantify the production effect of the disease challenge, and second a proposed intervention strategy and the costs thereof.

Recently, regulatory changes in the United States have eliminated growth-promoting and other non-therapeutic uses of antibiotics, but have continued allowance for most therapeutic uses under increased veterinary supervision. However, market-driven restrictions on all uses of antibiotics in poultry production have created challenges in the control of bacterial and protozoal infections and maintaining welfare of farmed poultry. This has led to new challenges in confronting increased early mortality, coccidiosis, and necrotic enteritis (NE) in broilers; coccidiosis and bacterial infections such as *Bordetella avium* in turkeys; and colibacillosis and NE in table egg layers. Solutions for these health problems will require changes to management and diet, use of non-antibiotic medications such as chemically synthesized coccidiostats, and alternative products such as probiotics. However, even with these changes, performance and health problems may exceed those found in conventional production schemes with unrestricted access to approved medications.

In conventional production schemes, appropriate antimicrobial uses includes proper pathogen diagnosis, knowledge of antibiotic properties, dosage, spectrum, interactions, and early initiation of treatment. The limited drug availability for poultry makes it imperative to combine an accurate diagnosis with antimicrobial knowledge to result in the most efficacious and cost-effective approach to disease treatment with minimal potential risk of antimicrobial resistance development and selection.

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Several poultry diseases have zoonotic potential. Some zoonotic diseases are rarely reported and others are commonly associated with human illness. The most high profile zoonotic diseases include H5N1 Gs/GD lineage and H7N9 Anhui lineage of avian influenza viruses, and foodborne pathogens such as *Salmonella* and *Campylobacter*. Educating poultry workers with respect to zoonotic pathogens and their modes of transmission is an important step toward disease prevention.

Principles of Disease Prevention, Diagnosis, and Control

Stephen R. Collett and John A. Smith

Summary

Disease challenges have evolved in step with the evolution of industrial-scale poultry production, and the principles of disease prevention, diagnosis, and control have, and must continue to, evolve as well. Disease management has shifted from classically recognized acute diseases of individual animals to management of both clinical and subclinical disease in populations. Morbidity and mortality are no longer the primary metrics monitored, and the emphasis has shifted to economic performance through the entire production chain, product quality, and animal welfare. Environmental considerations, food safety, marketing claims, and the like increasingly impact decision-making. Recognition of the roles of management, environmental stressors, and population ecology have been added to the traditional medical disciplines, and biosecurity and risk management have assumed equal importance to practical diagnosis and treatment in the job of the poultry practitioner.

Flock Health

Disease is the antithesis of health but neither state is easy to define in production animals. Health is defined in the human individual as a state of physical, mental, and spiritual well-being. It is impossible to apply this definition to an animal, and production animals have in the past been classed as healthy if they were free from clinical disease and performing to standard. Although individual animals are frequently described as healthy or diseased, these terms are not mutually exclusive. The impact of disease challenge on productivity is apparent long before clinical signs of disease appear. Production animals are expected to perform at their genetic potential and to achieve this they need to be physically and mentally well, or stress free.

Stress has been defined as a non-specific response of the body to any demand made upon it. From a physiological point of view this can be restated as the metabolic response of the body to external factors that impact well-being (33). Stress is cumulative and only impacts performance measurably once the aggregate of each individual stress exceeds the host's coping mechanisms. An interesting study (48) has shown that the degree to which an adverse stimulus or stress will negatively impact bird performance is directly proportional to the existing stress load. Any stress will impact productivity once the stress threshold is surpassed. In a production system where animals are expected to produce at genetic potential, the definition of health needs to be expanded to freedom from "dis - ease" or stress.

Disease prevention and control strategies tend to be too focused on addressing the *precipitating* cause, and too little attention is given to the *predisposing* causes of disease. In intensive animal agriculture environmental disease determinants often decide the economic outcome of infectious agent challenge. The focus of flock health management has consequently shifted. Initially aimed at avoiding mortality because of an inadequate immune response, health management is also now directed at avoiding an exaggerated or inappropriate immune response because it may depress productivity. The task of the veterinarian has shifted from the prevention, diagnosis, and control of specific disease conditions in the individual bird, to preventing and limiting the consequence of more complex multifactorial disease outcomes in order to maximize the productivity of the flock.

Resistance and Resilience

An animal's *resistance* to disease can be defined as its capacity to prevent an overwhelming infection by a disease-causing organism. Disease resistance is determined by immune competence and health status at the time of challenge. Since stress negatively impacts health it also negatively impacts resistance. Ironically the process of mounting an effective immune response is itself a stress because of the demands made on the immune system, and the consequence of the resulting fever response. An immune response, adequate to contain disease, can be considered as the cost of health. There is a delicate balance between too little and too much since an inappropriate immune response, whether inadequate or excessive, will depress performance.

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The *resilience* of an animal is a measure of its capacity to continue to perform while preventing a disease challenge from causing an overwhelming infection. As with resistance, resilience is negatively impacted by poor health but in this case the negative impact of the resulting stress is more significant. The chemical messengers (cytokines) released in response to a disease challenge depress production directly by influencing metabolism and indirectly by suppressing appetite and feed intake (48). While immune response is crucial to maintaining health, the consequence of an immune response is depressed productivity.

The skin and respiratory, urogenital, and gastrointestinal tracts form the interface between foreign (antigenic) material and animal cells (self). To protect the bird from disease the immune system has to develop exquisite sensitivity as to whether foreign antigens are friend (nutrients or normal flora) or foe (pathogenic). An inappropriate immune response to gastrointestinal antigens will for example have a negative impact on feed efficiency. The fever response induced by foreign antigens will depress feed intake, while the inflammatory response damages the gut lining, thus reducing the nutrients available for production. The capacity of an animal to fight off a disease challenge while avoiding the negative impact of the induced immune response on productivity (resilience), depends on how close the prevailing level of stress is to the bird's stress threshold. The success of any health program thus hinges on balancing immunity and health to maximize resilience. There is a dynamic interface between nutrition, immunity, and productivity. The aim of any production veterinarian should be to optimize feed utilization by modulating the immune response: enhancing the protective response to prevent clinical disease, while at the same time, suppressing the acute phase or fever response.

Population Dynamics

Like human medicine, traditional veterinary medicine is focused on the study of the disease process in individuals. In modern flock medicine where the emphasis is on prevention, diagnosis, and control of disease in finite and confined populations, the focus shifts to the epidemiology of the disease. Since health and disease are not mutually exclusive, individual birds within the flock will at any point in time be in various stages of health/disease (Poisson distribution). At what point is a flock diseased or healthy? Productivity gives a good estimate of an individual's state of well-being and welfare. Similarly, a flock that is performing to standard is assumed to be healthy, based on the fact that they act and produce as an equivalent non-stressed sibling would do in a laboratory situation. This approach unfortunately takes little cognizance of the flock variance, since flock performance indicators are based on flock averages. Population variance or range is a much better indicator of flock health.

In the past, intensive agriculture has been production driven, and contribution measured in terms of performance. In today's market-driven enterprise, value is regarded as a function of quality, yield, and cost of production with the emphasis shifting from performance to profit through the chain of realization. In this scenario the simplest strategy for improving productivity is to reduce within and between flock variance. By reducing variability and thus eliminating the extremes, it is possible to improve the quality, speed, and cost of production. Improved uniformity translates to improved productivity and hence profitability. Health (difference between stress level and stress threshold) is probably the single most important determinant of flock uniformity. Within a group of animals the threshold and level of stress experienced by each individual will vary. The relative efficiency of a production manager to minimize in-house environmental variation, and therefore host-, agent-, and environment-dependent stress, is reflected in flock uniformity.

Challenges of Disease Prevention, Diagnosis, and Control in Modern Poultry Production

Since the goal of a poultry operation is to *convert feed into food as economically as possible*, it is critical to manage both the risk and consequence of disease challenge. While the biological potential for *feed conversion* is governed primarily by intrinsic or genetic determinants, in an intensive production system it is the extrinsic determinants, including nutrition, minimization of stress by management, and disease that ultimately decide the efficiency of the operation in both biological and financial terms. Capital investment in the housing's environmental-control capability, and the effective operation of these controls, is fundamental to economic success. Even subtle disease challenge such as vaccination with live respiratory agent vaccines can compromise efficiency if exacerbated by environmental disease determinants.

Viral diseases are challenging to control because there are no effective treatment options, while bacterial, protozoal, and parasitic diseases present a challenge because the treatment options are either no longer available, or no longer effective. The approach to controlling diseases within these two categories is very different.

The molecular structure of a virus particle is relatively simple, making immunological recognition very acute and the control of *known* viral diseases possible through immunization. Provided the immune system has been primed by vaccination, immunological protection against

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viral disease challenge is usually highly successful. Emerging and re-emerging viral diseases arise when novel or immunologically distinct viruses are introduced into naïve populations (45). In the absence of prior exposure, immune recognition and activation is delayed and the extent of the primary immune response is frequently inadequate to prevent clinical disease (42). Under such conditions virus replication and spread occurs rapidly with potentially devastating consequences (24). While the majority of emerging viral diseases in humans are the result of exposure to novel viruses, it is the emergence of variant strains that pose the biggest threat to the poultry industry (81). Although controlled environment housing and good biosecurity practices have been highly effective in preventing the introduction of novel viruses, increased population density and vaccination have likely enhanced the emergence of variant strain viruses. The high population densities provide the opportunity for antigenic shift through gene mutation and recombination, while vaccination creates positive selection pressure for the variant strain viruses (73).

Bacteria and protozoa are, in contrast to viruses, structurally and immunologically complex, making protection through vaccination much less successful. Although a great deal of research effort is, and has been, focused on developing effective immunization strategies for these diseases, antibiotics and chemotherapeutics have remained the primary means of control (26); a point well illustrated by the continuing difficulties experienced in the EU with the systematic withdrawal of in-feed antibiotics (32). It is no coincidence that the downward trend in prophylactic (in-feed) antibiotic usage has been matched by an increase in therapeutic use (54). Many expert committees blame the use of in-feed antibiotics in food animal agriculture for the proliferation of antibiotic resistant strains of bacteria, and for the increase in prevalence of antibiotic resistant infections in humans (44). This is undoubtedly providing the impetus to ban in-feed antibiotic use, even though a link to increased antibiotic-resistant bacterial disease in humans has not been conclusively established (29). Consumer pressure to remove antibiotics from the food animal nutritionist's arsenal is, however, winning the battle and the trend toward re-emergence of previously controlled bacterial and protozoal diseases will likely continue. The industry must adapt in order to remain competitive. Refer to the section of this chapter on Disease Prevention and Control in Antibiotic-free Production for further discussion.

The Principles

Disease prevention and control involves the three interrelated processes of bioexclusion, surveillance, and biocontainment. Disease prevention is difficult, expensive, and requires total commitment because it invariably involves eradication. Eradication programs are appropriate when the economic consequence of the disease is so devastating that it is economically advantageous to implement such drastic control measures. It is only feasible if there is an effective means of *detecting* infection, *containing* the infection, and *preventing dissemination* of the disease causing agent (70). There are three categories of disease for which eradication is an appropriate means of control: those that significantly threaten public health, those that have a devastating effect on bird performance, and those that severely compromise product quality. With diseases of this nature, control effort is focused on the complete elimination of the agent from the environment (70). This places the emphasis on preventing contact between the agent and the host (bioexclusion). Early diagnosis and containment is in this case the contingency plan for failure in bioexclusion.

In contrast to eradication, control programs are aimed at limiting disease challenge to a tolerable level. There is a subtle shift in emphasis from prevention, through bioexclusion, early detection, and elimination, to reducing the *consequence* or economic impact of the disease, i.e., damage control. Although monitoring and surveillance are still used to gather prevalence data, the primary focus is to measure the level of protection and challenge, not the mere presence of the disease. The principles of prevention through biosecurity still apply, but in a disease control program, the focus shifts to limiting the extent and consequence of exposure. In reality, many of the biosecurity measures taken to eradicate the more devastating diseases provide a solid foundation for the control of the erosive diseases, and immunization is usually used to bolster host resistance.

Disease challenge management must be considered to be an integral part of any poultry business risk management program. It involves the development and implementation of a stringent biosecurity plan which comprises a hierarchy of components directed at preventing or limiting the risk and consequence of disease. Economic analysis is a critical step in biosecurity plan design, since resource allocation must match risk. Although it is difficult to accurately determine the precise risk and consequence of a disease challenge, it is possible to rank disease challenge according to relative risk (37).

No disease control or prevention/eradication program would be successful without diligent diagnostic surveillance. To support an eradication program, surveillance must be sufficiently intense to detect the *source case* of an outbreak so that biocontainment through quarantine and slaughter can be carried out before disease spread occurs. The difficulty lies in confident early detection since this requires frequently testing a large sample of the population. The heavy economic burden of such intense surveillance is difficult to carry especially when

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the probability of a disease outbreak is low. Potentially devastating diseases such as highly pathogenic avian influenza (HPAI) can be effectively eradicated provided adequately robust bioexclusion, surveillance, and biocontainment programs are in place. The fact that some strains of the virus have public health connotations has helped to justify sufficient commitment to surveillance, the linchpin between bioexclusion and biocontainment.

For disease control purposes, a surveillance program is aimed at identifying when disease prevalence changes are sufficient to initiate corrective action. The difficulty is in distinguishing *common cause* (background variation) from *special cause* (a disease effect). Surveillance for the purpose of disease control, or more appropriately flock health management, remains an art. There are no specific tests that can be carried out to determine the health status of a flock, thus placing the emphasis/burden on skilful clinical assessment. Flock health monitoring systems involve a combination of clinical observation, active and passive surveillance via laboratory testing, and necropsy findings. The sample size and frequency constraints of these procedures severely limit sensitivity, thus emphasising the need for careful sample selection and attention to detail. The focus should be on identifying and eliminating subtle disease challenge since even a mildly exaggerated or inappropriate immune response will compromise performance.

In contrast to respiratory disease where early signs of disease are outwardly apparent and relatively easy to detect, low grade gastrointestinal disease is much more insidious. Breeding and selection for performance has downregulated the clinical signs of intestinal disease; i.e., birds continue to eat and drink at normal levels even when gastrointestinal disease is quite advanced. Early changes in intestinal absorptive capacity, normally indicated by litter moisture changes because of compromised water balance, may be masked by litter buffering capacity and good ventilation. Similarly, accelerated cellular sloughing usually indicated by the presence of orange mucus in the feces, is to a degree masked by high feed through-flow rates.

Biosecurity

In poultry production biosecurity includes all procedures implemented to reduce the risks and consequence of introducing an infectious disease into a flock. These preventative measures must be practical, enforceable, and cost-effective and thereby form an integral part of the production system. Since the implementation of biosecurity carries a cost, it is necessary to relate this cost to the risk and consequence of infectious disease. Unfortunately there is no way of accurately defining the relative risk and financial consequence of disease exposure or, for that matter, the effectiveness of preventative measures. Clearly the development of a cost-effective biosecurity system must entail a calculated estimate of these parameters.

A comprehensive biosecurity program comprises a hierarchy of *conceptual, structural*, and *operational* components directed at preventing infectious disease transmission from: bird-to-bird, house-to-house, site-to-site, complex-to-complex, operation-to-operation, region-to-region, company-to-company, or country-to-country.

Every event in the production process that involves movement across the house/site/farm/complex boundary creates risk of contact between an infectious organism and the host. Avoidance is the best form of prevention. Where the event is unavoidable, biosecurity measures need to be implemented to alleviate risk. This can be achieved by reducing the frequency of the transgression, or the probability of the event resulting in colonization or infection.

Conceptual Biosecurity

This is the primary level of biosecurity and involves the location of a poultry operation and its various components. *Physical isolation is the most effective means of limiting disease risk* and should therefore be the primary consideration in establishing a new complex or farm. This physical separation will limit the use of common vehicles and facilities, preclude visitation of personnel not directly involved with the operation, and reduce the possibility of indirect spread of disease by vermin, wild birds, or wind. Farms should not be located adjacent to a public road, especially in an area that has a high density of poultry.

Structural Biosecurity

The second level of biosecurity includes *farm layout, perimeter fencing, drainage, change rooms*, and *housing design*. Long-range planning and programming of the operation, whether large or small, is very important and should consider movement patterns of various vehicles and equipment, work traffic of regular and holiday caretakers and special work crews, feed delivery and storage, and the system for moving eggs and flocks from the farm. Biosecurity should be considered when the farm is being designed and the production programmed, rather than after it is developed and serious trouble is evident.

Procedural Biosecurity

The third level of biosecurity comprises *implementation and control of routine procedures intended to prevent the introduction (bioexclusion) and spread (biocontainment) of infection within a complex or enterprise*. These activities can be adjusted at short notice to respond to disease emergencies, and constant review of these procedures is necessary.

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Risk

The success of a disease control program hinges on the ability to identify and then address the risk of infection. Disease risk in a flock situation is characterized by the probability of point infection and subsequent spread occurring. Aggregate risk is the sum of each individual risk of adverse health effects in an exposed population. The spread and consequence of point infection is influenced by several factors referred to as disease determinants.

Disease Determinants

An infectious disease is the result of a complex interaction between several factors. *Any factor that influences the risk and consequence of disease challenge* is thus a disease determinant. They have traditionally been classified as: primary or secondary, intrinsic or extrinsic, and host, agent, or environment associated. The latter best describes infectious disease in intensive poultry production units. In an intensive poultry production system the house environment, agent, and host determinants are largely under the control of the manager. The management thus becomes the most important disease determinant influencer.

Risk Assessment

This involves determining the probability of exposure to an infectious agent, the probability of that exposure resulting in infection and spread of the disease, and the consequence of the disease outbreak. For disease control purposes it is appropriate to evaluate each part of the production process in terms of the *probability* or *chance* of the process or event causing infection, and the *frequency* with which that event occurs.

$$\textit{Risk} \text{ of infection} = \textit{probability} \text{ of the event causing infection} \times \textit{frequency} \text{ of the event.}$$

Limiting the frequency of an event, that carries any form of health risk, is the obvious first step in any flock health program.

Establishing the degree of risk requires further analysis. The probability of infection occurring after exposure is influenced by the resistance of the host and the challenge dose and virulence of the organism.

$$\text{Risk of infection} = \frac{\text{challenge dose} \times \text{agent virulence} \times \text{challenge frequency}}{\text{host resistance}}$$

The probability of infection occurring can thus be reduced by improving host resistance through immunization and stress reduction, reducing the challenge dose through biosecurity, cleaning, and disinfection, or reducing organism virulence by medication or competitive exclusion.

Host Resistance

Bird resistance to disease challenge is primarily governed by the efficiency of its immune response. An appropriate immune response, adequate to contain infectious disease and minimize its impact on productivity, is the cost of health. An inappropriate (excessive or inadequate) immune response will depress performance unnecessarily. Inherent resistance to disease challenge varies amongst individuals, and baseline variance is due primarily to genetic differences and thus invariably demonstrates normal (Poisson) distribution within a flock.

Immune suppression as a result of stress, non-specific disease challenge, or disease of the immune system, will reduce both individual immunity and flock immunity. Since the impact of individual stressors is cumulative, the "poor doers" in the flock will be more adversely affected by stress or disease challenge when compared with the best birds in the flock. The distribution of resistance within stressed flocks thus becomes skewed and flock immunity drops dramatically because of the presence of highly susceptible individuals within the population.

Disease Challenge

(Dose × Virulence × Frequency)

Challenge dose is the number of organisms that an individual bird is exposed to and agent virulence is the inherent capability of the agent to infect the host (infectivity) and cause disease (pathogenicity). Because the challenge dose required to cause disease in an individual varies, the infective dose 50 (ID_{50}) is traditionally used as an estimate of agent virulence. *ID_{50} is the challenge dose required to infect 50% of the birds in a specific population*. Although the ID_{50} helps in estimating the risk of infection for the average bird in a flock, it is in fact the challenge dose required to infect the least resistant bird in the flock that is important when designing a flock health program. A chain is only as strong as its weakest link. Once one bird in a flock becomes infected or diseased, the process of agent replication increases the challenge of exposure (dose and possibly agent virulence) for other birds in the flock. The level of challenge escalates with each infection until even the most resistant birds in the flock are at risk.

Epidemiology

Epidemiology is the unbiased study of the interrelationships between the various factors (disease determinants) that affect the frequency and distribution of disease in a population. Since the prevalence and consequence of any infectious disease involves a complex interaction between several disease determinants it is critical to have a thorough understanding of epidemiology (causal relationships between exposures and outcomes) in order to design an effective flock health or biosecurity program.

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For flock health management purposes, each disease must be analyzed first in terms of its *relative risk*, to determine whether it is necessary to implement control procedures and second in terms of its *epidemiological characteristics*, to ensure optimum resource allocation. The important epidemiological characteristics for disease control purposes include:

- Source of infection. Although an infected bird is the obvious source of the agent, the shedding pattern, host range, mode of transmission, and farming practices will vary and ultimately determine the relative importance of a particular source.
- Transmission. While within flock spread might be the result of direct bird-to-bird contact, indirect contact through contaminated objects (fomites) can accelerate the rate of transmission within a flock and increase the extent of transmission to other noncontact birds/flocks. This type of transmission is commonly referred to as horizontal or lateral transmission. This is in contrast to vertical transmission where the disease agent is transmitted from parent to offspring. While vertical transmission may occur as a result of eggshell contamination, some disease causing agents are able to reside inside the egg or embryo and spread by transovarial transmission.
- Spread. The incubation period, replication rate, resilience, and virulence of the disease agent will determine the course of the disease within an individual (acute, sub-acute, or chronic) and the spread of the disease within a flock (defined population). An acute disease caused by a resilient organism with a short incubation period and high replication/shed rate will, for example, spread very rapidly in a susceptible flock.
- Susceptible host. The host range of a disease agent (species, breed, type) is important in control program design. The proximity of species that are not susceptible, is irrelevant to control.
- Predisposition. Several host, agent, and environmental disease determinants can enhance the detrimental outcome of exposure to a disease-causing agent. Any environmental stress could for example compromise the immune system and predispose to infection. Similarly, host factors such as breed, sex, size, and age, and agent factors such as concomitant infection with different organisms, or immune suppressive disease, can predispose birds to infection.
- Prevalence. The prevalence of a disease is directly proportional to the risk of challenge. Endemic diseases (those that are always present in the area under consideration) are difficult to prevent while those that are exotic (do not occur in the area under consideration) or occur sporadically as an epidemic are easier to contain and eradicate through surveillance and biocontainment.
- Morbidity. This term is used to describe the number of birds in a flock that show clinical signs of disease at a point in time (specific) or at the peak of the epidemiological curve (general) and is usually expressed as a percentage. The morbidity rate will be high in rapidly spreading diseases while the morbidity tends to be low in diseases that spread slowly.
- Mortality. The percentage of birds, in a finite population, that are expected to die during a particular disease outbreak.
- Recovery. The course of a disease is influenced by a multitude of factors (disease determinants). Epidemiological statistics on the expected outcome of a disease outbreak, aid in determining what course of action it is best to take to limit the current and future financial risk of that particular disease.

Disease Prevention: Bioexclusion

Preventing or reducing *disease challenge* requires that there is a *systematic approach* to eliminating or decreasing the number of disease causing organisms within the bird's environment. This is achieved through the implementation of cost-effective procedures to prevent pathogen movement across physical or imaginary barriers demarcating *protection zones* around the bird. The establishment of zone boundaries should be based on sound epidemiological principles while making use of existing physical and geographical barriers.

Global Perspective: Top Down

The poultry industry has become a global industry. Poultry and poultry products are shipped internationally on a daily basis. The World Trade Organization (WTO) is an intergovernmental organization that regulates international trade. The WTO's Sanitary and Phytosanitary Agreement seeks to harmonize sanitary and phytosanitary measures on as wide a basis as possible, and references the World Organization for Animal Health as the relevant organization for animal health. The World Organization for Animal Health was formerly known as the Office International des Epizooties and still goes by that acronym (OIE). The OIE currently represents 181 member countries, including the United States of America, and is led by the World Assembly of Delegates, consisting of representatives from each member country. It is important to understand the workings of the OIE as it pertains to disease prevention, diagnosis, and control. In order to trade internationally in poultry and poultry products, control measures implemented at farm level must ultimately comply with the organization's stipulated requirements. The reader is referred to the official OIE

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website (http://www.oie.int/standard-setting/terrestrial-code/access-online/) for details of the rules and regulations as laid out in the OIE Terrestrial Animal Health Code (Terrestrial Code), but in summary the objectives of the OIE are to:

- Ensure transparency in the global animal disease situation by reporting detected disease.
- Collect, analyze, and disseminate veterinary scientific information on animal disease control.
- Encourage international solidarity in the control of animal diseases by providing technical support to member countries requesting assistance with animal disease control and eradication operations, including diseases transmissible to humans.
- Safeguard world trade by publishing health standards for international trade in animals and animal products that member countries can use to protect themselves from the introduction of diseases and pathogens, without setting up unjustified sanitary barriers.
- Improve the legal framework and resources of national veterinary services.
- To provide a better guarantee of food of animal origin and to promote animal welfare through a science-based approach. The OIE works in conjunction with the Codex Alimentarius Commission (CAC) to improve the safety of food of animal origin and is viewed as the leading international organization for animal welfare.

Country Perspective: Responsible Trade Through Risk Reduction and Disease Containment

The movement of animals or animal products across country borders carries a risk of disease spread. The OIE plays an important role in establishing international agreement on the application of sanitary and phytosanitary measures. This so-called Sanitary and Phytosanitary (SPS) Agreement of the World Trade Organization provides definitions and describes the OIE in-house procedure for settlement of disputes. It also provides guidelines and principles for conducting transparent, objective, and defensible risk analyses for international trade. *The principal aim of import risk analysis is to provide importing countries with an objective and defensible method of assessing the disease risks associated with the importation of animals, animal products, animal genetic material, feedstuffs, biological products, and pathological material*.

OIE Listed Diseases

Diseases are included on the OIE list based on international prevalence and capacity for spread, resultant morbidity and mortality, zoonotic potential, and emergent properties. The details of the criteria and decision process are outlined in Chapter 2.1.1 in the Terrestrial Animal Health Code (7). The following avian diseases are included in the OIE List (8): avian chlamydiosis, avian infectious bronchitis, avian infectious laryngotracheitis, avian mycoplasmosis (*Mycoplasma gallisepticum*), avian mycoplasmosis (*M. synoviae*), duck virus hepatitis, fowl typhoid, highly pathogenic avian influenza and H5 and H7 low pathogenic avian influenza in poultry, infection with influenza A viruses of high pathogenicity in birds other than poultry including wild birds, infectious bursal disease (Gumboro disease), Newcastle disease, pullorum disease, turkey rhinotracheitis, and West Nile fever.

Region or State Perspective: Zoning and Compartmentalization

Due to the difficulties in controlling the disease status and management practices of poultry flocks across the vast expanse of large countries like the United States, the Terrestrial Code makes allowance for zoning and compartmentalization. Compartment, as defined by the Terrestrial Code, means *an animal subpopulation contained in one or more establishments under a common biosecurity management system with a distinct health status with respect to a specific disease or specific diseases for which required surveillance, control and biosecurity measures have been applied for the purpose of international trade* (6). By defining subpopulations based on flock health status, member countries are able to limit the damaging effect of a listed disease outbreak on international trade without exposing the importing country to the risk of disease spread. Compartmentalization applies to a subpopulation separated by biosecurity procedures, while zoning applies to a subpopulation separated on a geographical basis. The details of what is required to establish these subpopulations will vary according to the disease in question and the requirements of the trading partners. These details are ideally decided prior to the disease outbreak. Of particular interest is the epidemiology of the disease, environmental factors, applicable biosecurity measures (including movement controls, use of natural and artificial boundaries, commercial management, and husbandry practices), and surveillance and monitoring. To establish a zone or compartment within its territory for international trade purposes, the veterinary services of an exporting country should clearly define the subpopulation as stipulated in the Terrestrial Code. These claims must be communicated to the veterinary services of an importing country and supported by detailed documentation published through official channels.

Since the borders of a zone are based on natural, artificial, or legal boundaries, they can be established relatively easily and made public by the veterinary services through official channels. Compartments are a little

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more difficult to define in that they must be established based on biosecurity procedures. This involves developing a partnership between the company and the veterinary authority to develop clearly stipulated responsibilities. To meet the requirements for a compartment the biosecurity plan, operating procedures, and management practices must be adequate, documented, and evidence of compliance documented.

The plan must demonstrate adequately robust disease surveillance, animal identification, and traceability. This requires that detailed records are kept on bird movement, flock production, feed source, disease surveillance results, chick source, visitor's log, flock morbidity and mortality, vaccination and medication, and personnel training. Risk mitigation also requires that the biosecurity plan is regularly audited, reviewed, and adjusted when necessary.

Disease Status: Classification of Diseases for Biosecurity Purposes

The allocation of resources to the prevention of diseases that have a major biological and financial impact is relatively easy. First, the control measures are the *cost of doing business*; freedom from the disease in question is a perquisite to doing business. Second, through eradication the cost of the disease is usually totally recoverable. In contrast, designing a disease control strategy for diseases that are likely to occur with a high degree of certainty but have less of a financial impact, is a lot more difficult (37). The process begins with clearly defining the estimated cost that the disease presence may incur and the potential benefits that the options for control may provide. Unfortunately there are several unknowns in health related matters, and it is consequently impossible to perform detailed and accurate cost-benefit analysis to ensure optimum resource allocation. Instead, partial farm budgeting is commonly used to compare the economic efficiencies of the various control options, including nonaction. In such instances immunization and its related biological and financial impact is the *cost of health*. One of the more difficult but important factors to quantify is the degree of productivity recovery that the control option provides, since disease losses are in this case seldom if ever totally recoverable.

For biosecurity purposes, diseases should initially be grouped into those that are exotic and those that are endemic to the region as this helps to optimize resource allocation to biosecurity. In the case of foreign diseases the emphasis is on reducing the risk of disease through prevention and eradication. In the case of endemic diseases the emphasis is on limiting the consequence of the disease.

The success of an eradication program hinges on good biosecurity and early detection of disease. In the United States the following diseases are usually prevented by eradication:

- Bacterial diseases: pullorum disease (*Salmonella enterica* serovar Pullorum), fowl typhoid (*Salmonella enterica* serovar Gallinarum), salmonellosis (*Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Typhimurium), avian mycoplasmosis (*M. gallisepticum*, *M. synoviae*, *M. meleagridis*, and *M. iowae*), avian chlamydiosis (*Chlamydophila psittaci)* and avian tuberculosis.
- Viral diseases: highly pathogenic avian influenza (HPAI), low pathogenicity avian influenza (LPAI) of H5 and H7 types, velogenic viscerotropic Newcastle Disease (vvND), West Nile fever, duck virus hepatitis, and duck virus enteritis.

The National Poultry Improvement Plan (NPIP) is a voluntary cooperative United States federal–state–industry program initially developed to control and eventually eradicate pullorum and fowl typhoid. Over the years this program has expanded to provide established programs with specific procedures for not only pullorum and fowl typhoid, but also *M. gallisepticum*, *M. synoviae*, *M. meleagridis*, *S. enteritidis*, and avian influenza (HPAI and H5 or H7 LPAI), as well as other programs for general sanitation monitoring. Compliance with these programs provides official certification of control of these specific diseases by a company. In order to be eligible for indemnity in the case of depopulation for reportable avian influenza, commercial producers must participate in the NPIP Avian Influenza Monitored program. Among other monitoring requirements and response plans, this program now requires documented and audited minimum biosecurity practices. The NPIP biosecurity principles include requirements for a biosecurity coordinator; documented training of personnel; functional lines of separation for each house; perimeter buffer areas for each farm; personal protective equipment; wild bird, rodent, and insect control; equipment and vehicle sanitation; mortality disposal; manure and litter management; replacement poultry; feed, water, and litter supplies; monitoring; and auditing. Refer to the official NPIP website (http://www.poultryimprovement.org/documents/StandardE-BiosecurityPrinciples.pdf) for details on these programs.

Biosecurity program design begins with the identification of critical control points or epidemiological unit boundaries at which bioexclusion practices can be implemented. For the purposes of disease control an epidemiological unit is a group of birds with a defined epidemiological relationship that share approximately the same likelihood of exposure to a pathogen.

Primary Control Zone: Poultry House and Hatchery

Bioexclusion begins at the boundary of the smallest epidemiological unit within the company, in this instance

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the poultry house. The birds in the house form an epidemiological unit because they share a common environment, common management practices, and have approximately the same likelihood of exposure to a pathogen. In addition the roof and walls of the house provide a well-defined barrier to entry and an ideal site for the implementation of critical control procedures. From a biosecurity standpoint every crossing of the house perimeter (event) should be considered as a potential means of pathogen transfer or disease risk.

The process of risk reduction begins with an all-in all-out placement strategy, so that decontamination of the house environment by thorough physical cleaning followed by chemical disinfection and/or "downtime" is possible between successive placements. After placement the emphasis shifts to first limiting the *frequency* of any house perimeter crossing (event), and second at reducing the *probability* of pathogen transmission and infection if the event is unavoidable or essential.

The Poultry House

Management of the House Environment in Disease Prevention. While it is important to keep disease out, it is equally important to prevent the house environmental conditions from causing discomfort or stress. Traditional thinking, stimulated by widespread acceptance of *Koch's Postulates* in the 1900s, overemphasizes the importance of infectious agents in the disease process. As production systems have evolved, environmental and host disease determinants have played a more obvious role in the disease process, emphasizing the multifactorial nature of disease. The prevalence of specific infectious disease entities has declined as knowledge and control measures have improved. In contrast, the predisposition to and prevalence of noninfectious disease has increased with intensification and genetic change. The distinction between infectious and noninfectious disease has become somewhat blurred in intensive agriculture, and a more fully encompassing epidemiological approach to disease diagnosis and control has become necessary.

The poultry house environment, with all its intricacies, is a crucial disease determinant since stress of any kind stimulates a cascade of physiological and biochemical changes which erode host resistance and productivity (69). Stress lowers the minimum dose of infective agent required for development of infection and increases the risk of infectious or noninfectious challenge developing into clinically detectable disease. The risk and consequence of infectious disease spread within the population is increased by the presence of stress because susceptible individuals act as amplifiers for the infectious organisms and thus increase the challenge dose to which the pen mates are exposed. While the introduction of a noninfectious disease to a flock may also lower individual resistance, there is no risk of spread (70). The influence of the house environment on viral disease of poultry has been reviewed (3).

Turnaround-time and Downtime. Turnaround time is the time lapse from the start of depletion/transfer to the start of subsequent placement. Downtime, which is of greater significance, is the time between the removal of all poultry, poultry by-products, and litter to the start of the next placement. The process of bioexclusion is pointless if the production system does not start off disease or pathogen free. The risk of pathogen carry over from one production cycle to the next is directly linked to the time interval between the removal of one flock and the subsequent placement of the next flock. Pathogen attrition occurs with time and the chance of pathogen carry-over from one grow-out cycle to the next is reduced by extending downtime. The longer the bird-free period, the greater the reduction in disease challenge. In a low challenge situation or if prevailing conditions preclude the removal of litter from the house, an extended turnaround time can be used to substitute for clean-out and disinfection.

In the United States, true clean-out and decontamination is seldom practiced at the broiler level and extended turnaround times (minimum of 14 days) are commonplace. Decontamination of the house through clean-out and disinfection hastens the attrition rate of pathogens within the house environment and therefore serves to reduce the need for long downtime. While the process of clean-out and disinfection carries a cost, it reduces turnaround time and hence improves return on investment. The decision as to whether to implement a clean-out and disinfection program or to reuse litter is complex and should involve a detailed analysis of the fixed versus variable cost benefit, the level of disease challenge, the nature of the prevailing diseases, the type of housing, stocking densities, and so on.

Decontamination: Clean-out and Disinfection. This procedure is designed to reduce the risk of disease through the physical removal of all poultry, poultry by-product and litter, and the sequential washing, disinfection, and possibly fumigation of all the houses. The relative importance of this process increases as the length of turnaround time diminishes. Decontamination is a sequential process which requires careful planning, execution, and control. As outlined in Chapter 5 of the American Association of Avian Pathologists (AAAP) publication *A Practical Guide for Managing Risk in Poultry Production* decontamination involves five steps: removal of debris, detergent application, washing with water, drying, and disinfecting (56).

After depopulation, the litter or droppings should be removed. Once the bulk of the litter has been removed as much of the remaining solid material as possible should be brushed out of the house before the washing

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process begins. With development of huge specialized poultry farms, proper and economical disposal of litter and poultry manure has become a serious problem. There is no clear-cut answer. A general recommendation is to remove it far enough from the buildings so that insects will not crawl or fly back into the houses, and to dry it, compost it, or spread it onto fields and work it into the soil. If cleaning is done while chickens are still present (cages), remember that contracted personnel, trucks, and equipment may recently have been on another farm where a disease outbreak occurred.

In some cases, the physicochemical properties of a pathogen may dictate that some extra precautions (wetting down or soaking with disinfectant, delaying removal, burying, burning) be taken with litter, even though expensive. Any treatment of manure or litter must consider residual effects of the applied compounds on plant life when treated manure is spread on the land. For most disease agents, composting of litter or droppings is sufficient. Whatever is done, one must be aware that wherever litter is spilled or piled, it remains as a pathogen reservoir for varying lengths of time.

In the case of outside runs such as turkey and game bird ranges, the topsoil should be scraped off and hauled some distance from the site. Sunlight and soil activity combine over a long period to destroy most pathogens. Anything that can be done to aid the destruction process is helpful. Removal of organic residues, such as leaf beds and manure accumulations, helps to reduce the danger for future broods. It is best to rotate the ranges or dirt yards so that they stand idle for one complete flock cycle.

Washing begins with *blow-down*, a process by which water (preferably hot) and detergent sprayed though high-pressure nozzles is used to wet the surfaces and remove most of the dirt and dust from the house. The detergent helps to dissolve the organic biofilm and aids the cleaning process. This is followed by *cleaning* with water (preferably hot) sprayed at high pressure to remove residual dust and dirt. If washing is not possible, dry-cleaning must be thorough and includes scraping and sweeping or vacuuming surfaces, corners, ledges, nests, and feeders.

Once the house is physically clean and free of organic matter the process of disinfection can begin. The house should be allowed to dry to prevent dilution of the applied disinfectant by residual water. Disinfection involves the application of correctly diluted disinfectant to all internal surfaces of the house by low-pressure spray (preferably as foam to increase contact time). The concentration and volumes of chemical applications must be correct to ensure adequate success. Dry-cleaning will significantly compromise the disinfection process. The amount of disinfectant used on dry-cleaned surfaces must be increased over that required for washed surfaces.

Disinfectants. Many effective disinfectants are sold under a variety of trade names; follow the manufacturers' recommendations. A disinfectant is a physical or chemical agent that destroys vegetative forms of harmful microorganisms, usually on inanimate objects but sometimes on the animals (10).

Disinfectants are regulated by the Environmental Protection Agency (EPA) under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA); Title 40 of the Code of Federal Regulations (CFR). Individual states also have regulations which may be stricter than the federal regulation. The Worker Protection Standards (WPS) are a specific portion of FIFRA (Title 40 CFR Part 170) which requires the protection of employees from agricultural pesticides (including disinfectants). Supervisors of individuals who will be applying disinfectants must read the label on the disinfectant closely and look specifically for references to the WPS. If the labeling refers to WPS, compliance is mandatory. Copies of *WPS How to Comply* may be obtained from local cooperative extension offices.

Complete discussions of various disinfectants and sterilization methods should be consulted (10, 17, 46, 56). Additional references on disinfectants and their use (28) and textbooks on pharmacology and therapeutics should be consulted. The virucidal activities of several commercial disinfectants against vvND have been determined. A list of commercial disinfectants approved for use against avian influenza virus is available from the EPA (United States) (5).

After disinfecting the house, all subsequent processes and movements should be controlled to prevent recontamination. This requires that "clean" and "dirty" areas are clearly demarcated. Stringent sanitary practices are frequently ineffective because disease is tracked in after the buildings and equipment are cleaned and disinfected, or because some step in the total program was omitted.

Feed bins should be emptied and cleaned between grow-out/production cycles and special care must be taken to ensure that the inside is totally dry before new feed is delivered.

Water lines should be drained, cleaned, and disinfected. It is important to strip the lines of biofilm before disinfecting the system. The water line biofilm is composed of both a minera1 and organic component so the cleaning process entails dissolution of the mineral component (acid or alkali) and destruction of the organic fraction (oxidation and disinfection) (41, 55).

Built-Up Litter and Uncleaned Buildings. Commercial producers require that chicks and poults are delivered disease free. To maintain this status, it is preferable to place these healthy new flocks in cleaned and disinfected buildings with fresh clean litter. This is an expensive and time consuming task. Litter material is becoming scarce and litter disposal requires detailed nutrient management

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program compliance. Rearing of several successive flocks on the same (built-up) litter has become an economically acceptable practice with broilers, where the life span is very short and single ages of birds per farm permit complete depopulation at the end of each brood. Cleaning and disinfecting of houses is in such instances reserved for disease outbreak control.

While young poults are usually placed in cleaned and disinfected buildings with fresh clean litter the litter in turkey grow-out buildings is frequently used for several successive flocks. Rearing of meat birds on reused litter has become commonplace with the development and use of litter-processing machinery. This equipment is used after flock depletion to break up or remove caked litter to ensure that a deep friable and absorbent layer of bedding material remains for the next brood. This practice of reusing litter will unfortunately result in the accumulation of microbial pathogens and parasites within the litter and is strongly discouraged in egg-producing operations.

Culling. Culls are birds that are removed from the flock for humane reasons because they are injured, diseased, or poor performers. The practice of using hospital pens to separate sick birds from the main flock should be discouraged as they act as a source of infection for the rest of the flock. Instead birds that are injured, diseased, or dying should be humanely destroyed and removed from the chicken house as soon as possible to avoid unnecessary suffering and disease spread. Such culling must be done judiciously, performed in a humane manner, and started from placement.

Mortality. Any dead bird which is left in the poultry house poses a serious threat to flock health. The carcass undergoes decomposition with the production of millions of decomposition bacteria and potentially pathogenic organisms within the carcass. These are released when the carcass breaks. Occasionally toxins may be formed in the carcass and cause problems for the flock. Dead birds should be removed at least daily and categorized per house according to the likely cause of death. If daily mortality is abnormally high (more than 1/1000 in broilers and more than 0.3/1000 in breeders) further investigation is indicated.

Nest and Egg Hygiene. The most important consideration in hatching egg sanitation is to manage the flock so that eggs are clean when gathered. It is crucial to keep the litter dry in order to prevent soiling of nests, nest material, and eggs. Table-egg breeding stock are traditionally raised on slatted or sloping wire-floor houses which greatly reduces the number of dirty eggs. Broiler and turkey breeders do not perform as well on these floors, so combinations of part slat and part litter are used to aid in litter management.

Automatic nest boxes are generally speaking more biosecure than manual collection nest boxes. The plastic mats lining the automatic nest boxes are less likely to cause egg contamination and the eggs spend much less time in the nest box. It is essential to keep the nest box environment as clean as practically possible. Dirty or contaminated nest boxes can result in egg contamination, vertical transmission of disease agents, and infection of the hen's oviduct. The shell of an egg laid by a healthy breeder hen is warm, moist, and clean when it first makes contact with the nest box shavings. Debris will adhere to the moist surface and, as the egg cools, particles that are small enough, such as microorganisms, may be drawn in through the pores before the cuticle has had time to dry. During oviposition there is a tendency for the distal section of the reproductive tract to prolapse. In many instances this delicate moist tissue actually makes contact with the nesting material and is therefore very easily contaminated. Microorganism contamination of the nest box can thus be the cause of infectious reproductive disorders and peritonitis.

It is essential that the nest boxes are thoroughly cleaned of all organic material, and disinfected during terminal disinfection of the laying houses. If wood shavings are used as nesting material, there should be a very low proportion of sawdust, the shavings must be dry, free of contaminants including fungus and preferably even fungal spores. Ideally the shavings should be fumigated with formalin prior to use to ensure there are no live microorganisms present. The nesting material should be "topped up" with clean material every two weeks to keep it filled to a depth of 5–10 cm and ideally replaced (and the nest box disinfected) monthly. Any extraneous material should be removed from the nest box as soon as possible—broken eggs, fecal material, and so on.

The house environment is from a biosecurity point of view, classed as a dirty area and the eggs need to be removed from this environment as soon as possible. If manual collection is practiced, eggs need to be collected as frequently as possible—at least four times a day. Each egg collection must be a complete process so that each nest box is emptied. The operator's hands need to be washed before commencing with egg collection and every effort must be made to keep hands clean during collection to prevent contamination of the eggs. Every effort must be made to ensure that the hatching eggs do not get wet. Eggs and egg trays must be dry-cleaned (compressed air) prior to fumigation to remove all the dust and debris which has accumulated during collection.

Very dirty eggs and floor eggs must not be used as hatching eggs. They should be collected separately and must not be placed on the egg trays that are used for collection of clean nest eggs. It is important that the floor eggs are stored away from the nest eggs to avoid cross contamination.

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Properly handled, nest-clean hatching eggs will produce suitable chicks and fumigation or sanitation may not be necessary. If a sanitation procedure is desired, in order to derive maximum benefit from any disinfection procedure the eggs need to be sanitized or fumigated within two hours of being laid, that is, immediately after collection. Effective formalin fumigation of hatching eggs is a proven method of reducing eggshell contamination with the vegetative and spore forms of bacteria and fungi. While formalin fumigation has been the backbone of most egg hygiene programs in the past, the classification of formalin in 2004 as a known human carcinogen by the International Agency for Research on Cancer has made its use a lot more arduous. Stringent health and safety regulations apply even in the United States where the EPA has classed formalin as a probable human carcinogen (9, 28).

Egg washing is routine practice in the commercial egg industry. These table-eggs are washed with warm (43–51.8 °C) detergent solution and then sanitized with a chlorine compound, quaternary ammonia product, or other sanitizing agent. It is critical that the washing water is at least 16.6 °C higher than the egg itself but not higher than 54 °C. While this procedure is often employed successfully with turkey hatching eggs, it is seldom used in the broiler industry. If hatching egg washing is attempted, a brush conveyer machine that uses continuous-flow water is preferable and very careful supervision and meticulous management is essential to avoid contaminating rather than sanitizing the eggs. It is also important to consider water quality. If for example the iron content of the wash water exceeds 5 ppm, a serious egg spoilage problem is likely. A complete review of egg sanitizing agents is presented by Mackenzie (56) and Scott and Swetnam (67).

After fumigation the eggs should be transferred to the egg storeroom. Control of temperature and relative humidity during storage is critical to the survival of the embryo. Temperature and relative humidity fluctuation during storage reduce embryo viability and cause condensation and wetting of the shell surface (sweating). This increases the chance of egg contamination and vertical transmission of bacteria and fungi. The egg storage room should be maintained at a constant 15–20 °C with a relative humidity 75%. Movement of eggs in and out of the storeroom should be done as quickly as possible to avoid excessive fluctuation in temperature and humidity.

Feed and Drinking Water. The potential for feed or waterborne challenge occurs every time the birds eat or drink, so the frequency of challenge is very high. This means that even a low level of contamination poses a high risk for introduction or spread of disease. Contamination of feed with fecal pellets from rats, mice, and other rodents is particularly worrying. First, they are likely carriers of dangerous pathogens such as *Salmonella* species. Second, the fecal pellet provides a concentrated source of pathogens in a package that birds are highly likely to selectively pick out and consume.

Litter scratched into feed and water troughs and feed spilled into litter increases intake of litter and litter-borne disease agents (e.g., more coccidial oocysts and less coccidiostat are ingested, and a clinical infection may result). If poultry are permitted to consume litter, considerable mortality and depression can occur from impaction of the gizzard, and litter fragments may cause enteritis by mechanical irritation.

Feed troughs should have some type of guard to keep poultry out and should not be overfilled so that feed is spilled into litter. Feeders without guards permit defecation into feed, which encourages spread of diseases shed in feces. Wet feed in litter provides a good medium for growth of molds, which can cause liver, kidney, immune system, and other damage to the well-being of poultry. Growing and laying cages for egg production flocks in light- and temperature-controlled houses eliminate most of the problems associated with litter. Many good automated feeding and watering systems are available commercially, but sometimes these are not installed or oriented as the manufacturer intended, and consequently health problems develop.

Roost areas over screened or slatted dropping pits are common in floor-laying and breeder hen houses to keep chickens away from their feces (Figure 1.1). Screened roost areas are also desirable in rearing houses for layers and breeders to prevent piling by the birds and excessive fouling of litter with feces, which in turn leads to packing



Figure 1.1 Slat floors aid in the control of intestinal diseases and parasites. Droppings fall through open spaces and out of reach of the flock.

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Figure 1.2 Nipple drinkers are effective in preventing microbiological contamination of clean water and help maintain dry litter conditions.

and caking. Feeders and drinkers over the pits keep the birds on the roost area much of the daytime as well as at night, so most droppings collect out of reach. Spilled water also falls under the roosts, so the litter area stays drier.

Drinkers are frequently set or hung over the litter area. In this case, drinkers should be managed so that spillage onto the litter is minimized. Drinkers can be put into two basic categories: those that provide a constant reservoir of water, which is maintained automatically (troughs, cups, and hanging plastic bells), and nipple drinkers (Figure. 1.2), which supply water on demand when activated by a bird. Drinkers that provide an open reservoir of water must be cleaned and disinfected regularly to prevent the buildup of potentially pathogenic organisms in the water supply. These drinkers are also more prone to spillage and the associated problems of wet litter. Starting day-old birds is somewhat easier with drinkers that have an open and visible water reservoir. The advantages of nipple drinkers are found in the significant improvement they offer in providing water free of organisms commonly found in the poultry house environment and in decreased water spillage.

Feed and Water Medication. Facilities for quick treatment by medication in water or feed should be provided in case birds become sick. When thousands of birds are grouped in one pen, segregation and treatment of individuals is impractical so mass-medication is essential.

Feed medication is not the best method of treatment because sick birds have little or no appetite and are unable to compete for feed. Water medication is better because the sick will still frequently drink. Mass-medication, while not completely successful in curing the sick, may hold the disease in check until the host can respond with a successful immune response. Provision should also be made for mass vaccination through drinking water, as this is an accepted and successful labor-saving practice. If drinking water is chlorinated or otherwise treated, the sanitizing agent may destroy the vaccine, so provision must be made to permit the use of untreated or distilled water for mixing and administering water vaccines.

Several methods can be used to reduce, remove, or neutralize chlorine in chlorinated water supplies. The only practical method for dealing with this problem on poultry farms is to add protein to the water when mixing water vaccines. A common practice is to add 1 cup of nonfat dried milk to 50 gallons of water in tanks or canned liquid nonfat milk mixed with vaccine in a proportioner.

If a building is constructed with a bulk water tank for gravity-flow watering devices, the tank should be of plastic, or lined with some nonreactive protective substance and be readily accessible for cleaning and for mixing medicaments. If the watering devices are operated on high pressure, the pipe leading into the pen should have a bypass system with proper valve arrangement so that a medicament proportioner can be installed quickly when needed. A metering device to measure feed and water consumption is useful to keep track of the health of the flock.

Bulk feed delivery, metal bulk storage tanks, and automatic feeders are common in modern poultry operations. These reduce the possibility of rodent contamination, because feed is always in closed tanks rather than in bags or open bins, but the system leads to difficulties when short-term emergency medication in feed is desirable and the bulk tank is full. Two alternative systems are useful: an additional smaller bulk tank may be installed just for emergency medicated feed, or a small dispensing tank may be interposed between the bulk tank and feed troughs so that emergency medicated feed can be put in the smaller tank by hand.

House Access: People and Equipment. People and especially visitors pose the greatest biosecurity risk to any poultry operation. Their mobility, duties, curiosity, ignorance, indifference, carelessness, or total concentration on current profit margin, make them one of the most likely causes of disease spread. Rarely is this because they become infected and shed the disease agent, but rather because they track in infectious diseases, use contaminated equipment, or manage their flocks in such a way that spread of disease is inevitable. At least one avian disease pathogen (Newcastle disease virus) has been found to survive for several days on the mucous membrane of the human respiratory tract and has been isolated from sputum. It is a sound principle of disease prevention that no employee of a commercial unit should

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have any contact with non-company poultry, pet, or hobby birds, at home or elsewhere. The backyard flock maintained without regard for disease control can perpetuate a disease that constitutes a threat to a large productive industry. The greatest hazard to commercial producers that is created by fancy breeds and backyard flocks is the possible perpetuation of diseases that have been eradicated from the industry.

Disease outbreaks in a community have been known to follow the path of a careless visitor. If visitors do not enter premises or buildings, they cannot track in diseases. The easiest and most effective means of reducing this risk is to reduce the frequency of visits to those that are essential. When it is necessary to enter the house steps must be taken to reduce the probability of inadvertently transporting infectious agents into the house. Shower-in facilities with dedicated clothing and footwear are the optimum solution, but are rarely available in commercial production in the United States. As an absolute minimum any person entering the house should don coveralls, a hair net, gloves, and boot covers to reduce the risk of disease transfer. While it is not practical to change protective clothing between houses on the same farm, special attention must be given to hands and feet as they are the most likely means of infectious agent transfer. Any equipment brought into the house while birds are present or after cleaning and disinfection should follow similar rules. In particular, shared or borrowed equipment should be avoided if possible, and if not, it must be thoroughly cleaned and disinfected prior to entry.

When moving from one house to another it is best to change boot covers or use house dedicated footwear. Footbaths might work well when the boots or boot covers are clean and the disinfectant is clean and at the correct concentration. Footbaths are however notoriously difficult to manage and frequently end up enhancing disease transmission not preventing it. When using house dedicated footwear it is best to set up a step-over partition barrier just inside the entrance to the house. It is thus possible to maintain a clear barrier between "clean" and "dirty" by stepping over the partition barrier into a new pair of shoe covers or into a "house dedicated" pair of boots on entering the house and stepping out of them on exiting the house.

Bird contact is invariably made with the hands so it is essential to pay close attention to hand hygiene. Using disposable surgical gloves is the best option but hand washing and disinfection between houses is acceptable. It is preferable to have hand washbasins with running water and soap/liquid soap next to each entrance/exit. Where this is not possible there should at the very least be a hand sanitizer dispenser appropriately placed for use on entrance and exit.

Personnel that frequently visit many different types of poultry enterprises, farms, and farm units such as veterinarians, managers, supervisors, and company owners are high risk for disease transfer. Apart from needing to set an example they must be meticulous in following procedural biosecurity practices to prevent spreading disease. For such personnel a "no shoes touch the ground" policy is recommended. The vehicle should be parked in a secure area away from exhaust fans and as far as practical from the houses. Shoe covers are carried in the vehicle, donned before exiting the vehicle, and doffed as the vehicle is re-entered. Outer boots or shoe covers are used to enter the houses as described above. This practice, coupled with the use of hairnets, coveralls, and exam gloves that are doffed prior to re-entering the vehicle will help control contamination of the vehicle interior. Procedural biosecurity is as much a culture as it is a discipline.

The source of a new or dreaded disease is often puzzling. World trade and travel are becoming more commonplace. It is not uncommon for a person to leave one farm in the morning and be visiting another farm or place of business in another part of the country or another continent on the same day. Some disease agents can survive that time frame easily. All who travel should be cognizant of this and guard against introduction of disease into their own flocks or onto the premises of clients, competitors, friends, or fellow producers when returning from a trip. Protective footwear and clothing are not readily available in all countries and poultry areas. Personnel traveling internationally should be advised to use clothing and footwear on the trip which will not be worn to farms upon return home. Requiring a waiting period of several days before international visitors or those returning from international trips are allowed to visit farms is a prudent practice.

Many poultry farm procedures require sporadic use of specialized crews (e.g., blood testing, beak trimming, vaccinating, inseminating, sexing, weighing, and moving birds from one location to another). These crews travel about the poultry community handling many flocks and must be regarded as a potential source of infection. Thus, they should take stringent precautions to safeguard the health of every flock with which they work.

House Access: Animals. No animals should be allowed into the poultry house. All openings in the outer structure of the house must be sealed so as to exclude animal entry into the house. Dogs and cats, like rodents, are capable of harboring enteric organisms that are infectious to poultry. When these pets are not confined to the household area, but are allowed to roam among the poultry, they constitute a serious health hazard. Such pets are just as capable of tracking contaminated material on their feet and in their hair as people.

Wild birds are capable of carrying a variety of diseases and parasites. Some cause infection or illness in the wild

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birds themselves, while with others, the birds act as mechanical carriers. Every effort should be made to prevent their nesting in the poultry area, and to exclude free-flying wild birds from the houses. Poultry raised on range or with access to the outdoors are especially vulnerable to infections carried by wild birds. For this reason and for improved sanitary practices, the trend has been to house poultry in closed or partially closed bird-proof houses. However, the advent and growth of the free range and organic industries threaten to compromise carefully designed national programs to eradicate devastating diseases like HPAI.

Imported zoological specimens destined for zoos are not a direct contact threat because the zoos are located in cities, but they should be considered as a potential source of introduction of an exotic disease or parasite. Exotic ornamental pet birds constitute a real hazard because they become widely dispersed and may be purchased by poultry workers. On numerous occasions, exotic birds in or destined for pet stores have been found infected with a virulent exotic form of Newcastle disease virus, which in at least one instance was the source of a serious and costly outbreak in poultry. Stringent entry quarantine requirements to apprehend and destroy infected birds provide a good barrier against the introduction and dissemination by carrier birds, but failures can occur (illegal smuggling), and producers should be wary of such personal pets. Domestic pigeons can also be a source of dangerous strains of Newcastle disease virus.

Rodents contaminate feed and litter with their excrement. They are particularly hazardous to *Salmonella* control, because they are frequently infected with these organisms and can perpetuate the disease on a farm. The house should be monitored for signs of rodent presence. Regular baiting of rodent stations and breeding areas must be enforced. Housekeeping must be of such a standard as to deter the vermin from settling. This can be achieved by the removal of rubble and waste materials from the house, the avoidance of feed spillage, elimination of tall grass and other harborages in the vicinity of the houses, and the regular rotation of chemical control products and traps used. For more detail on rodent control the reader is referred to Chapter 9 of the AAAP publication entitled, *A Practical Guide for Managing Risk in Poultry Production* (78).

House Access: Insect, Mite, and Tick Control. Many insects act as transmitters of disease. Some are intermediate hosts for blood or intestinal parasites, others are mechanical carriers of disease through their biting parts. They also act as reservoirs of disease, in that they can transfer an infectious agent from one flock to the next. Litter beetles are not only a major pest in the poultry house environment but can play a vital role in the spread or carry-over of disease. The control of litter beetles is based primarily on the spraying of insecticide between flocks and during the clean-out process. The beetles migrate into the walls and out of the house as soon as the birds and feed are removed, so applications should be made as soon as possible after depletion. Insecticide resistance is a problem, so frequent assessment of efficacy and rotation of products is necessary. Flies and mosquitoes can be a problem in disease transmission in layers, breeders, and birds on range. Mites and ticks can also pose a threat to flock health. Control measures need to be directed primarily at the breeding areas of these insects.

The EPA defines a pesticide as any substance intended for the preventing, destroying, repelling, or mitigating of any pest. A pest can be any insect, animal, plant, or microorganism. Insecticides destroy animal parasites such as lice, mites, ticks, and fleas. They also destroy other undesirable insects (flies, beetles, ants, and sow bugs) in the environment. The limited number of available commercial parasiticides, their active chemical properties, limitations, tolerances, and various applications, are discussed in detail in Chapter 26. See also Chapter 32 for toxic effects of some insecticides. For more detail on insect control the reader is referred to Chapter 8 of the AAAP publication (64).

Building Construction. An apron of concrete at the entrance to a poultry house helps prevent tracking of disease into the unit. Rain and sunshine help keep the apron cleaned and sterilized. A water faucet, boot brush, and covered pan of disinfectant available on the apron for disinfecting footwear are further aids in keeping litter and soil-borne diseases out of the house. Boots must be thoroughly cleaned before the wearer steps into the pan of disinfectant. The disinfectant is useless, however, unless renewed frequently enough to ensure a potent solution at all times.

Optimally, all surfaces inside the building should preferably be of impervious material (such as finished concrete) to permit thorough washing and disinfection. It is impossible to sterilize a dirt floor! Unfortunately, broiler and broiler breeder houses in the United States are typically constructed with dirt floors and porous walls such as unfinished concrete blocks or plywood, with many cracks and crevices, making disinfection difficult.

The Hatchery

The building and equipment in which the fertile egg is converted to a day-old chick, poult, or other fowl and the equipment used to process and deliver it to the farm must be clean and sanitary. An individual hatched from a pathogen-free egg will remain pathogen-free only if it hatches in a clean hatcher, is put in a clean box, and held in a clean room where it can breathe clean air, and is then hauled to the farm in a clean delivery van.

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Design and Location. A hatchery should be located away from sources of poultry pathogens such as poultry farms, processing plants, necropsy laboratories, rendering plants, and feed mills. It is not good practice to retail poultry equipment and supplies from a hatchery building, because this draws producers and service workers who may introduce contaminating material.

A good hatchery design has a one-way traffic flow from the egg-entry room through egg-traying, incubation, hatching, and holding rooms to chick-loading area. The cleanup area and hatch-waste discharge should be off the hatching room, with a separate load-out area. Each hatchery room should be designed for thorough washing and disinfecting. The ventilation system is equally important and must be designed to prevent recirculation of contaminated and dust-laden air. Hatcheries with poor floor designs and faulty traffic patterns are highly contaminated compared with those with one-way flow (36).

Importance of Good Sanitation. Techniques have been devised for evaluating the sanitary status of commercial hatcheries by culturing fluff samples (83), detecting microbial populations in hatchery air samples (27, 36, 50), and culturing various surfaces in the hatchery (52). To minimize bacterial contamination of eggs and hatching chicks, hatchery premises must be kept free of reservoirs of contamination, which readily become airborne (51). Trays used for hatching should be thoroughly cleaned with detergent and hot water and then disinfected before eggs are placed in them. This can be done by dipping in a tank of suitable disinfectant (see Disinfectants), disinfectant spray, or fumigating with formaldehyde in the hatcher. Trays and eggs are frequently fumigated together immediately after eggs are transferred to the hatcher. Fumigation is sometimes done during the hatch (at about 10% hatch), but concentrations low enough to avoid harming the hatching chick probably serve only to give the down a pleasing yellow color. As chicks hatch, the exposed embryo fluids collect bacteria from contaminated shells, trays, and ventilating air. The combination of the nutritious fluids and warm temperature forms an excellent environment for bacteria and they multiply very rapidly (36). The cleaner the air and environment the less likely the navel is to become infected (omphalitis).

Breeder Codes. The breeder code is a designation used to denote the source of hatching eggs. It usually denotes breeders of the same age on the same or different farms, all breeders on a particular farm, or any other grouping. There is a tendency to keep breeders in larger flocks and to avoid as much as practicable the mixing of hatching eggs from flocks of many different microbial, nutritional, and genetic backgrounds. Keeping chicks of different breeder codes separate ensures that all have more nearly the same level of maternal antibodies against the same diseases, which may permit a more uniform response to vaccines applied to chicks the first two to three weeks of life when maternal antibodies have a protective effect. Segregating chicks by breeder sources also contributes to better size uniformity and reduces the impact of any vertically transmitted pathogens.

Occasionally, a disease is believed to be egg transmitted from a breeder flock to the offspring. When this occurs, the disease nearly always appears in several offspring flocks derived from the same breeder flock(s) and delivered to different farms. A hatch of chicks is frequently divided into deliveries to several farms, and if a disease occurs in only those delivered to one farm it indicates that the disease is farm associated and not hatchery or breeder-flock associated.

Chick Sexers. Unless the output of one hatchery is so great as to demand them full time, chick sexers may go from one hatchery to another, which introduces the possibility of carrying disease. Most sexers are aware of this hazard and are eager to follow proper biosecurity procedures. If sexers must also service other hatcheries, facilities should be provided so that their equipment can remain at the hatchery. They should have a clean area in which to change clothes and wash themselves and their equipment and should have clean protective garments to wear. Their habits should be at least as clean as those of the hatchery crew.

Surgical Procedures. Beak trimming is commonly practiced in breeder flocks, meat turkeys, and cage layers. Proper beak trimming promotes maximum performance. Done improperly, it provides a portal of entry for normally nonpathogenic organisms like *Staphylococcus aureus* or primary pathogens like *Erysipelothrix rhusiopathiae*. Similarly, other surgical procedures, such as removing wattles, combs, or toenails of certain toes, must be done as aseptically as possible.

Storage Facilities. Hatching eggs are frequently stored in a cool room (about 15–20 °C) at the hatchery until set. Cool rooms should be clean and free of mold and bacteria and periodically disinfected to prevent recontamination of shells. Holding hatching eggs too long or under improper storage temperature, humidity, and environment can result in poor quality chicks. Clinical histories indicate that infection in young chicks may sometimes be traceable to fungus-contaminated hatching eggs; infections have been produced experimentally by contaminating shells with fungus spores (82). Whenever cold eggs are moved into a warm, humid atmosphere, moisture condenses on the cold shells (called "sweating"). This moisture provides a medium for the growth of bacteria and fungi already present on the shell or from contaminated warm air around the eggs. Cold eggs should, therefore, be warmed

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(preheating) to room temperature in clean, low humidity air before placing them in an incubator.

Secondary Control Zone: Farm or Site

The company farms or sites constitute the next logical zone or compartment for disease control. For this purpose, the farm and not the house, is defined as the epidemiological unit. First, the farm has a defined boundary and second, because the houses are in close proximity, the birds on the farm share a *defined epidemiological relationship* (common environment, with common caretakers and management practices) and thus have approximately the same likelihood of exposure to a pathogen.

The boundary of the farm/site serves as a physical (fenced) or imaginary (non-fenced) line of access control to the secondary contro1 zone. The farmer should enforce full biosecurity with no uncontrolled access from the start of the disinfection process—site is *closed*. The farmer should enforce general biosecurity from the start of transfer/depletion with access only granted to necessary vehicular traffic—site is *open*. The farmer should enforce routine control from the point of last bird removal—site is *fully open*. In the event of a disease outbreak, the site should remain *closed* until the responsible veterinarian declares the site clean.

Isolation

Not all producers follow the same disease control practices. A close neighbor may disregard sound principles and be burdened with diseases until forced out of business by economic pressures. Disease agents present on his premises may be blown or carried by various vectors and fomites to adjacent premises. Until a disease has been eradicated from a flock like this, it serves as a reservoir and potential source of infection for future flocks on the same premises and those on adjacent premises. The closer houses or premises are to one another, the more likely it is for disease to spread.

Highly concentrated poultry production areas frequently deteriorate into problem zones of disease of one type or another. Farms are so close together that the area forms an epidemiological unit from a disease perspective. Within these areas there are several different age groups of birds, many managers, each vaccinating, treating, or exposing birds without regard to the programs of others. In such situations a system of a single age of fowl, permitting complete depopulation at the end of each rearing or laying cycle goes a long way to solving the problem. This is even more successful if coordinated area depopulation and restocking is practiced.

One Age of Fowl per Farm

Removing carriers from a flock and premises is an effective way of preventing a recurrence of some diseases, but it is impossible or impractical for others. The best way to prevent infection from carrier birds is to remove the entire flock from the farm before any new replacements are added and to rear young stock in complete isolation from older recovered birds on a separated farm segment or preferably on another farm and in an isolated area. This practice is often called "all-in, all-out production."

Where birds of different ages exist on a large farm, depopulation seems drastic, but considering mortality, poor performance, and endless drug expense, it could be the most economical solution. Where only one age of bird is maintained, depopulation occurs each time pullets or poults are moved to the layer or breeder premises, each time the broilers or turkeys are moved to slaughter, and each time the old layers or breeders are sent to market. Should a disease occur, the flock can be quarantined, treated, and handled in the best way possible until its disposal. Depopulated premises are then cleaned out, washed, and disinfected, and left idle for at least two weeks before new healthy stock is introduced.

Functional Units

For certain economic reasons (breeding farm or small specialized market trade), it is not always possible to limit the entire farm to a single age of poultry. In such instances, it should be divided into separate quarantinable units or areas for different groups of birds (rearing area, pedigree unit, production groups, and experimental birds) (Figure 1.3). Each area can periodically be depopulated, cleaned, and sanitized. Much stricter security procedures for personnel, bird, and equipment movements are necessary for this type of operation. A very rigid monitoring system is also essential to detect any disease early enough to bring it under control while it is still confined to one quarantinable segment.

Farm Environment

The farm or site must be maintained so as to minimize the breeding areas and any overt protection given to wild birds (especially waterfowl), vermin, predators, or other organisms. The grass must be kept short and the aprons free of grass and weeds. Vermin are vulnerable to predation when crossing these exposed areas.

Water must not be allowed to accumulate on site in open pools. Drainage must be sufficient to remove excess water especially during storms and clean-out. Stagnant water is an ideal breeding ground for insects and other organisms. No rubble or waste debris should be stored on site and equipment must be stored in such a way as to avoid offering shelter or protection to unwanted creatures.

Farm Access Control

The site should ideally be completely fenced with sufficient deterrents to access by predators, vermin,

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Figure 1.3 This isolated breeding farm benefits from several fundamental disease prevention and control principles. It is isolated from other poultry farms, is surrounded by forest land, and is divided into quarantinable sections separated by woods as well as distance.

and unauthorized people. There should preferably be only one access point into the site. This entrance should be protected by gates which should be locked at all times and access control must be exercised by site personnel to limit vehicle, equipment, and people movement.

Only poultry considered to be part of the site flock must be allowed on site and they must be confined to the house or free-range enclosure. No domestic or wild animals must be allowed within the perimeter fence and wild birds must be actively discouraged from the site through the control of any activity that may attract (feed spillage) or harbor (nesting) these birds.

People. Farm/site personnel should ideally be the only people permitted on site and even they must not have had contact with other avian species for at least two days prior to entering the farm. Only essential visits by authorized personnel such as mechanics, managers, working crews, and so on should be allowed on site. They must not have had contact with other non-company poultry or domestic birds for at least two days and must observe the prevailing biosecurity procedures for house access if entering a poultry house.

All non-essential visits by company employees and all non-company personnel visits must be authorized by the relevant authority (live production manager or veterinarian). No such visitor must have had contact with other poultry or domestic birds for at least two days.

The visitation sequence to sites should always be from youngest flock age to oldest flock age. In the event of a disease outbreak, the disease control should always supersede the age sequence, that is, affected flocks must always be visited last, even after visiting an older healthy flock.

A visitor and vehicle register must be maintained to record all visitors (defined as a person not working on site on a daily basis) and all vehicle movements onto and off the site. Such records should include the reason for and the duration of the visit.

In situations where a "shower-in, shower-out" policy is in place, the shower unit is the crucial point separating the site from the outside environment. The shower complex must therefore be unidirectional with the shower unit in-line. All transit or personal clothing and personal items must be stored on the external side of the shower. Any item not suited to washing must not be taken onto the site unless they can be fumigated or suitably decontaminated. Anyone or anything entering the shower unit must be thoroughly cleansed prior to exiting onto the site side of the unit.

After showering, or if there is no shower-in policy, any person entering the farm/site must don site-dedicated protective wear: coveralls, hairnets, and protective footwear or plastic shoe covers. Hands should be cleaned with running water and soap prior to entering and on leaving the site.

The purpose of protective clothing is to provide site personnel with a standard uniform that has not had outside contact or contamination and therefore poses no disease risk to the poultry. The protective clothing colors can also be used to distinguish between departments and the various biosecurity zones.

The office should be a separate room and must only be accessible from the site side. Nothing should enter this room until it has passed through the designated cleaning and disinfection procedures.

Specialist crews and people performing specialist tasks are frequently called upon to visit more than one site per day and sometimes not in the prescribed visitation

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sequence. Such crews, and their equipment, pose a serious disease risk to the site so they need to be particularly vigilant with regards following biosecurity protocols.

Vehicle Access. To reduce the risk of disease agent transmission it is best to prohibit vehicle access to the site. Unfortunately, it is often necessary for vehicles to drive onto site, for example to deliver feed, propane, or egg collection supplies (trays, racks, boxes) and to pick up eggs. If possible, all vehicles entering the site should be suitably disinfected prior to entry. This means that the vehicle must pass through a full spray bay, which has the capacity to deliver a coarse to fine spray of disinfectant over the entire vehicle to ensure total wetting of the exterior. The disinfectant used must be applied at the recommended dosage rate and should not be unduly corrosive or damage the painted surfaces of vehicles. A vehicle wheel dip should be built into the spray bay to ensure that all vehicles entering and leaving the site at any stage of the production cycle pass through this dip. Any vehicle that carries live birds, non-wettable exposed cargo (shavings), or with no roof or side protection for the driver (tractor), must have a full undercarriage spray. The spray bay must be designed to spray the entire vehicle including the undercarriage. Vehicle drivers must not leave their vehicles whilst on site unless the cab has been suitably disinfected on entering the site and the driver has gone through the correct access control procedures applicable to personnel. Site dedicated vehicles must not leave their area of dedication except for repairs, servicing, and fueling. On return, site dedicated vehicles must be completely disinfected at the point of reentry.

Equipment. All equipment entering a site should be suitably decontaminated by a detergent wash, disinfectant spray, and/or fumigation. Some equipment does not lend itself to fumigation or wetting and such items (cell phones, beepers, vaccine syringes, pens, etc.) must be suitably cleaned at point of dispatch to remove gross contamination, or stored in a sealed plastic bag, or the exposed surfaces may be wiped with a moist disinfectant cloth.

All site equipment must be sanitized during the clean-out process. Site equipment must be dedicated to a site, or have at least a 14-day outside storage period to reduce the risk of disease spread. House equipment such as chick fonts, feeder or scratch pans, crates, plastic sheeting, partitions, nest boxes, and so on should not leave a site to be used on another site.

Placement Transfers and Depletion

All placements, transfers, and depletions must be synchronized to ensure that sites are placed in a suitable sequence within complexes and operations. All placements and transfers require that the live birds are kept for some time within the company's transport equipment and therefore, all vehicles and equipment must be cleaned and disinfected between loads. This should be done at the point of origin for placements and transfers and at the point of delivery and again at the complex/site entrance for depletions.

Egg Room

The egg room is a holding room for eggs prior to dispatch to the hatchery. The eggs originate from the houses on site (dirty area) and eggs should preferably be fumigated prior to entering the egg room (clean area). Although the egg room is part of the site, no eggs or buggies placed into an egg room should be taken back on site. The external door is the physical demarcation of the site side of the egg room and must only be opened for the purpose of removing filled egg buggies. It is preferable to wheel the egg buggies leaving the egg room through a wheel-dip containing a suitable disinfectant to reduce the chance of spreading a disease agent off site.

The egg truck, egg buggies, and egg trays form an important epidemiological link between all the company farms (broilers and breeders) via the hatchery. It is thus essential to implement and enforce strict controls at this interface. All buggies and egg trays coming from the hatchery must enter the site through the fumigation room to ensure decontamination. The egg room must be cleaned and disinfected at least once a day, preferably straight after eggs are dispatched to the hatchery, that is, when the room is empty.

Fumigation Room

Fumigation is the process of decontamination of an object through the use of a gas compound. Since gases can penetrate tiny holes, this form of disinfection is ideal for most objects that are otherwise difficult to clean. The fumigation room must have two accesses – one on the site side and the other to the outside. The external access must be used for loading all objects that need to be taken onto site. The site access must be used for receiving fumigated goods onto site and for dispatching potentially contaminated goods from site. Only one access must be open at any given time. Nothing should be allowed to be taken onto site unless it has been showered (soap wash), disinfected, or fumigated. Certain exceptions do however exist and include live birds and non-wettable cargo such as shavings and feed.

Dead-Bird Disposal

All dead birds should be taken to a designated collection point on the farm/site and: (1) stored in suitable containers in a cool environment (shade or refrigerator) so as to delay the rate of decomposition, avoid ground contamination through leakage and spillage and prevent predation,

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(2) mortalities must be disposed of on a daily basis, either on site through incineration, pickling, pit, or tank (Figure 1.4A), composting (Figure 1.4B) or burial, or off site through burial, composting, central depots, or rendering plants (61), and (3) mortality collection vehicles must not enter any site and must always follow strict visitation sequences (young to old and healthy to diseased) and disinfection procedures.

On-site disposal is generally preferable due to the hazards associated with entry of disposal vehicles to the site and to other farms associated with transport of the carcasses. Transport vehicles should be completely sealed to prevent leakage of liquids and to exclude insects, rodents, and scavengers. On-site disposal areas must also be secure from insects, vermin, and scavengers.

Tertiary Control Zone: Complex

An epidemiological unit may also refer to groups of birds that share a communal animal handling facility. The sites/farms within a complex will for example share a hatchery, feed mill, and processing plant and thus form an epidemiological unit. Similarly, production processes within the complex such as pullet rearing farms, breeder or laying farms, and broiler farms also form separate epidemiological units. Depending on the level of biosecurity these areas can be demarcated and classified as tertiary control zones.

Tertiary control zones are frequently set up around high-value sectors of the operation because resource allocation to biosecurity is easier to justify. Grandparent stock are for example substantially more valuable than broiler breeders which are, in turn, more valuable than broilers so the implementation of tertiary control zones becomes easier to justify as one moves up the production pyramid. *Tertiary critical control points* (transit facilities) may be established beyond the outer confines of the site perimeter to reduce the risk of disease agent transmission. Control procedures such as showering or merely changing into protective clothing at this point and using site/zone dedicated transport to move to the site significantly reduce the chance of disease transmission. The tertiary control zone is seldom fenced so the access control boundary is usually imaginary.

Complex Environment

In rare situations a group of sites within a geographical location can be protected as a single unit by the erection of an enclosure (perimeter fence) with access control (transit facility). In other situations the "boundary" may

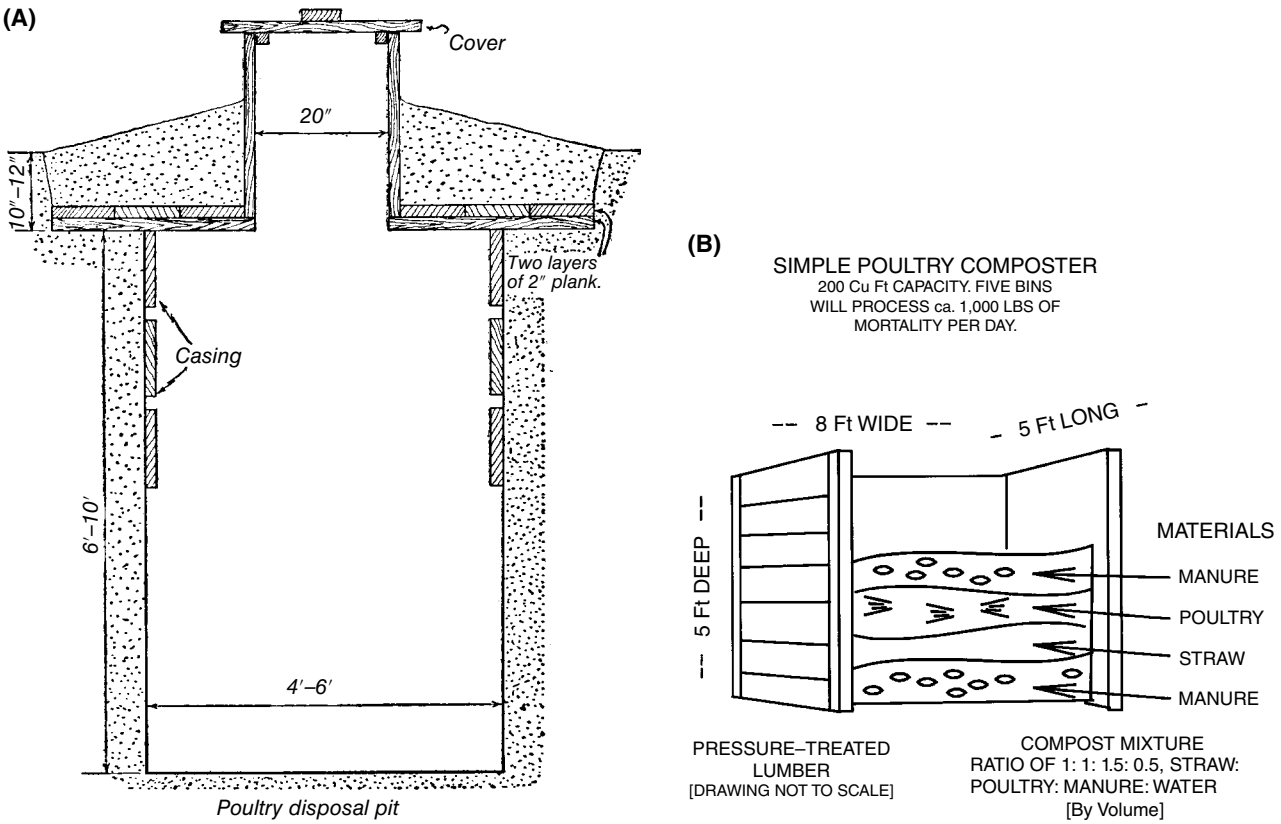


Figure 1.4 (A) Poultry disposal pit. Such a pit can be made any size that is convenient. (B) A simple above-ground poultry carcass composter of 200 ft^3 (5.7 m^3) capacity. Five such bins will process 1000 lb (455 kg) of carcasses per day. (Courtesy Poultry Science Dept., University of Maryland).

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be "operational" in nature. Such a complex does not merely exist because two or more sites are on the same location but rather as a means of implementing bioexclusion procedures. The entrance to such a complex is referred to as a transit facility, and the complex, a disease free area or control zone. A complex must always be considered to be closed in terms of the enforcement of biosecurity control measures and dedicated vehicle transport used within this control zone.

Although a complex can be a large expanse of land, the same principles to housekeeping on the site are applicable. The land around each site must be maintained so as to minimize breeding sites and overt protection given to vermin, predators, and other organisms. The grass outside the site perimeter fence must be kept short and free of any rubble and debris.

Complex Access: Transit Facility

The transit facility is the entrance to the complex and serves as a biosecurity critical control point to reduce the risk of disease. Site and complex personnel should ideally be the only people within the complex. Only essential visits by authorized personnel such as mechanics, direct managers, working crews, and so on should be allowed onto a complex. All non-essential visits by company employees must be authorized by the veterinarian. All non-company visits must be authorized by the relevant authority. In the event of a disease outbreak, the veterinarian is responsible for the imposition of additional control measures appropriate to the disease.

Access to the Complex

Procedures for people, vehicle, and equipment access to a complex are the same as those for a farm. Anybody intending to visit any part of the tertiary control zone (complex) must comply with transit facility controls. This should involve a clear separation between clean and dirty areas/items. Anybody or anything entering the complex should ideally be "decontaminated" by washing with soap and water. People entering the complex should at the very least leave all personal clothing and personal items in the transit facility and change into complex clothing. Complex dedicated vehicles should be used to move between the transit facility and the farms/sites and a visitor and vehicle register similar to those at farm level must be maintained.

Diagnosis: Monitoring, Surveillance, and Confirmation

Judicious use of cost items like antibiotics and non-antibiotic feed additives makes both scientific and economic sense and begins with *accurate* and *early* diagnosis.

Monitoring and Surveillance

Monitoring and surveillance are both terms used to describe the ongoing collection of data to describe the prevalence and severity of disease in a population. A *monitoring* program is usually designed to accumulate statistically reliable disease prevalence data over time, to indicate a change in the incidence or severity of a disease. A *surveillance* program is in contrast usually designed to collect prevalence data from a readily available sector of the population (potential sample bias) with the *primary purpose of implementing timely corrective action* when there is a perceived increase in incidence of a disease. As flock size and production intensity increases, management control becomes more remote, so monitoring and surveillance programs become more important.

With eradication programs implemented to control diseases of catastrophic nature, the objective of the surveillance program should be to detect the *source case* of an outbreak so that biocontainment through quarantine and slaughter can be initiated before the disease spreads. If the goal is less than eradication the degree of deviation from normal prevalence necessary to stimulate corrective action needs to be set at such a level so as to differentiate *common cause* (background variation) from *special cause* (a disease effect).

Several parameters such as the sample size necessary to detect specific levels of prevalence can be calculated by equation and it is important to realize the significance of this in program design.

For disease eradication and trade purposes it is often necessary to demonstrate freedom from infection (absence of the pathogenic agent) in the country, zone, or compartment (company). It is not possible to prove with 100% confidence that a population is free from infection (unless every member of the population is examined simultaneously with a perfect test with 100% sensitivity and specificity). So a surveillance system to demonstrate freedom from infection should be designed to predict with an acceptable level of confidence that infection is below a specified level of prevalence in the target population. Any evidence of infection at any level in the target population does however automatically invalidate any freedom from infection claim.

For disease control purposes surveillance is used to determine the distribution and occurrence of infection or immunity within a zone or compartment. In this instance surveillance is designed to collect data on several variables relevant to flock health, including prevalence or incidence of infection, morbidity and mortality rates, flock immunity as indicated by frequency distribution of antibody titres, farm production records, and so on.

Poultry flock health tracking requires that flocks are monitored for disease at regular intervals. A change in prevalence over time indicates a change in incidence

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which signals the need for corrective action to prevent disease spread. Unless monitoring includes true random sampling, results cannot be taken to be absolute measures of disease incidence and prevalence, but may serve as adequate indicators for intervention.

The following formula provides a simplified method of estimating the number of animals that need to be tested for the probability of selecting at least one diseased animal, in a finite population of birds to be greater than a predetermined confidence level (commonly 95%).

$$n = \left[1 - (1-p)^{1/d}\right] \times \left[N - d/2\right] + 1$$

Where: n = sample size, N = flock size, p = probability of selecting at least one diseased animal, and d = the number of animals affected for the desired level of prevalence.

Although this method of sample size determination is widely used, its accuracy is based on several assumptions. Violation of the assumptions (that the disease is present at a certain minimum prevalence, the diagnostic test used is 100% sensitive and 100% specific, sampling is performed with replacement, and the data is collected by simple random sampling) renders the estimate inaccurate (21, 23). A more accurate determination of sample size is given with a computer program like "FreeCalc". This program uses trial and error to calculate the exact sample size required for a specified probability, can be used on finite populations and takes account of test imperfections (21).

Sample frequency must be calculated based on the epidemiology of the disease under consideration. With *M. gallisepticum* for example the index case could produce infected eggs within 17 days but peak shedding occurs when colonization peaks at 3–6 weeks after flock exposure (39, 40, 66). After flock exposure to *M. gallisepticum* there is a latent phase of 12–21 days in which less than 5% of the flock has a detectable antibody response (57). To prevent vertical transmission the monitoring system must be capable of detecting infection at the 5% level with 99% confidence. The sample size (n) that must be tested to have 99% confidence in determining whether MG is present at a prevalence of 5% in a flock of 7,000 birds can be estimated by calculation as 90 birds. To prevent infected eggs from entering the hatchery it would be necessary to sample flocks every two weeks (assuming 100% sensitivity for the test system). The testing interval can be extended by two weeks where hatchery tracking systems allow infected egg removal from the setters.

Performance Parameters

Metrics generally used to judge overall health, which encompasses vaccine program efficacy, are percent hatch, culls at the hatchery, 7-day mortality, 14-day mortality, final flock livability, feed-conversion efficiency, rate of gain, condemnation, egg production, and egg quality. Many of these metrics have standards or comparative histories established through each company's own historical data or, in the United States at least, national reporting services such as AgriStats (AgriStats, Fort Wayne, IN), and government reporting services such as the poultry slaughter reports published monthly by the National Agricultural Statistics Service (NASS), Agricultural Statistics Board, US Department of Agriculture. An additional metric that can be used over time is antimicrobial and antiparasitic drug usage. Although this is influenced by many things, including management changes and climatic shifts, monitoring usage is an essential for evaluating overall health and vaccination program efficacy.

Examination of Field Birds

Health surveys (11, 47) that include extensive gross and microscopic evaluation of necropsy specimens, and controlled challenge studies (59) to measure a relative protection level, are both useful in assessing vaccine program effectiveness. Perhaps the most frequent controlled challenge work done is measurement of passive protection of broiler chicks from hens hyperimmunized to infectious bursal disease (59). Trends in program efficiency may be identified over time if sufficient groups of chicks are sampled.

Serologic Monitoring

Serologic monitoring (71) is only useful in production medicine if adequate samples have been analyzed over time in order to establish a normal baseline for a specific program, in a specific location, in a specific bird, using specific and consistent application techniques, with samples run consistently by a specific laboratory. After a baseline is established, flocks can be identified that have serologic profiles above or below the established baseline.

In broiler and turkey production flocks, an effective monitoring program can be the regular sampling and testing of blood as they are slaughtered at the processing plant. This serologic monitoring will establish a baseline of antibody titers that are the result of both vaccination and field challenge. Changes in the usually observed antibody titers may indicate a decrease in the efficacy of vaccine administration or an increased field challenge by a particular pathogen. A regular serologic monitoring program is also helpful to determine whether a flock has been exposed to a new pathogen, not previously present in the region.

Serologic monitoring of layer flocks should be performed before the flock is placed in the layer building,

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with periodic serologic monitoring throughout the production cycle. This type of program will assess both the efficacy of vaccine administration and the disease challenge the flock experiences in the field. Breeder flocks should be monitored in the same way as layer flocks and, in certain instances, breeders can be revaccinated during production to boost the maternal antibody titers of their progeny if they are found to be low.

Interpretation of Serologic Data

It is usually impossible to differentiate between antibodies that are produced by vaccination versus those induced by field exposure to a given infectious agent. The only difference that may be observed is that the antibody titer following a field challenge may be higher than that observed following vaccination. A valid interpretation of serologic results requires a complete knowledge of the flock's vaccination history.

It usually takes poultry 1–3 weeks to produce detectable levels of antibodies in their serum. It is possible, therefore, to collect blood during the middle of a disease outbreak and not be able to detect any antibodies to the causative disease agent. If this same flock is tested 2 weeks later, however, serum-antibody levels will be high. A useful practice in establishing a disease diagnosis is to take acute and convalescent serum samples from the flock as it is undergoing an unknown disease challenge. Typically, the acute serum sample collected during the initial phase of the disease outbreak will be negative for antibodies to the suspected disease agent. The convalescent serum sample, taken shortly after the flock has recovered, if positive, will provide a definitive diagnosis when interpreted in conjunction with the clinical signs and lesions of the case. An important concept in the interpretation of serologic results is that a single positive serologic test only indicates that the flock was exposed to that disease agent during its life.

Different laboratories often conduct serologic tests using different reagents or techniques. Because of this, comparing antibody titers (a titer is a measure of the level or concentration of antibody in the serum) reported from different laboratories may be confusing. It is best to use one laboratory for a given test so that a familiar range for negative, low, or high titers is established. With experience and training, production managers can become skilled at the interpretation of serologic results.

Flock Profiling

Today's disease problems often represent the sum of various subclinical disorders occurring at different times throughout the life of a flock. Acquisition of the fullest understanding of this sequential collection of serologic and other data concerning multiple pathogens requires disciplined and careful organization. The systematic, graphic presentation of this data is commonly called a "flock profile." The establishment of such profiles is facilitated by enzyme-linked immunosorbent assay (ELISA) technology, because a single basic test system is used to monitor for a broad array of diseases.

There is value in correlating ELISA profiling data with flock performance (71), and with gross and microscopic pathology data (53). Baseline profiles can be established both as targets for vaccination goals and as a base from which deviations from the norm may be demonstrated when a field problem is subsequently encountered. Several flock-profiling kits and systems are now commercially available. Their value is enhanced when good data retrieval and graphic presentation of data (Figure 1.5) is combined with the diagnostician's veterinary skills and experience in assimilating medical information and establishing a plausible diagnosis.

Diagnostic Procedures

Many satisfactory diagnostic and necropsy methods exist. The goal of the necropsy is to determine the cause of impaired performance, signs, or mortality by examining tissues and organs, and to obtain the best specimens possible to carry out microbiologic, serologic, histopathologic, or animal inoculation tests. It is important that in the process, infectious materials do not endanger the health of humans, livestock, or other poultry. By proceeding in an orderly fashion, possible clues are less apt to be overlooked, and tissues will not be grossly contaminated prior to examination. Remember that a blood sample or tissue specimen determined later to be superfluous can always be discarded.

A key to good poultry diagnosis is the art of "seeing the forest as well as the trees." Try to identify the most significant flock problem(s), rather than becoming engrossed in individual bird disorders. The techniques and procedures necessary to make an accurate diagnosis and identify specific disease agents are found in succeeding chapters of this book and in reference manuals such as *A Laboratory Manual for Isolation Identification and Characterization of Avian Pathogens* (80), *Avian Disease Manual* (18), *Avian Histopathology* (1), and *Color Atlas of Diseases and Disorders of the Domestic Fowl and Turkey* (62). *Avian Hematology and Cytology* (22) should be consulted for detailed information on avian blood elements and methods for preparation and study. New information is continually being presented in journals such as *Avian Diseases*, *Avian Pathology*, and *Poultry Science*, in the proceedings of several regional poultry disease conferences, and in other avian pathology and science journals.

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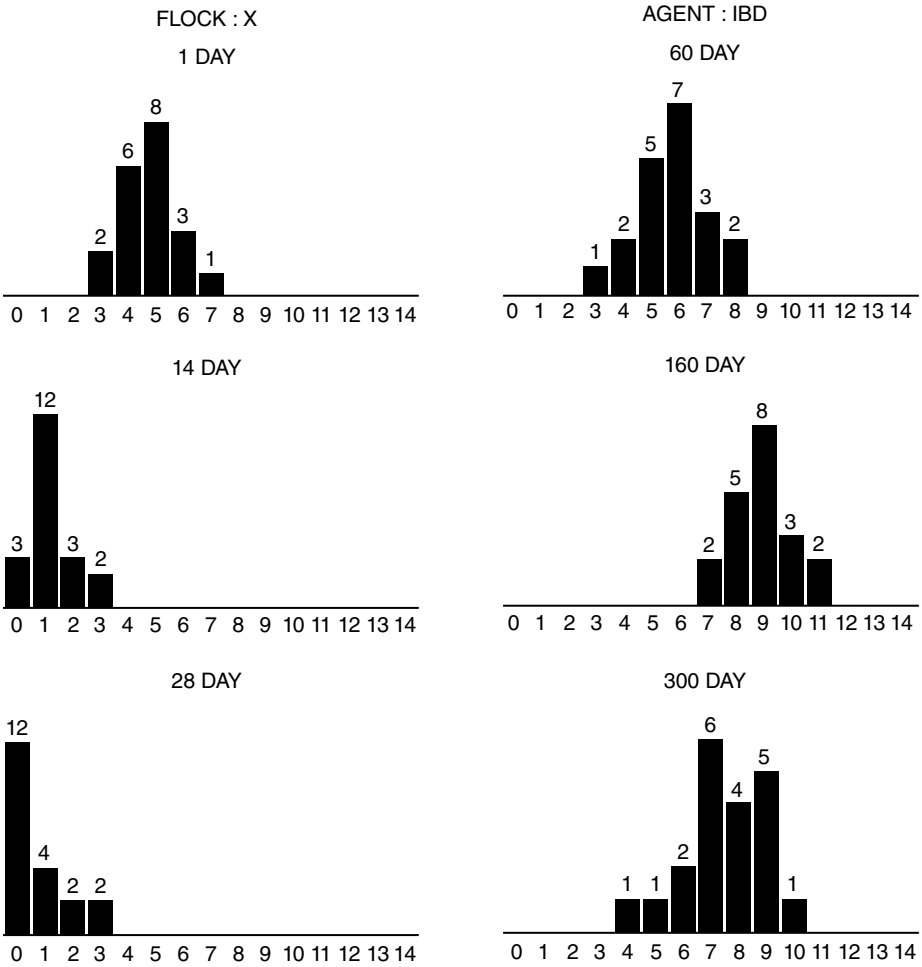


Figure 1.5 Temporal graphic distribution of infectious bursal disease (IBD) enzyme-linked immunosorbent assay (ELISA) group titer levels at 1, 14, 28, 60, 160, and 300 days of age for an IBD-vaccinated broiler breeder flock. Numbers on the X-axis represent group titer levels obtained by ELISA. Titers of 0 are group 0; 1–350 are group 1; 351–1,500 are group 2; 1,501–2,500 are group 3; 2,501–3,550 are group 4; etc., with titers of 12,500 comprising group 14. Numbers above each bar represent the number of samples reacting at each level on the indicated day of age.

Case History

The pathologist who has not seen the farm or the flock before attempting to diagnose the problem and recommend corrective measures is at a disadvantage. This can be partially overcome by getting a complete history of the disease and all pertinent events leading to the outbreak. Knowledge of management factors such as ventilation; feeding and watering systems; accurate records of egg production, feed consumption, feed formulation, and body weight; lighting program; beak trimming practices; brooding and rearing procedures; routine medication and vaccination used; age; previous history of disease; farm location; and unusual weather or farm events may make the difference between diagnosis of the flock problem and the finding of a few miscellaneous conditions in a sample that may or may not be representative. Duration of the signs, the number of sick and dead, and when and where they were found dead can be important clues.

External Examination

Look for external parasites. Lice and northern fowl mites (*Ornithonyssus silviarum*) can be found on the affected chicken. If red mites (*Dermanyssus gallinae*) or blue bugs (*Argas persicus*) are suspected, examination of roosting areas and cracks and crevices in the houses and around the yards must be made, because these species do not stay on birds. See Chapter 26 for diagnosis and identification of external parasites.

The general attitude of live birds and all abnormal conditions should be noted carefully. It is very important to observe evidence of incoordination, tremors, paralytic conditions, abnormal gait and leg weakness, depression,

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blindness, and respiratory signs before the specimens are killed. It is very helpful to place birds in a cage where they can be observed after they have become accustomed to the surroundings and perform at their best. It is sometimes advisable to save some of the affected birds to observe possible recovery from a transitory condition (transient paralysis), respiratory infection, chemical toxicity, feed or water deprivation on the farm, or overheating during transport to the laboratory.

Examination should be made for tumors, abscesses, skin changes, beak condition, evidence of cannibalism, injuries, diarrhea, nasal and respiratory discharges, conjunctival exudates, feather and comb conditions, dehydration, and body condition. These are all useful clues.

Blood Samples

Blood specimens may be taken at this time or immediately after the bird is euthanized. Venipuncture of the brachial vein is usually the simplest and best method for obtaining blood from turkeys, chickens, and most fowl under field conditions, especially when the bird is to be returned to the flock. Ducks are bled from the saphenous vein near the hock. Expose the vein to view by plucking a few feathers from the ventral surface of the humeral region of the wing. The vein will be seen lying in the depression between the biceps brachialis and triceps humeralis muscles. It is more easily seen if the skin is first dampened with 70% alcohol or other colorless disinfectant. To facilitate venipuncture, extend both wings dorsally by gripping them firmly together in the area of the wing web with the left hand. Insert the needle into the vein of the right wing holding the syringe in the right hand (Figure 1.6). The needle should be inserted opposite to the direction of blood flow. For quick and accurate bleeding, it is essential that the needle be sharp. A very slight vacuum should be developed intermittently to determine when vein or heart puncture has occurred. After vein puncture, a steady slight vacuum should be continuous to withdraw blood. If the vacuum is too great, the vessel wall may be drawn into the needle and plug the beveled opening. It is sometimes necessary to rotate the needle and syringe to be sure the beveled opening is free in the lumen of the vessel.

For most serologic studies, the serum from 2 mL blood is adequate. The blood should be removed aseptically and placed in a clean vial, which then is laid horizontally, or nearly so, until the blood clots. An occasional sample may require a long time to clot. This is especially true of turkey blood. Clotting can be hastened by adding a drop of tissue extract, made by killing and pooling a number of 10–12-day-old chicken embryos, grinding in a blender, and freezing for future use. After the clot is firm, the vial may be returned to the vertical position to permit serum to collect in a pool at the bottom. Plastic vials are also



Figure 1.6 Obtaining a blood sample from the wing vein.

available for blood collection. The clot does not adhere to the vial, and special positioning during clotting is unnecessary. Frequently, the serum from fat hens will appear milky due to lipids. Placing vials in an incubator will hasten the separation of the blood clot and serum. A fresh blood sample should never be refrigerated immediately after collection, as this will hinder the clotting process. Sera should not be frozen if agglutination tests are to be performed as this frequently causes false-positive reactions.

If an unclotted blood sample is required, it should be drawn into sodium citrate solution at the rate of 1.5 mL 2% solution/10 mL fresh blood, or deposited in a vial containing sodium citrate powder at the rate of 3 mg/1 mL whole blood, and the mixture should be gently shaken. One way to prepare tubes for collecting sterile citrated blood is to add the proper amount of 2% sodium citrate solution to the collecting tubes ahead of time and then sterilize the solution and evaporate the moisture in an oven.

Blood-collecting vials containing the anticoagulants heparin or EDTA can also be obtained commercially from laboratory supply companies. For certain types of serologic tests, fresh blood can be absorbed on the tips of filter paper strips, dried, and sent to the diagnostic laboratory, where antibodies can be recovered for testing by placing pieces of the treated paper into saline solution.

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If a blood parasite or blood dyscrasia is suspected, smears of whole blood should be made on clean glass slides previously warmed to promote rapid drying. For staining techniques, see Campbell (22). A drop of blood for a wet mount or smear may be obtained from very small chicks by pricking the vein on the posteromedial side of the leg or by pricking or cutting the immature comb.

Killing Birds for Necropsy

Several methods can be used to kill fowl, and each has certain advantages. The objective is to kill the bird instantaneously so it will not suffer in the process. Cervical dislocation and decapitation are considered humane methods of poultry euthanasia by the American Veterinary Medical Association (AVMA) (4). For large breeders and turkeys in which cervical disarticulation is not feasible, a purpose-designed captive bolt gun may be used. Specimens selected for diagnosis may also be killed by intravenous injection of euthanasia solutions or by placing the bird in a chamber filled with carbon dioxide (CO_2) or a mixture of 30% CO_2 and either nitrogen or argon. Local availability of a source of these gasses may limit utilization of this technique. Other methods of euthanasia can be found in a report of the AVMA (4). The method selected will depend upon the existing situation: species, size, and number of birds to be necropsied or sacrificed; tissues, fluids, and cultures to be taken; and so on.

Necropsy Precautions

If there is reason to suspect that birds to be necropsied are infected with disease that may be contagious for humans (chlamydiosis, erysipelas, or equine encephalitis), stringent health precautions are essential. The carcass and the necropsy table surface should be wet thoroughly with a disinfectant. Good rubber gloves should be worn and care should be taken that neither the pathologist nor assistants puncture the skin of their hands or inhale dust or aerosols from tissues or feces. It is advisable to wear safety glasses and a fine-particle respiratory mask to prevent inhalation of contaminated dust. All laboratory personnel who may come in contact with carcasses, tissues, or cultures should be informed of their possible infectious nature and precautions to be taken.

With some notable exceptions (see sections on the specific diseases), most commonly encountered poultry disease agents are not considered pathogenic for humans. Nevertheless, it is wise to wear rubber gloves at all times while performing necropsies. For a review of poultry diseases in public health, see Galton and Arnstein (35). Adequate instruments for routine work are necropsy shears to cut bones, enterotome scissors to incise the gut, a necropsy knife to cut skin and muscle, and a scalpel for fine examination of tissues. These should be supplemented with forceps, sterile syringes, needles, vials, and petri dishes for collecting blood samples and tissue specimens as the situation dictates.

Necropsy Technique

Internal Organs

The specimen is laid on its back and each leg in turn drawn outward away from the body while the skin is incised between the leg and abdomen. Each leg is then grasped firmly in the area of the femur and bent forward, downward, and outward until the head of the femur is broken free of the acetabular attachment so that the leg will lie flat on the table (Figure 1.7A).

The skin is cut between the two previous incisions at a point midway between keel and vent. The cut edge is then forcibly reflected forward, cutting as necessary, until the entire ventral aspect of the body, including the neck, is exposed (Figure 1.7B). Hemorrhages of the musculature, if present, can be detected at this stage.

The poultry shears are used to cut through the abdominal wall transversely midway between keel and vent and then through breast muscles on each side (Figure 1.7C). Bone shears are used to cut the rib cage and then the coracoid and clavicle on both sides (Figure 1.7D). With some care, this can be done without severing the large blood vessels. The process may also be done equally well in reverse order, cutting through the clavicle and coracoid and then through the rib cage and abdominal wall on each side. The sternum and attached structures can now be removed from the body and laid aside. The organs are now in full view and may be removed as they are examined (Figure 1.7E, F). If a blood sample has not previously been taken and the bird was killed just prior to necropsy, a sample can be promptly taken by heart puncture before clotting occurs. Large veins leading into the leg may be incised, allowing blood to pool in the inguinal region for subsequent collection.

Laboratory Procedures

Bacterial Cultures

If gross lesions indicate bacterial cultures are needed, they can be made from uncontaminated surfaces of the viscera without searing the surface. If contamination has occurred, the surface of the organs should be seared with a hot spatula or other iron designed for that purpose before inserting a sterile culture loop. Care must be taken not to sear and heat the tissue excessively. It is often desirable to transfer large tissue samples aseptically to a sterile petri dish and take them to the microbiology laboratory for initial culture in cleaner surroundings.

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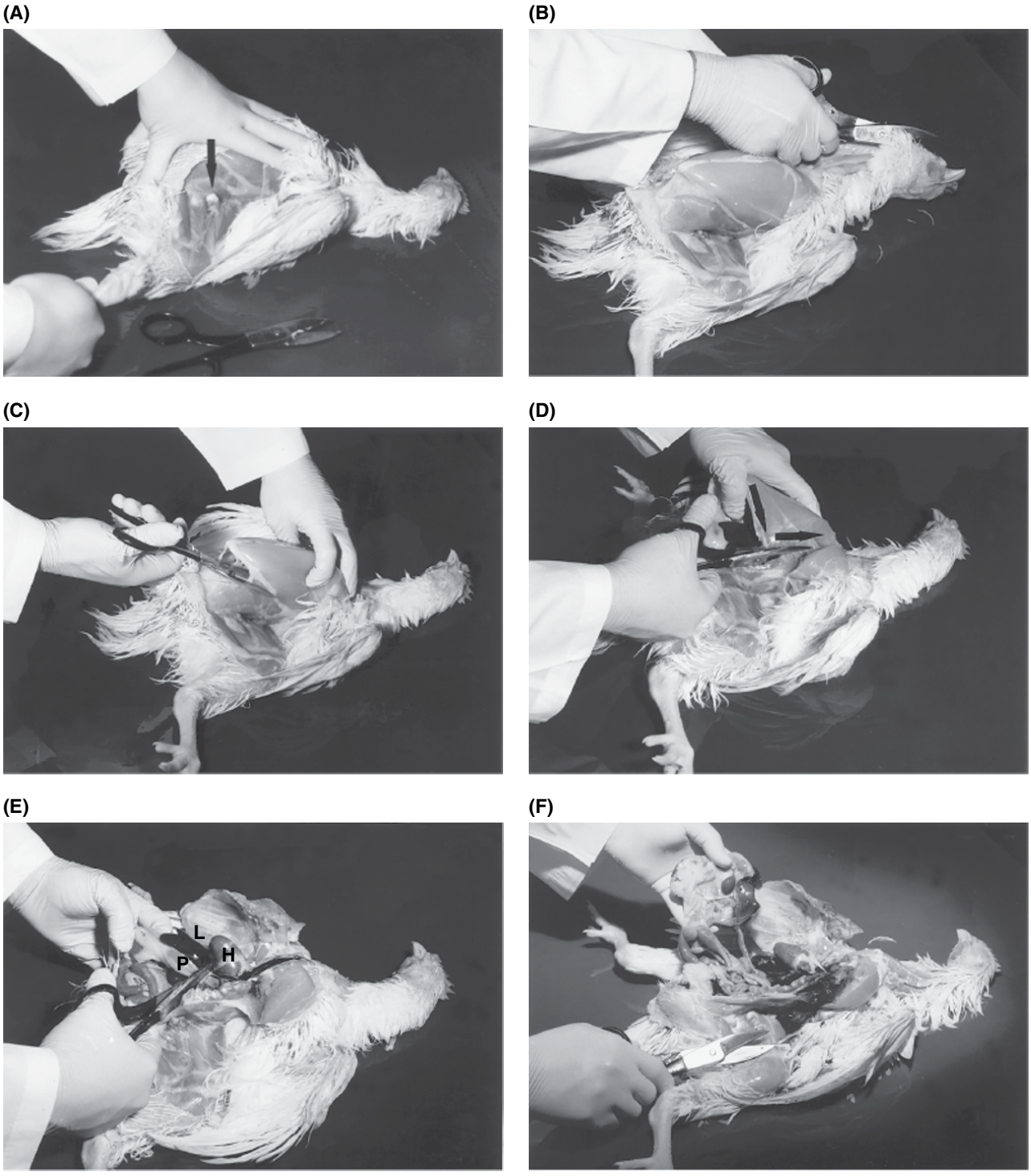


Figure 1.7 Each pathologist will develop their own systematic technique for conducting a necropsy. A sturdy pair of poultry shears is usually sufficient to conduct a necropsy. Other instruments such as scissors, forceps, and scalpel may be helpful in collecting small or delicate samples. A knife may be needed to cut through joints and bone. The illustrated technique will aid the beginner. (A) The skin and fascia between the leg and abdomen are cut, and the legs are pulled and twisted to disarticulate the head of the femur (arrow) from the hip. (B) The skin from the vent to the beak is incised and reflected. (C) The body cavity is entered at the ventral tip of the sternum. The incision is made at the margin of the pectoral muscle and continues through 2–3 ribs. A similar incision is made on the opposite side of the breast. (D) The shears are reoriented (arrows), and the incision is continued through bone and muscle to the thoracic inlet. The breast is broken over to the opposite side (or removed) exposing the viscera. At this point of the necropsy, microbiological samples are collected. (E) The intestinal viscera are freed by cutting through the esophagus and vessels of the liver just anterior to the proventriculus and liver. Heart (H), liver (L), and proventriculus (P) are indicated. (F) The intestines can be removed by gentle traction, which tears mesenteric and air sac attachments. The lungs, heart, and kidneys remain in the body cavity for later examination.

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Respiratory Virus Isolation

If a respiratory disease is suspected and virus culture or bird passage is desirable, an intact section of lower trachea, the bronchi, and upper portions of the lungs is removed aseptically with sterile scissors and forceps and transferred to a sterile container. Other tissues (air sac tissue) can be added aseptically to the sample or transferred to other sterile containers for separate study. The trachea can now be incised. If exudate is present, it can be added to the preceding collection or saved in separate vials. Similar procedures can be followed for initial virus isolation from various parenchymatous organs.

Salmonella Cultures

All other visceral organs should be examined for abnormalities (microabscesses, discoloration, swelling, and friability). If abnormalities are observed, inoculum from the affected tissues should be transferred to suitable solid or liquid media for culture before the intestinal tract is opened. Once opened, gross contamination of other organs with gut contents is almost certain to occur. If *Salmonella* infection is suspected, selected sections of the gut are removed with sterile forceps and scissors and placed directly into a sterile petri dish for later culture. For routine examination, a single section comprising the lower ileum, proximal portions of the ceca and cecal "tonsils," and proximal portion of the large intestine may be used. All are minced or ground aseptically to produce an inoculum. Additional areas of the intestinal tract or tissues of other visceral organs may be added to the gut collection or cultured separately. Alternatively, sterile swabs may be used to obtain samples from the exposed gut lining for *Salmonella* cultures. See Chapter 2 of *A Laboratory Manual for Isolation and Identification of Avian Pathogens* (80) for detailed culture technique.

Gross Necropsy

After necessary cultures have been collected, a thorough gross examination of all tissues should be performed. Enlargement of the liver, spleen, and kidney should be evaluated. A clear indication of hepatomegaly is rounded liver margins. The intestine may be examined for inflammation, exudates, parasites, foreign bodies, malfunctions, tumors, and abscesses. The various nerves, bone structure, marrow condition, and joints can now be examined. The sciatic nerve can be examined by dissecting away the musculature on the medial side of the thigh. Inside the body cavity, the sciatic plexus is obscured by kidney tissue. These nerves can best be exposed by scraping away the tissue with the blunt end of a scalpel. Nerves of the brachial plexuses are easily found on either side near the thoracic inlet and should be examined for enlargement. Examination of vagus nerves in their entirety should be made, or otherwise short enlargements may be missed.

The ease or difficulty with which bones can be cut with the bone shears is indicative of their condition. The costochondral junctions should be palpated and examined for enlargement ("beading") and the long bones cut longitudinally through the epiphysis to examine for abnormal calcification. Rigidity of the tibiotarsus or metatarsus should be tested by bending and breaking to check for nutritional deficiency. A healthy bone will make an audible snap when it breaks. Bones from a chicken deficient in vitamin D or minerals may be so lacking in mineral elements that they can be bent at any angle without breaking.

Joint exudate, if present, can be sampled after first plucking the feathers and searing the overlying skin with a hot iron. After searing, the skin may be incised with a sterile scalpel and exudate removed with a sterile inoculating loop or swab. Paranasal sinus exudates can be removed and examined in a similar manner.

Exposure and Removal of the Brain

Removing the intact brain is not easy, since meningeal layers are attached firmly to bony structures in some places. The following technique can be performed quickly and is satisfactory for examination and removal of the brain in most instances. Remove the head at the atlantooccipital junction and remove the lower mandible. Sear the cut surface and trim away excess loose tissue. Reflect the skin forward over the skull and upper mandible and hold it firmly in that position with one hand. Sterile instruments should be used for the succeeding steps if a portion of the brain is desired for animal inoculation, virus isolation, or fungal or bacterial culture.

With the sterilized tips of heavy-jawed bone shears or strong surgical scissors, nip just through the bone to the cranial cavity on both sides of the head, beginning at the occipital foramen and proceeding forward laterally to the midpoint at the anterior edge of the cranial cavity (Figure 1.8A). Lift off the cut portion of bone and expose the entire brain (Figure 1.8B).

If a portion is needed for culture or animal inoculation (e.g., avian encephalomyelitis virus suspect) and also one for histopathologic examination (e.g., vitamin E deficiency), cut the brain medially from anterior to posterior along the midline with a sharp, sterile scalpel blade. With sterile, sharp, curved scissors, cut the nerves and attachments carefully from one of the brain halves while the head is tipped upside down, so that the loosened portion falls into a jar of formalin as it is freed (Figure 1.8C). The second half can now be removed aseptically (but without concern for preservation of tissue structure) to a sterile petri dish or sterile mortar and pestle. Be careful not to contaminate brain tissue intended for virus isolation with instruments that have been in contact with formalin. The separate halves may also be removed in reverse order (Figure 1.8D). If all of the brain is required for either purpose, proceed with proper precautions for the

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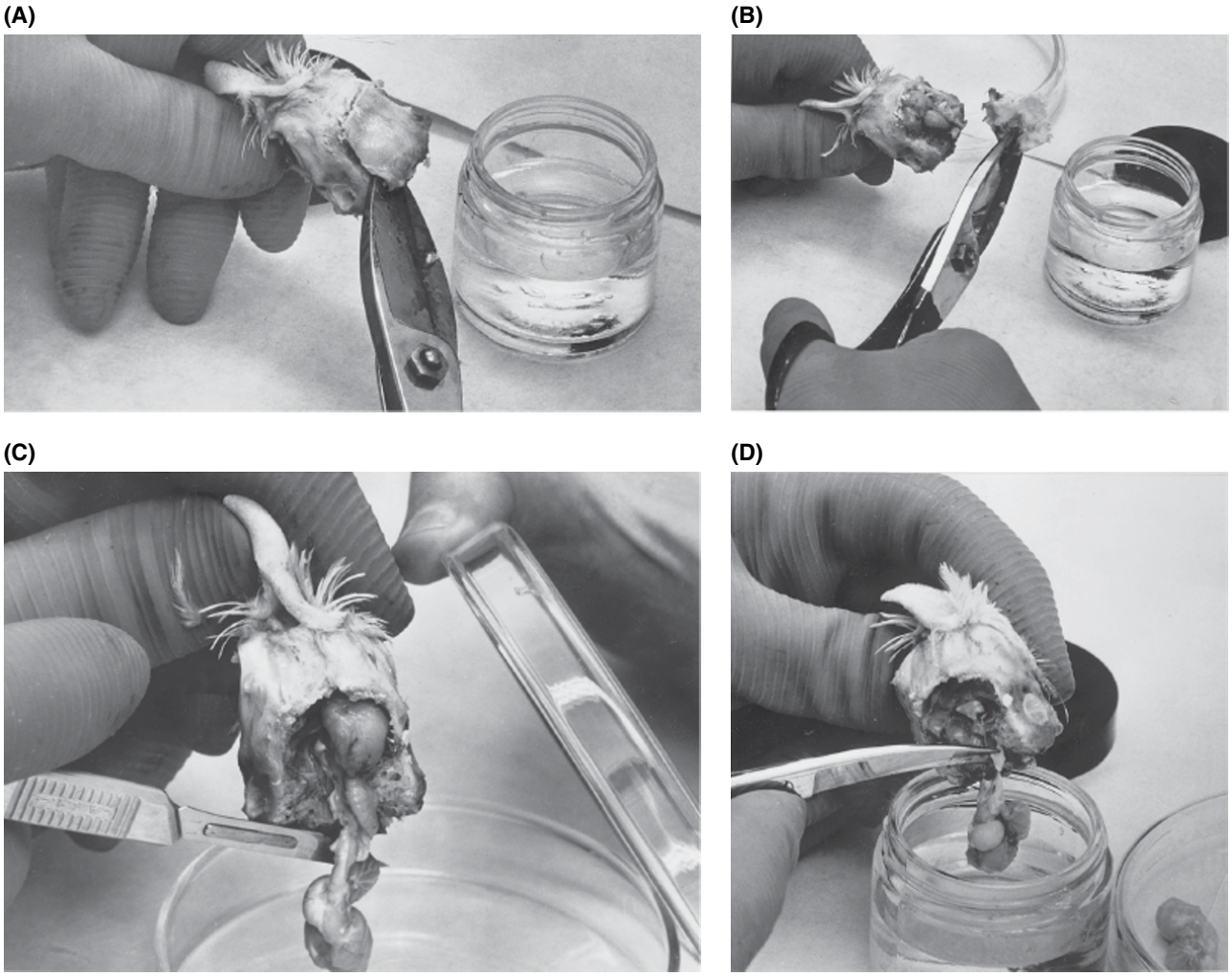


Figure 1.8 With a little practice, the brain can be removed with a minimum of trauma. (A) Incise bone all the way around the periphery of the cranial cavity with heavy bone shears. (B) Remove loosened portion of the bony skull. (C) Incise brain longitudinally with sterile, sharp scalpel and remove one-half for sterile culture technique. (D) Remove second half by dropping it into 10% formalin for histologic techniques.

purpose intended. If the brain is destined only for sectioning, it may be fixed in situ and then removed. Large brain portions should be incised longitudinally to permit good penetration of fixative.

Tissues for Histopathologic Examination

For good preservation, the tissue pieces from killed birds should be saved immediately after death, especially gut, brain, and kidney tissues, which deteriorate rapidly. Specimens should be small to allow quick penetration of fixative, gently incised with a sharp scalpel or razor blade to preserve tissue structure, and preserved in 10 × their own volume of 10% formalin or other fixative. Bone pieces should be sawed with a sharp bone saw unless thin or soft enough to cut with scissors or scalpel.

Lung tissue usually floats on the surface of the fixing solution because of trapped air. Satisfactory fixation can be accomplished by placing absorbent cotton over the tissue, which serves to keep it immersed. Methods to exhaust air from air spaces in lung tissue by creating a vacuum over the fixative can be used but are less satisfactory and may result in artifacts.

If eye tissue is to be saved for sectioning, the whole eye should be removed and all ocular muscles trimmed off the globe to allow for rapid penetration by the fixative.

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Any tissue held too long in formalin fixative becomes excessively hard. If processing is to be delayed, tissues should be transferred to 70% alcohol after 48 hours in fixative. Textbooks on histologic techniques (60, 61, 74) should be consulted for detailed procedures.

Disposing of the Specimen

If a disease infectious for humans is suspected, the carcass should be autoclaved, incinerated, or otherwise rendered incapable of causing infection to laboratory or other personnel. Similar precautions should be followed during disposal of carcasses infected with a virulent poultry pathogen that presents a health hazard to the industry. The necropsy area, instruments, and gloves should then be cleaned, washed, and disinfected.

Communication

Flock owners are not always interested in technical data. They want to know what the problem is and what should be done to correct it and/or how to prevent reoccurrences. Sometimes technical data are necessary to clarify the diagnosis, but the report should be in language and terms that they will understand. A minimum of complicated scientific and medical technology words should be used. When medical terms are apt to be confusing, they should always be explained in lay terms.

The report should include the necropsy findings, results of laboratory studies, (histopathologic, serologic, and cultural), diagnosis (temporary or final), and conclusions and recommendations. The owner is seeking professional advice. The veterinarian should give their best conclusions and recommendations based on the facts available. A verbal report or telephone call to the flock owner, manager, or service worker soon after completion of the necropsy and initial tests is highly advisable. A tentative diagnosis can be offered pending further confirmation.

Disease Control: Biocontainment

Disease control strategies are designed to reduce the consequence of disease challenge by limiting challenge (bioexclusion), enhancing bird resistance (immunization), and preventing spread (quarantine). In the case of eradicable diseases quarantine is usually followed by emergency slaughter. Control measures are implemented routinely for diseases that are endemic to the epidemiological unit and sporadically when there is an unexpected epidemic disease outbreak.

The word quarantine has several different meanings: (1) enforced isolation of animals that may have been exposed to a contagious or infectious disease, e.g., when entering a country, (2) a place in which animals spend a period of isolation to prevent the spread of disease, and (3) the period of time during which animals are kept in isolation to prevent the spread of disease. For live bird and product importation, quarantine is routine. To prevent the introduction of disease into a country, region, zone, or compartment it is essential that potentially infectious material is kept in isolation until they have been shown to be clear of the disease(s) in question.

In a production setting quarantine is the first step of biocontainment and it involves the immediate enforced isolation of birds that have been exposed to a contagious disease. First, the movement of anything into, onto, from, or through the area of control must be restricted and monitored. The extent of the control zone will depend on the risk associated with the disease but usually involves the house, farm, site, or complex within a particular company. If the disease is of national or regional importance the control zone is usually a circle with a ten kilometer radius around the affected farm. It is important to establish the extent of the disease outbreak through disease monitoring, first within the quarantine zone then in a demarcated surrounding contact zone. In the case of foreign/notifiable diseases the relevant veterinary authority assumes control. In the United States each state will have a predetermined emergency response plan carefully designed to handle all the relevant details of containment and eradication.

Chemoprophylaxis

Prophylactic medication in the form of in-feed medication and, in specific cases, water medication may be used to reduce the risk of disease. For example, chemoprophylaxis is used routinely in the control of coccidiosis worldwide. Judicious use principles dictate that chemoprophylaxis with antibiotics for bacterial diseases should be reserved for situations in which a specific bacterial disease is judged highly likely to occur in the absence of chemoprophylaxis, and other measures such as biosecurity and immunization have proven inadequate.

Immunization

Immunization through vaccination is a commonly used method of reducing the risk (increased ID_{50}) and consequence (reduced pathogenicity) of bird or flock exposure to a disease causing agent. Vaccination is the practice of administering live and/or killed vaccines, which have been modified to minimize disease manifestation yet maximize immunity. The primary purpose of immunization is to raise the ID_{50} of the flock in order to prevent clinical disease following subsequent challenge. While some vaccines are given to protect that individual bird against disease, others are given to pass the protection on to the next generation, and others are given to prevent disease in the hen and subsequent transmission of the disease to the chick.

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Vaccines and vaccine programs vary widely in their effectiveness, and this is frequently by design. Some vaccines are designed to incite high levels of immunity to protect birds in the face of aggressive endemic disease challenges, such as, vvND. Some vaccines may cause a mild form of the disease themselves but are deemed appropriate and useful because of the risk associated with eventual infection of the deadly field pathogen. Vaccine selection and how they are programmed frequently becomes an exercise in risk management and cost efficiency. Local conditions must always be considered when evaluating and critiquing a vaccination program.

A second reason for the vaccination of poultry flocks is to hyperimmunize hens to maximize maternally derived antibody passed through the egg to the progeny. Chicks frequently receive up to three weeks of protection from maternal antibodies allowing their immune system to mature to a level capable of eliciting an efficient active immune response if exposed to a potentially harmful virus or bacteria. Antibodies are not always completely protective but for viruses such as IBD, many areas of the world have found maternal antibodies a very useful tool in IBD prevention and control. The effect of maternal antibodies on the efficacy of modified live virus vaccines in young chicks must be considered, and varies with the disease. For example, maternal antibody is highly effective in blocking active immunization with homologous IBD, moderately effective in blocking lentogenic Newcastle disease virus vaccines, and much less effective in blocking infectious bronchitis virus vaccines.

The success of vaccination does not rest solely with the manufacturing or research of vaccines. More important is the maintenance of the cold chain, protection of the vaccine from the elements, and the correct application of the vaccine to the bird. All vaccines must be stored at the correct temperature. Most vaccines require refrigeration at 2 °C to 8 °C. Some vaccines, mostly killed oil vaccines, can be safely stored at room temperature. Some vaccines need to be stored at temperatures below 0 °C. Vaccines are adversely affected by exposure to sunlight and heat. Vaccines must be administered using suitably cleaned equipment and be given to every bird in the defined epidemiological unit.

Types of Vaccines

Poultry vaccines are typically characterized as live or inactivated. General characteristics of vaccines are summarized in Table 1.1 (20). Live vaccines are available for numerous viral, bacterial, and coccidial organisms. Techniques used in the development of live vaccines have varied widely. Table 1.2 shows some of the most common methods used to generate an acceptable live vaccine candidate and examples of each method.

Live vaccines are widely used throughout the world because they are effective when mass applied, and they are relatively economical. Immunity from live vaccines is generally short-lived, particularly following initial exposure. Some exceptions to this exist for vaccines such as laryngotracheitis, fowl pox, and Marek's disease.

For live vaccines to work as they were designed, they must be stored, mixed, dosed, and applied appropriately. Storage of live vaccines is generally in a dark, refrigerated area. Liquid nitrogen freezing of live vaccines preserves and prolongs cell culture viability that is essential for

Table 1.1 General characteristics of live and inactivated vaccines for poultry.

Live vaccines	Inactivated vaccines
Smaller quantity of antigen. Vaccination response relies on multiplication within the bird.	Large amount of antigen. No multiplication after administration.
Can be mass administered—drinking water, spray.	Almost always injected.
Adjuvanting live vaccines is not common.	Adjuvanting killed vaccines is frequently necessary.
Susceptible to existing antibody present in bird.	More capable of eliciting an immune response in the face of existing antibody.
In immune bird, booster vaccination is ineffective.	In immune bird, additional immune response frequently seen.
Local immunity stimulated (i.e., trachea or gut).	Local immunity may be re-stimulated if used as a booster but poor if not a secondary response.
Danger of vaccine contamination (e.g., egg drop syndrome, reticuloendotheliosis virus).	Little danger of vaccine contamination.
Tissue reaction commonly referred to as a "vaccine reaction" is possible and frequently visible in a variety of tissues.	No microbe replication; therefore, no tissue reaction outside that which is adjuvant dependent.
Relatively limited combinations—due to interference of multiple microbes given at the same time (e.g., infectious bronchitis, Newcastle disease virus, and laryngotracheitis).	Combinations are less likely to interfere.
Rapid onset of immunity.	Generally slower onset of immunity.

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Table 1.2 Methods of generating live vaccine candidate.

Method	Example
Virulent organism inoculated to a less susceptible target tissue or at a controlled dose	Laryngotracheitis–cloacal route
Naturally occurring mild pathotype	*Mycoplasma gallisepticum* F strain
Egg passage of virulent parent	Infectious bronchitis—Arkansas strain
Temperature-sensitive mutant of virulent parent	Turkey coryza vaccine—*Bordetella avium*
Chemically derived mutants of virulent parent	M-9 Fowl cholera vaccine
Tissue culture/passage of virulent parent	Laryngotracheitis
Combination of egg passage and tissue culture passage of virulent parent	Infectious bursal disease—Lukert virus
Plaque selected "clones" of parent virus	Newcastle disease virus—cloned Lasota vaccines
Selection of subpopulations or organisms based on replication characteristics *in vivo*	Precocious strains of *Eimeria* spp.
Relatively virulent organisms given at an age that minimizes disease	Avian encephalomyelitis

cell-associated vaccines such as Marek's disease vaccines. Licensed live vaccines have an expiration date printed on the vial that, if stored according to label directions, ensures that the appropriate minimum dose is maintained through the dating period. Shelf life varies widely with live vaccines but most generally are licensed with 18 months to 2 years dating. Mixing directions also vary widely, but many recommend the use of a water stabilizer such as powdered skim milk. Water stabilizers minimize some of the negative effects of residual chlorine, metals, pH, and high temperature on the reconstituted virus. Cell-associated Marek's vaccines generally have very specific diluents aimed at maintaining cell culture viability through the time period between reconstitution and inoculation. The dose needed to get an appropriate immune response from a live vaccine is frequently dependent on the virus, genetic background of the bird, age of the bird, existing circulating antibody within the bird, and the method to be used when applying a vaccine. Vaccines generally are licensed based on protection studies performed in a specific pathogen free (SPF)-type leghorn bird, without any circulating antibody to that particular agent, at the youngest age on the label, and at the minimum titer expected at the end of the dating period allowed for each given vaccine. With all these variables, it is not difficult to imagine why clinical veterinarians and other health professionals may adjust dosages of live vaccines according to local field conditions. Severe vaccine reactions or insufficient protection can result from misjudging any of these variables. As a final note, poultry house conditions and local disease risks need to be taken into account when optimizing the use of live vaccines.

A second type of live vaccine is emerging with the development of genetically engineered, live virus and bacteria vectored vaccines and gene deletion mutants of a pathogenic parent organism. The recombinant vaccines are made using live virus or bacteria as a vector to transport the gene coding for the protective antigen of a second infectious agent, for which immunity is desired. Examples of live virus-vectored vaccines include recombinant fowl pox virus vaccines expressing genes to protect against H_5N_2 avian influenza (14), Newcastle disease virus (19), and IBD virus (12), and baculovirus-expressing IBD virus (77). Commercially licensed live virus-vectored vaccines currently available and widely used in the United States include herpesvirus of turkeys (HVT) expressing IBD virus antigens, Newcastle disease virus antigens, infectious laryngotracheitis virus antigens, fowl pox expressing Newcastle Disease virus antigens, infectious laryngotracheitis virus antigens, and *M. gallisepticum* antigens. There are also licensed HVT-avian influenza and pox-avian influenza constructs available, but use of these vaccines is only under permit from the USDA (United States Department of Agriculture). Bacteria-vectored vaccines described in poultry include bacteria such as *E. coli* (43) and *Salmonella* spp. (63) expressing antigens from coccidia and *E. coli*, respectively. Vaccines to reduce *Salmonella* infection, made from a gene deletion mutant of *Salmonella typhimurium* (30), and an *E. coli* gene-deleted vaccine are commercially available.

These recombinant and gene deletion mutant vaccines have been shown to be relatively protective, when compared to controls, against pathogenic challenge under experimental conditions. This type of vaccine may offer advantages where the spread of traditional vaccines to susceptible populations cannot be properly managed. Additionally, these technologies allow for diagnostic differentiation of vaccine from virulent field challenge. This property may be useful when utilized in eradication programs such as laryngotracheitis. Regulatory considerations when acquiring a federal license for vectored vaccines include demonstrating the genetic and phenotypic stability of recombinant viruses or bacteria and documenting any alterations in the host range or tissue tropism of the recombinant organism, as compared to the parent organism (58).

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Inactivated vaccines or killed vaccines used in poultry are generally whole bacteria or virus preparations combined with an adjuvant that are designed for subcutaneous or intramuscular injection. They are frequently, but not always, used in commercial egg layer and breeding birds to stimulate long-lasting immunity and/or antibody levels to specific antigens. Inactivated vaccines generally consist of two distinct components, often referred to as aqueous and adjuvant phases, emulsified into a homologous liquid. The aqueous phase contains the antigen, and the adjuvant generally enhances the bird's response to this antigen. The ratio of antigen to adjuvant differs greatly depending on the vaccine. This ratio generally is determined by factoring in the properties of the adjuvant(s), the antigen(s), viscosity, immune response, and tissue reactivity. Mineral oil is the most commonly used adjuvant, although aluminum hydroxide is a common alternative in notoriously reactive inactivated vaccines such as fowl cholera and infectious coryza. Adjuvant technology continues to grow, and vegetable, fish, and animal oils used as adjuvants offer some opportunities for lower viscosity, immunogenic vaccines. Injection of humans that are administering these inactivated vaccines should be avoided. Serious injuries have been reported from accidentally injecting vaccine into a finger or hand. The site of injection can become swollen, red, and painful, and the function of the area may be affected. Victims should seek medical treatment at once and inform attending physicians of the organism(s) and adjuvant contained in the inactivated vaccine.

DNA vaccines are a new type of vaccine that evolved in the late 1990s. These vaccines can achieve both humoral and cell-mediated immunity, are similar to live vaccines, and have the relative safety associated with inactivated or vectored vaccines. DNA vaccines have been used successfully experimentally in poultry for avian influenza and Newcastle disease in chickens (34, 65) and duck hepatitis B in ducks (76). Although promising, DNA vaccines have both technological and economical challenges to overcome before they are commercially viable.

Vaccine Delivery Systems

Improper vaccine application is the most common reason vaccines and vaccine programs fail. With the success and growth of the poultry industry throughout the world came tremendous challenges in efficient and economic application of poultry vaccines. The most commonly used application techniques in commercial poultry include *in ovo* at 17–19 days of embryonation, subcutaneous or intramuscular injection at day of hatch, spray in the hatchery, intraocular or nasal drop in the hatchery or on the farm, spray on the farm, through the drinking water on the farm, wing web stab, and subcutaneous or intramuscular injection on the farm.

In Ovo Vaccination. *In ovo* vaccination is performed during the process of transferring incubating eggs in the hatchery from the setter to the hatcher. Vaccine is injected just under the membranes at the floor of the air cell. Depending on the embryo age at transfer, generally between 17 and 19 days of incubation, approximately 25–75% of the vaccine (0.05 mL in most cases) is injected into the area of the neck and shoulder. In the remaining 25–75%, vaccine is administered into the extra embryonic compartment (38). The most common vaccine administered *in ovo* in the United States is Marek's disease vaccine; IBD vaccine, reovirus vaccine, and the various Marek's-vectored vaccines are also commonly given by this route. The original experiments on *in ovo* vaccination with Marek's disease vaccine showed that chicks were protected earlier than those vaccinated after hatch (68). However, in the United States, where more than 80% of broiler chickens are vaccinated against Marek's disease *in ovo*, the primary reason for its acceptance has been the labor savings when compared to day-old injection (75). Using an egg injection system (Embrex Inovoject® Egg Injection System, Research Triangle Park, NC), one machine with three people generally inoculates 20,000–30,000 eggs per hour (Figure 1.9). This method of vaccination leaves a hole in the egg for the final few days prior to hatch and in poorly sanitized hatcheries has resulted in poor early livability due to bacterial or fungal contamination while in the hatcher. Hatcheries must be acutely aware of their aspergillosis levels to run an egg injection system successfully (79).

Subcutaneous or Intramuscular Injection at Day of Hatch. Day-old vaccination, most commonly using Marek's disease vaccine, is generally accomplished by giving 0.2 mL of vaccine subcutaneously under the skin at the back of the neck or 0.5 mL intramuscularly in the leg. The automatic vaccination machines used in many parts of the world generally are designed for the neck injection. A skilled operator can vaccinate about 1,600–2,000 chicks/hour. A 20-gauge needle generally is used, as smaller gauge needles restrict the flow in cell-associated vaccines. Needles should be changed several times during the course of the day to prevent damage from burred or bent needles. Improper positioning of the chick or a bent needle can result in damage to the neck muscles or vertebrae. A dye is frequently mixed with the vaccine to allow visualization of the vaccine under the skin after injection. A quality check of technique generally means examining each bird in several boxes, 100 to a box, after vaccination looking for colored dye under the skin. The most frequent cause of missed birds is the operator trying to go too fast, resulting in chicks being pulled off the needle before proper deposition of vaccine.

Spray in the Hatchery. Spray vaccination of birds in the hatchery generally is done using a spray box that is triggered each time a box of chicks is placed inside or an in-line spray

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Figure 1.9 A modern hatchery with an egg injection system for *in ovo* vaccination.

cabinet that sprays boxes as they move down a controlled speed conveyor line in an automated hatchery. Both methods, frequently used to deliver Newcastle disease virus, infectious bronchitis virus, or coccidiosis vaccine, attempt to mimic eye drop vaccination. Spray vaccination in the hatchery generally works well if the droplets generated have a particle diameter of approximately 100–150 microns. Particle size is very important. Low relative humidity may decrease the particle size by the time it reaches the bird, resulting in too fine a spray. Fine spray, generally something less than 20 microns in diameter, can travel deep into the respiratory tract, resulting in excessive vaccine reaction if using a respiratory disease vaccine. Although there is some variability, Newcastle disease virus and infectious bronchitis virus vaccines are often delivered in 7 mL of distilled water per 100 chicks. Coccidiosis vaccines generally use more distilled water, approximately 20–25 mL per 100 chicks. Birds preening themselves and each other immediately following spray vaccination is thought to be important to the resulting vaccination response, although little data exists to support this concept.

Spray Vaccination on the Farm. With the increased acceptance and use of closed watering systems and the increased cost of labor required to effectively vaccinate through the drinking water, spray vaccination of respiratory vaccines, such as Newcastle disease virus and infectious bronchitis virus, has become increasingly popular. This method of vaccination frequently uses spray equipment adapted from insecticide and pesticide application technologies. As with the hatchery spray vaccination, the method is designed to mimic eye drop vaccination but allows the vaccinator to avoid handling each bird in the poultry house.

Distilled water generally is used to reconstitute the vaccine(s). Although the volume of water used varies depending on the spray machine selected, five gallons of water per 20,000 birds vaccinated is a good general recommendation. It generally is preferred to vaccinate a flock first thing in the morning. Fans should be turned off, if possible, and the lights should be as dim as the vaccinator can allow and still walk through the house. In floor houses, if another person is available, one person can split the flock while the vaccinator slowly sprays one side at a time. If possible, running fans should be minimized for the 15 minutes following vaccination.

An effective spray vaccination technique allows exposure of birds to aerosolized vaccine for approximately 5–10 seconds. This is best accomplished by spraying a relatively coarse spray, in the range of 100–150 microns, and walking slowly through the poultry house. A visual evaluation of a spray pattern can be done with each vaccination. Look for an even distribution and consistent projection. A crude estimation of droplet size may be made using the analogies listed in Table 1.3 (72).

Table 1.3 Visual analogy to droplet size diameter.

Analogy	Diameter (microns)
Wet fog	25–40
Visible droplets	50
Misty rain	50–100
Light rain	200–400

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Intraocular or Nasal Drop in the Hatchery or on the Farm. Intraocular or nasal drop is a highly effective but labor-intensive method used to deliver respiratory disease vaccines for diseases such as laryngotracheitis. This method generally involves depositing approximately 0.03 mL of reconstituted vaccine in the eye or nares. Both techniques generally require the vaccinator to pause briefly as the vaccine disappears in the appropriate opening. A dye colored diluent helps to visualize the vaccine and allows a quality check on technique by looking around the nares or eye for dye. Frequently some dye can be seen by looking in the bird's mouth around the choanal cleft or edges of the tongue.

Drinking Water Vaccination on the Farm. A very common and useful technique in commercial poultry has been to apply vaccine through the drinking water. Proper preparation of the watering system to be used through removal of all disinfectants, such as chlorine, should be done two days prior to vaccination. It is best to buffer the system by flushing it with a weak solution of powdered skim milk, generally one cup powdered skim milk to 50 gallons of water (25). This type of buffer generally is also used while administering the vaccine.

Best results are achieved through a process that creates a mild degree of thirst by eliminating access to drinking water for approximately two hours prior to the vaccination procedure. This time varies widely. Climatic conditions may necessitate longer or shorter time periods. Emptying the drinking system and then charging the water lines with vaccine-laden water ensures that the first birds to drink receive a dose of vaccine. The total time required to administer the vaccine is a balance between the gradual deterioration of vaccine titers in the water system against adequate time for all birds to get a drink. Two hours generally allows all birds, even those lower in the social order, adequate time to get a drink of water containing vaccine. This technique requires constant adjustment as the climate changes.

Wing Web Stab. Wing web vaccination requires individual bird handling but can be done relatively rapidly. There are two commonly used wing web application tools. The first is the traditional small plastic handle approximately 3 cm long that has two solid stainless steel prongs, approximately 2 cm long, with a bevel on each prong toward the needle end. The prongs are dipped into an open container of vaccine between each bird. The second is a Grant inoculator, a syringe-like tool with a self-contained reservoir for vaccine, most often fowl pox or fowl cholera, through which a needle passes loading a new dose of vaccine for each bird inoculated. Both tools are designed to deliver approximately 0.01 mL on the needles to the bird's wing web. The wing web is an area that has relatively few feathers, bone, or muscle. The vaccinator loads the applicator and sticks the needle(s) completely through the skin on both sides of the web, originating from the underside of the wing. There is little or no bleeding, and vaccine has been inoculated through the needle holes. Wing web vaccination technique can be checked by returning to the vaccinated flock 7–10 days after vaccination and palpating the wing web area for nodular scabs or granulomas. These areas created by the vaccine are commonly referred to as "takes." Proper vaccination technique frequently results in 95–100% take.

Subcutaneous or Intramuscular Injection on the Farm. Subcutaneous and intramuscular injections are frequently used in breeder pullets and commercial egg-laying pullets prior to egg production. These vaccines are generally recommended for use at least four weeks prior to the onset of egg production to minimize any adverse effect the handling or the vaccine may have on egg production performance. Subcutaneous vaccination is most frequently performed using a ½inch, 18-gauge needle, in the neck. The area halfway between the head and the shoulder is optimal and allows the vaccinator to lift the skin away from the neck muscle and insert the needle, pointed toward the body of the bird, into the subcutaneous area and deposit the vaccine. Intramuscular injection generally is performed using a ½ inch, 18-gauge needle to inject vaccine into the breast or leg muscle. Breast muscle injections are safest when the vaccine is deposited in the superficial pectoral muscle 2–3 cm lateral to the keel bone. If the needle is kept at a 45-degree angle to the bird, any accidental injections into the body cavity or liver can be avoided (49). Leg vaccination generally is done in the lateral gastrocnemius muscle. Both intramuscular injection sites may result in residual emulsion being present for an extended period of time (31). A residual deposit in muscle depends on many factors including the antigen and the adjuvant found in the vaccine. Care should be taken to determine the intended use of meat before injecting intramuscularly.

Vaccine Failure

Numerous factors can cause a vaccine failure. One of the most common causes of vaccine failure is the inappropriate administration of the vaccine. Certain live vaccines, such as Marek's disease vaccine, are easily killed, and failure to follow the manufacturer's recommended handling practices will result in the inactivation of the virus prior to administration. Viable vaccines administered in the drinking water can, likewise, be destroyed before they reach the bird if they are mishandled or if water sanitizers have not been removed from the water prior to the addition of the vaccine. Vaccines that are administered by intramuscular or subcutaneous injection can also fail if vaccinators do not deliver the vaccine to the appropriate vaccination site.

Although the most common cause of vaccine failure is an inadequacy or error in vaccine delivery, numerous

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instances of vaccines simply not providing adequate protection have occurred. In some cases, the field strain of an organism is of very high virulence, and the vaccine strain is highly attenuated. In this situation, the flock may be effectively vaccinated, but the immunity is insufficient to protect against disease completely. Many infectious agents have several different serotypes, and vaccine failure may be the result of the antigens in the vaccine serotype being different and not providing protection against the particular serotype of the agent causing the field challenge. It is not uncommon for a vaccine break to occur with infectious bronchitis virus when the field challenge is of a serotype different from that of the vaccine used (13).

Management conditions play an important role in the prevention of vaccine failures. If infectious disease agents are allowed to build up on a farm over successive flocks without clean-out and disinfection, it is possible that the challenge dose of a particular infectious agent will be so great, or so soon, that a normally effective vaccination program will be overwhelmed. The immune status of the breeder flock also can be involved in a vaccine failure. If the breeder flock provides progeny with high levels of maternal antibodies, vaccination during the first two weeks of life may result in the vaccine being neutralized. The timing of the vaccination of young poultry with viable vaccines must always take the presence or absence of maternal antibodies into consideration.

Certain infectious disease agents and mycotoxins are immunosuppressive and may result in vaccine failure. Infectious bursal disease virus (Chapter 7), infectious anemia (Chapter 8), and Marek's disease virus (Chapter 15) are examples of agents that may cause severe immunosuppression in chickens. One mycotoxin, aflatoxin, has been shown experimentally to be immunosuppressive and has been implicated in decreased resistance to disease (see Chapter 32).

Handling Disease Outbreaks

Good poultry producers watch feed and water consumption and egg production at all times, but more important, they observe normal sounds and actions of the flock. They sense immediately when any of these conditions are abnormal and interpret them as signs of abnormal health. When this happens, it should be assumed that an infectious disease has gained entry and may be tracked elsewhere during the investigation period. A producer should not procrastinate for any reason when a disease threatens, or it may get completely out of hand before a diagnosis is made. In a modern poultry production system, any disease creates serious disruption in the economical operation of the farm and the plants processing products from it. The following steps should be followed when disease is suspected.

Take precautions against tracking an infectious disease that may be present, but investigate management errors immediately. A high percentage of so-called disease problems referred to laboratories for diagnosis are noninfectious conditions related to management: beak trimming errors; consumption of litter and trash; feed and water deprivation; chilling of chicks; injury from rough handling, automatic equipment, or drug injection; electrical failures; cannibalism; smothering; overcrowding; poor arrangement of feeders, waterers, and ventilators; inexpensive low-quality feed ingredients; ingredients causing feed refusal; improper particle size of feed ingredients; and rodent and predator attacks (2, 16). Bell (15) observed marked reduction in lay from water deprivation related to a beak trimming system that resulted in long lower beaks, making it difficult to obtain water when the level was low. These are conditions that do not require services of a diagnostic laboratory. External parasites (mites, lice, and ticks) can be determined by producers if they examine affected birds.

Quarantine the Flock

In the event that no management factors can be found, the next step is to set up a quarantine of the pen, building, farm unit area, or entire farm, depending upon its design and programming. If this emergency was anticipated when the farm was laid out and programmed originally, the quarantine will be a minor problem. If the basic principle of "a single age in quarantinable units" was disregarded in original farm planning, a disease outbreak can be an economic disaster. Separate caretakers should be established for affected birds or at least sick ones should be visited last.

Submit Specimens or Call a Veterinarian

The owner or caretaker should submit typical specimens to a diagnostic laboratory or call a veterinarian to visit the farm and establish the diagnosis. Owners should seek professional diagnosis, rather than trying to hide some disease because of possible public recrimination. Veterinarians and caretakers can and should help dispel this apprehension by maintaining high ethical standards and refraining from discussing one producer's problems with others. Yet, there comes a time when all producers must be apprised of a problem. Service workers frequently are requested to examine the flock, select specimens for the laboratory, and initiate first aid procedures until the veterinarian can be called or visited. If so, they should wear protective footwear and clothing when they enter the house. No other farm should be visited en route to the laboratory.

Special Precautions

In addition to causing serious losses in poultry, some diseases (chlamydiosis, erysipelas, and salmonellosis) are especially hazardous for humans. When these conditions

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are suspected or diagnosed, extra precautions must be taken to ensure against human infection. The proper government health authorities should be notified of chlamydiosis outbreaks, and all handling and processing personnel should be apprised of the disease, hazards, and necessary precautions.

In some states, certain diseases (*Mycoplasma* infections, avian chlamydiosis, and laryngotracheitis) must be reported immediately to the state animal disease control authorities so that proper investigation and action can be taken to protect the human population and the poultry industry. Common sense dictates that when a condition suggestive of an exotic disease, such as vvND, fowl typhoid, or avian influenza is encountered, the proper state and federal regulatory authorities should be informed immediately.

Nursing Care

Nursing care plays an important role in the outcome of a disease outbreak. Additional heat should be supplied to young chicks that begin huddling because of sickness. Clean and fresh (or medicated) water should be available at close range. Hopelessly sick and crippled birds should be killed in a manner to preclude or control the discharge of blood or exudates (see Diagnostic Procedures). Dead and destroyed birds should be disposed of immediately (see Dead-Bird Disposal).

Drugs

Therapeutic medication, if appropriate, should be prescribed by the veterinarian after the problem has been diagnosed. Therapy is not a sustainable method of disease control and should not be considered as an ongoing part of any biosecurity program. The flock response to medication merely provides the time necessary to investigate, design, and implement further control measures to avert further need for therapeutic medication.

No drugs should be given until a diagnosis is obtained or a veterinarian consulted. Beginning in 2017 in the United States, all medically-important drugs given to food animals by feed or water must be used under a veterinary prescription (for water) or a Veterinary Feed Directive (for feed). See Antimicrobial Therapy Including Resistance for a discussion of the types of drugs included in these categories. If the wrong drug is given, it can be a waste of money, or it may be harmful or even disastrous. If an infectious disease is found and corrective drugs are indicated, they should be used very carefully according to directions.

Strict regulations govern the use of drugs in mixed feeds for food-producing animals. A handy reference is the annually updated *Feed Additive Compendium* published by Miller Publishing Co., Minnetonka, MN. Feed manufacturers must have Food and Drug Administration (FDA) clearance to include drugs in mixed feeds. When treated flocks are to be marketed, a specified period (depending on the drug used) must follow cessation of treatment to allow dissipation of drug residues from tissues before slaughter. If the flock is producing table eggs when treated, the drug must be one permitted for use in laying flocks, or eggs must be discarded during, and for varying lengths of time after, treatment, which is a costly alternative.

If the flock is producing hatching eggs when it becomes infected and there is danger that egg transmission of the infectious agent from dams to offspring may occur (for example, salmonellosis, mycoplasmosis, reovirus, inclusion body hepatitis, and avian encephalomyelitis), eggs should not be used for hatching until the danger has passed. It should also be kept in mind that in fertile eggs, residues of drugs used to treat breeders occasionally may cause abnormalities in some embryos.

Disposition of the Flock

The flock should not be moved or handled until it has recovered, unless the move is to a more favorable environment as part of the therapy or for emergency slaughter if permitted. After treatment, if any, has been completed and the flock appears to be completely healthy, it may be marketed or moved to permanent quarters if such a move is part of the management program. Some healthy carriers may remain. If the flock is moved to another depopulated farm, this will present no problem except that occasionally a disease may flare up from stress of handling and moving. If the recovered flock is moved to a multiple-age farm, carriers can introduce the disease into susceptible flocks already there. If the recovered flock is already in permanent quarters having multiple ages, newly introduced flocks may be exposed and contract the disease, a common occurrence especially with respiratory and litter-borne diseases.

Disease Prevention and Control in Antibiotic-Free Production

John A. Smith, Martine Boulianne, Robert L. Owen, and Eric Gingerich

Summary

Regulatory changes in the United States have restricted non-therapeutic uses of medically important antibiotics, but continue to allow most therapeutic uses under increased veterinary supervision. More importantly, market-driven restrictions on all uses of antibiotics in poultry production have created challenges in the control of bacterial infections. Resulting problems in broilers are increased early mortality, coccidiosis, and

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necrotic enteritis (NE); in turkeys, coccidiosis and bacterial infections such as *Bordetella avium*; and in table egg layers, colibacillosis and NE. Management of these problems may require adjustments to management and diet, use of non-antibiotic medications such as chemically synthesized coccidiostats, and alternative products such as probiotics. Even with these interventions, performance and health problems may exceed those found in conventional production schemes with unrestricted access to approved medications.

Introduction

Current United States Regulatory Environment

In an effort to address the emergence of antibiotic-resistant bacterial pathogens in the human population, government authorities in numerous nations have enacted restrictions on agricultural uses of antibiotics. In 2017 the United States rescinded all growth promotion and feed conversion clearances for all medically important antibiotics. The Food and Drug Administration (FDA) list of critically important, highly important, and important classes of antibiotics includes all currently approved poultry drugs except the ionophores, bacitracin, bambermycins, and avilamycin (43). Therapeutic uses of currently approved drugs (including medically important antibiotics) are still permitted, albeit with increasing restrictions and oversight. For example, in the United States all over the counter (OTC) clearances for medically important antibiotics administered by feed or water were withdrawn in 2017. All such uses must now occur under a veterinary feed directive (VFD) for in-feed treatments, or by veterinary prescription for water treatments, and duration of use restrictions are being tightened. Clearances for prevention and control of disease are considered therapeutic uses and remain available in the United States with the same requirements and restrictions for a VFD or prescription. Non-medically important drugs remain OTC except for avilamycin, a non-medically important drug which was nevertheless cleared as a VFD drug. In operations where marketing schemes permit access to all remaining clearances, chemically synthesized anticoccidial drugs and non-medically important antibiotics can be used without restriction to control coccidiosis and NE, and approved antibiotics, including medically important drugs, can be used judiciously under veterinary supervision to treat, prevent, and control NE and other diseases. Consequently, these new regulatory restrictions should have only modest impacts on bird health and efficiency and should be manageable with minor changes to management practices. Industry veterinarians are called upon to address production shortfalls and these changes may impact that aspect of practice. Veterinarians are expected to observe judicious principles of use in employing these remaining clearances.

Marketing Restrictions on Antibiotics

A larger emerging issue is marketing strategies that more severely limit or entirely prohibit the use of all antibiotics in food animals. These marketing campaigns are becoming increasingly common in developed countries and can have major impacts not only on productive efficiency but also on poultry health. In the United States, these campaigns are conducted via product labeling and advertising strategies. Many are initiated by retailers who demand these restrictions of their supplier–producers, frequently under pressure from activist groups and social media, while others have been initiated by the producers themselves in an effort to distinguish their products and capture a niche market of affluent customers. These marketing strategies are quite varied in their restrictions, and the resulting impacts and necessary responses therefore vary as well. Regardless of the scientific merit or prudence of such marketing strategies, they have become a fact of life in many countries and veterinarians will be called upon to address the resulting health problems. The veterinarian must therefore be fully aware of and understand the restrictions imposed by a given marketing program in order to know what substances are prohibited or allowed and how to manage that specific program.

Levels of Restriction

In the United States, all food labeling and marketing claims are governed by federal regulation. All claims regarding production, product attributes, and so forth on labels of animal products must be approved by the United States Department of Agriculture (USDA), Food Safety and Inspection Service, and cannot be false or misleading. Perhaps the most restrictive labeling claim permitted is "certified organic production".

The next most restrictive category commonly seen on labels in the United States is "raised without antibiotics" (RWA) or "no antibiotics ever" (NAE) and similar claims. Regulators in the United States generally disallow claims such as "antibiotic free", "residue free", or "chemical free" as such claims generally cannot be conclusively proven and are considered misleading. If RWA/NAE statements are made on labels or in advertising, no antibiotics of any sort, whether deemed important in human medicine or not, can be used at any point in the life of the bird, by any route, including *in ovo* or at hatch. If a sick flock must be treated, the produce from that flock cannot be labeled as RWA/NAE and must be diverted to commodity product; the producer must be able to demonstrate to the regulator that systems are in place to reliably and completely segregate such treated produce. In the United States ionophores are considered antibiotics for labeling purposes.

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Consequently, marketing programs in the United States making RWA/NAE claims cannot use ionophores and the only options for coccidiosis control are coccidiosis vaccines or chemically synthesized non-antibiotic coccidiostats (i.e., those not derived from fermentation or other biological processes). Canada formerly did not allow chemically synthesized coccidiostats in "raised without antibiotics" programs and therefore Canadian producers making these production claims were restricted entirely to coccidiosis vaccines and nonpharmaceutical measures for the control of coccidiosis and NE. These vaccine-only programs have been difficult to manage on a commercial scale, and in August 2016 the Canadian restriction on chemically synthesized coccidiostats in RWA/NAE labeled production was rescinded. While there have been vaccine-only, completely nonpharmaceutical programs in the United States similar to the original Canadian program, these appear at present to be uncommon outside of organic production. As consumers and activists become more demanding, such extremely restrictive vaccine-only marketing programs may become more common in the future.

An intermediate level of antibiotic restriction has been adopted by a major international fast food retailer based in the United States (27) and many competitors have promulgated similar programs. This program cites the World Health Organization (WHO) definitions of critically important, highly important, and important antibiotics for human medicine. In practical terms, the three WHO categories together include essentially all agricultural antibiotics except ionophores, avilamycin, and bambermycins (49). This intermediate program prohibits any use of WHO critically important human antibiotics that have no veterinary approvals (i.e., no extralabel use of critically important antibiotics). It requires veterinary oversight (a veterinarian–client–patient relationship) for use of any so-called "dual use" human/veterinary drugs for treatment or prevention. It prohibits the use of WHO medically important antibiotics for growth promotion. Producers adhering to this program can use ionophores, avilamycin, bambermycins, and chemical coccidiostats, and the program allows treatment of sick flocks and prevention/control uses of veterinary-labeled drugs under veterinary supervision, without requiring diversion of the product. As with the new US federal restrictions on antibiotic use, programs such as this may have minor effects on productive efficiency, but animal health issues should be manageable with only minor deviations from standard commercial practices. Other programs continue to emerge, creating a complex and confusing situation for regulators, consumers, producers, and veterinarians.

At present most antibiotic-based marketing campaigns have not reached back beyond the hatching egg. Broiler hatching egg production has generally been less reliant on antibiotic use, and recent changes in regulation of antibiotics in the United States are not anticipated to have major impacts in this area. More severe restriction could increase issues however. Broiler production with RWA/NAE restrictions often entails increased neonatal mortality, and hatching egg quality and hygiene are important aspects of controlling this problem. Further restrictions on the use of antibiotics in breeder flocks could compromise health management and therefore hatching egg quality, exacerbating neonatal health problems in RWA/NAE broiler operations.

Economic Impacts and Metrics of RWA/NAE Programs in Broilers

It is common for RWA/NAE programs to include additional marketing claims such as an all-vegetable diet or bird density restrictions. Due to the wide variations on the theme of antibiotic restriction itself and these additional ancillary claims, it is difficult to precisely separate the results of varied restrictions on antibiotics from the effects (positive or negative) of these other differentiating attributes, and to cite conclusive figures for the impacts and costs of antibiotic restriction alone. One index of the impacts can be gleaned from a reporting service widely used by the United States broiler industry. AgriStats, Inc. is a United States-based company that gathers production information from over 95% of United States broiler producing companies to produce reports that benchmark individual companies' performance to industry average performance levels. Individual company numbers are confidential as companies see their information identified but are unable to identify the information from individual competitors. Subsets of the data can be created to analyze average results for items such as breed comparisons and nutrition and feeding programs, such as whether or not birds were fed an antibiotic. Before 2013, approximately 90% of birds grown in winter months were fed an antibiotic with a growth promotion claim. In late spring and through fall, the use of these medications typically declines to roughly 70% of broilers (15). By early 2017 fewer than 50% of all birds processed were fed antibiotics important in human medicine. The percentage of birds grown under RWA/NAE programs has also increased significantly, from 13.9% of all birds in December of 2015 to 38.7% of all birds in February of 2017 (15). Historically, broiler performance has shown a steady improvement over the last thirty years due to continually improving genetics, management technology, scientific feeding, and veterinary care. The anticipated annual gains have been tempered in recent years coinciding with the growth in antibiotic-restricted and RWA/NAE programs. For instance, in April 2017, for bird weights between 2.36 and 3.18 kg

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(5.2–7.0 pounds, the most common weight range for RWA/NAE production), the 21 complexes producing RWA/NAE birds suffered a 2.9% increase in caloric conversion (2,595 calories per pound versus 2,522) and 0.68% higher mortality (15).

Managing RAW/NAE Programs in Broiler Production

Primary Issues

The major issues in RWA/NAE programs in broilers are coccidiosis, NE/dysbacteriosis, secondary issues related to gut health and gut barrier function, and the removal of therapeutic doses of antibiotics from Marek's disease vaccines which may lead to increased neonatal infections. In addition, and especially when all-vegetable diet claims are included, litter moisture control becomes more difficult, which can lead to foot and hock burns, skin lesions, air quality issues (and concurrent respiratory system problems), as well as increased coccidial and bacterial challenges from the wet litter (4). Finally, although RWA/NAE producers and retailers almost universally claim that sick flocks must be treated for welfare reasons, the produce from treated flocks cannot be marketed as RWA/NAE. This situation creates very real pressure on managers and veterinarians not to treat sick flocks in these programs until losses become severe, resulting in economic losses, compromised animal welfare, and a serious moral and ethical dilemma for the veterinarian.

Early Mortality

Increases of about 0.5–1% above the normal average seven-day mortality of 1.4% (3) can be expected at the onset of RWA/NAE production. There are no simple nonpharmaceutical solutions to address this problem, but strict attention to details from the hen house through brooding can recoup the majority of this loss. Proper hen house lighting, ventilation, litter management, nest space, nest placement, nest management and hygiene, egg collection practices, gut health management, and pest control will help to minimize floor eggs and produce a nest-clean hatching egg. Farm egg coolers should be managed to provide clean storage with constant temperature and humidity, and egg pickup and transport procedures should be designed to minimize egg sweating. Egg disinfection is controversial in broiler production and should not be necessary for dry nest-clean eggs. Refer to the section of this chapter on nest and egg hygiene for further discussion. Hatchery cleanliness and maintenance become paramount. Hatcher and setter halls should have adequate ventilation capacity to supply the needs of the machines, and hall temperature and humidity should be regulated to maximize machine ventilation while minimizing the work that the machines must perform to maintain stable incubation parameters. Machine maintenance is critical to provide uniform target incubation conditions throughout the box, to minimize the hatch window and produce the highest quality chicks. The machine manufacturer should be consulted for advice and assistance on ventilation, maintenance, and operation of their specific machines. There should be careful and continuous monitoring of set and pull times to produce a clean, dry chick with a healed navel, that is neither "green" nor "overdone" and dehydrated. Scrupulous cleaning and disinfection of the egg trucks, egg coolers, incubator and hatcher halls, incubators, hatchers, egg trays, racks, hatcher baskets, processing equipment (including *in ovo* injection machines, separators, processing belts, reusable chick boxes, vaccination equipment, etc.), and chick delivery vehicles are essential. Refer to the section of this chapter on disinfectants for further information. Wash water temperatures and water, detergent, and disinfectant flow rates and usage in tray and basket washers should be monitored. The hatchery water supply should be monitored for bacterial contamination, especially for humidification in incubators and hatchers, as should the room ventilation systems. Fumigation of eggs and fumigation of hatchers may be necessary, especially in the early stages of transition to RWA/NAE production. Formaldehyde is effective but controversial, and there are other alternatives for fumigation such as hydrogen peroxide that can be helpful. Bacterial monitoring of hatchers, *in ovo* injection machines, spray vaccination equipment, vaccine mixing rooms, and all chick contact surfaces (trays, boxes, belts, etc.) after cleaning by means of swabs and contact plates is a useful means to check the effectiveness of cleaning procedures.

Proper brooding management becomes even more critical in RWA/NAE production. A useful acronym for assessing brooding practices is "FLLAWSS", for feed, lights, litter, air, water, sanitation, and security. Starter feeds are best crumbled for easy prehension, and multiple shallow supplementary feeders easily accessible by the chicks should be placed throughout the thermal comfort zone. Light levels of 30–40 lux are recommended throughout the brooding period, and spotlights on the feed and water sources can be an added advantage. Litter should be clean, warm, dry, absorbent, friable, and deep enough to insulate chicks from the floor. The air must be of proper quality in terms of dust, ammonia (less than 20 ppm), humidity (60–70% relative humidity [RH]), and carbon dioxide, and must be of the proper temperature (28–30 °C). Zoned heating units such as radiant heaters are useful to provide a range of temperature zones in which chicks can seek their own

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comfort levels. Clean water should be easily accessible near the feed and during brooding should be warmed to avoid chilling the chicks. Drinkers should be adjusted to proper height for access by the chicks and in the case of nipples the pressure must be regulated so chicks can easily trigger the nipples. Biosecurity and sanitation are as critical during brooding as at any time during rearing. Monitoring of proper brooding conditions is important. Tools such as infrared thermometers to check floor and air temperatures, rectal thermometers to check chick body temperature, light meters, and ammonia and carbon dioxide meters are useful not only to inspect conditions but also to illustrate to growers and caretakers where they are doing a good job and where improvement is needed. Assessing crop fill at 24 hours of age by palpation, with a target of at least 90–95% of chicks having detectable feed in the crop, is a good means of assessing overall preparation for brooding and early brooding conditions. The primary breeders have excellent rearing guides with extensive advice on brooding management.

Coccidiosis

Coccidiosis Vaccines and Vaccine-Only Programs

Live coccidiosis vaccines used in RWA/NAE programs, whether in vaccine-only programs or in rotation with chemical coccidiostats, should have high numbers of viable oocysts of at least the three major species for broilers (*Eimeria acervulina*, *E. maxima*, and *E. tenella*) and probably should contain *E. mitis* as well in many areas (47). *E. maxima* is especially critical (35). Proper refrigerated storage and usage within the expiration date is therefore important. Unattenuated and attenuated or precocious strains are both available, but neither is clearly superior under United States conditions. Methods of administration should be used that maximize initial uptake by the greatest number of chicks possible. Administration of the vaccines has been problematic, from both logistical and efficacy standpoints, as evidenced by the continued evolution of methods adopted to attempt uniform uptake. Feed and water administration were generally abandoned many years ago as logistically difficult and marginally effective. Administration via spray cabinets at the hatchery has been the standard since the 1990s, typically using large spray volumes up to three or more fold greater than those used for respiratory vaccines (often 21–24 mL per box of 100 chicks or even higher). Mechanisms such as stir bars or aerators are necessary to maintain oocysts in uniform suspension during spray administration. Wetting and chilling of the chicks with these high spray volumes must be managed, especially if used in tandem with a respiratory vaccine spray. Ancillary measures to increase preening of the sprayed vaccine, such as inclusion of dye in the spray, increasing light intensity, and maintaining quiet conditions and warm temperature (33–35 °C) in the chick holding area post-application prior to transportation have been recommended (7, 8). Nevertheless, spray vaccination still results in wide variability in uptake and subsequent shedding after the first cycle (34, 35). Gel discs containing the coccidiosis vaccine and sometimes nutritional supplements have been administered for consumption by the chicks in transport boxes, and eye spray and *in ovo* administration have been utilized, with none of these techniques significantly more efficacious than spray cabinet administration. Recently commercialized colored gel droplets that are preened by the chicks in the transport box show promising results (35). Excessive dosing is generally not the problem; rather it is the chicks not receiving an initial immunizing dose that are at much higher risk for coccidiosis or NE (35). An optimum vaccine preparation and a logistically simple administration method delivering a viable immunizing dose to a large percentage (more than 95%) of chicks on initial administration would greatly improve the efficacy of these vaccines, but such a method has yet to be devised.

A single infection will induce immunity to reinfection, although the extent varies depending on the species and dose (11). However, due to marginal initial immunization, and to the fact that better immunity develops from repeated low doses, coccidiosis vaccines rely on natural cycling of the administered *Eimeria* vaccine strains and possibly wild strains resident in the litter to develop immunity before the cycling of the wild strains reaches clinical levels (47). Some vaccine manufacturers recommend holding the chicks in a restricted space (the "brood chamber", usually representing one-third to one-half of the total space eventually allocated to growing the flock) for a specified period (typically about 12 days) to encourage early cycling. Exact recommendations vary with the brand of vaccine, and the manufacturer's directions should be followed. Excellent brooding conditions, especially proper temperatures and light levels that encourage chick foraging, and control of litter moisture are critical. It is conventional wisdom that excessively dry litter will decrease oocyst cycling and timely development of immunity, and that wet litter can result in excessive cycling and clinical disease. Managers in arid or very cold areas with low environmental humidity levels and ensuing dry litter (Western Canada) even add moisture to litter to encourage cycling. However, it has been shown that sporulation was optimum in dry litter (16% moisture), intermediate in moist litter (42% moisture), and worst in wet litter (62% moisture) (45). Nevertheless, the association of wet litter with diseases is probably accurate and litter moisture should be controlled. Proper cycling can be difficult to manage on a large commercial scale without some measure to control NE (i.e., prophylactic antibiotics or coccidiostat treatment programs),

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and problems can be expected on a percentage of farms when these additional controls cannot be used (21).

The risk of problems with coccidiosis and NE in vaccine-only programs often varies among farms within a production complex (21, 37). When RWA/NAE marketing needs are considerably less than the total capacity of the production complex, it is possible over time to identify lower and higher risk farms via trial and error and concentrate RWA/NAE production on the low risk farms, decreasing the incidence of issues (37). This process is part of the institutional learning curve and can be painful for several years, especially when a pathogenic *Clostridium perfringens* clone is present on the premises and persists from one production lot to the next (32). Some clinicians have noted a curious phenomenon in which performance on vaccine-only RWA/NAE farms tends to decline over time, even on low-risk, well-managed farms. Again, when part of the production complex's output consists of conventionally raised poultry production, placing a conventional flock (e.g., with ionophores or chemical coccidiostats and perhaps prophylactic non-medically important antibiotics) on those RWA/NAE farms results not only in excellent performance in that conventional flock, but also in several subsequent RWA/NAE flocks. The excellent performance in the first conventional flock is likely due to the fact that the farm environment has been seeded with vaccine strains of coccidiosis, which are sensitive to coccidiostats. The phenomenon of vaccine use restoring coccidiostat sensitivity in a flock is well documented (47, 48). The renewed performance in the subsequent RWA/NAE flocks is more difficult to explain; perhaps reducing resident coccidial challenge and/or alteration of the litter flora may be involved. When RWA/NAE production comprises the majority or all of a complex's output, these selective placement and rotational programs are not available and long-term management of vaccine-only programs can be difficult.

Since immunity from coccidiosis vaccination currently depends on controlled cycling of vaccine strains before wild challenge builds to critical levels, one might suppose that placement of vaccinated chicks on new, clean litter would be optimum, but this does not always appear to be the case under United States conditions, where reuse of litter is common. Clinicians in the United States have for years recognized "new house syndrome", in which coccidiosis and NE seem to be more prevalent and severe in new (or cleaned out) houses. In one report on risk factors for NE in a large vaccine-only program over a 30-week period, the odds of a NE outbreak were 2.6 times higher on new litter compared to built-up litter (38). Possibly, new litter is not as conducive to adequate cycling of the vaccine, or perhaps normal flora in the used litter is beneficial, or perhaps resident coccidial populations contribute to development of solid immunity.

A subunit coccidiosis vaccine has been registered in several countries outside the United States for administration to breeder pullets to provide maternal immunity to progeny. The vaccine is based on subunits forming the oocyst wall, and is therefore directed against the final sexual stages of the life cycle. It reportedly suppresses oocyst production while still allowing schizogony of any oocysts ingested from live vaccines or the environment, and is therefore hypothesized to allow the development of immunity via schizogony while controlling the early buildup of challenge in the house (36).

Chemically Synthesized Coccidiostats

In programs allowing chemically synthesized coccidiostats, control of coccidiosis and therefore NE becomes more feasible but is still not without difficulties. The number of chemically synthesized coccidiostats available in the United States is limited, most are old compounds, and some are relatively expensive compared to other options such as vaccines and ionophores. The currently approved list in the United States includes amprolium, clopidol, decoquinate, diclazuril, halofuginone, nicarbazin, robenidine, and zoalene. As of 2017, clopidol and halofuginone were not being marketed, and the supply of some of the others has been problematic at times due to limited sources and fluctuating demand. Although sulfonamides are chemically synthesized and can be used to control coccidia, they are classified as antibiotics and cannot be used in RWA/NAE programs. Chemical coccidiostats have more issues with development of resistance than ionophores or vaccines. The older chemicals in this order are listed from most to least likely to develop rapid resistance: quinolones (decoquinate), clopidol, robenidine, amprolium, zoalene, and nicarbazin (10). Indeed, the last three on the list have proven most durable, at least in terms of continued use in industry. Diclazuril and halofuginone were not marketed yet when this list was proposed, but most would place them fairly high on that list (i.e., likely to rapidly develop resistance). It appears that the more effectively the drug suppresses oocyst excretion, the greater the selection pressure applied to the population, the less the immune system is engaged, and the more rapidly resistance appears to emerge (46). Consequently, careful design of shuttle programs (using two or more drugs in one growing cycle) and rotational plans (changing drugs seasonally) over the long term is necessary to preserve drug efficacy. The producer may need to utilize less potent drugs more frequently, accepting some decline in productive performance in exchange for prevention of overt clinical problems with coccidiosis and NE, and resisting the typical response to change the coccidiostat whenever a modest rise in feed conversion is detected. Occasionally a failed drug must be abandoned for an extended period in the hope that prolonged rest and the use of unrelated

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compounds or vaccine in the interim will result in some restoration of sensitivity. While there is evidence that vaccine use can help restore drug efficacy (47, 48) the evidence for shuttle and rotational programs is less robust (10). Coccidiostat sensitivity tests in batteries may be helpful, but are expensive, time-consuming and, due to the limited number of isolates that can be tested, may not always be representative of the overall field situation (33). Nicarbazin, one of the more durable options, causes heat intolerance and can be used safely only in cooler seasons, and it must be initiated in the starter feed, as toxicity is likely if introduced in grower and later feeds, further limiting shuttle options. Nicarbazin can have severe impacts on egg production and egg quality in breeders and layers, so it must be used with care to prevent cross-contamination in mills that manufacture both broiler and breeder or layer feeds. Amprolium and zoalene are two less potent drugs that may compromise performance to some degree, but this aspect can also be used to some advantage as the coccidial leakage engenders some premunity. With proper management these "leaky" drugs are less likely to result in catastrophic failures, which have been known to occur with most of the other drugs excepting nicarbazin. These leaky drugs are generally best positioned in the earlier feeds, to take advantage of any immunity that might be generated and because early coccidial leakage has less economic consequences than late (40). Chemical coccidiostats, especially amprolium, can be used in shuttle programs or as scheduled prophylactic treatments with vaccines to reduce vaccine-associated problems, as was commonly practiced in the early days of coccidiosis vaccination (48). Prior to the use of spray cabinets for vaccine administration, it was common to treat vaccinated flocks with a low dose of amprolium approximately 10–12 days post-vaccination. However, it can be difficult to determine the dose and timing of such treatment to control vaccine issues without also circumventing immunity development and incurring NE, or creating performance issues related to delayed cycling. Development of immunity requires about 3–4 weeks whereas problems with NE tend to occur at 16–21 days, making timing of chemical treatment problematic.

Necrotic Enteritis and Dysbacteriosis

Nutritional Risk Factors

Necrotic enteritis is typically though not always associated with coccidiosis, so adequate control of coccidiosis will preclude most clinical problems with NE. Necrotic enteritis and dysbacteriosis may still be an issue in both vaccine-only and chemical coccidiostat programs even in the absence of clinical coccidiosis. Limiting substrates (both protein and energy) reaching the terminal ileum and cecum limits the proliferation of *C. perfringens*. Accordingly, use of well-balanced diets formulated with high quality ingredients is an important adjunct to success with RWA/NAE programs (5). Least-cost formulation, the use of lower priced, lower quality ingredients, sourcing of less commonly used alternative ingredients to which the birds are not accustomed, and frequent changing of raw materials to realize buying opportunities may all be problematic in these programs. Successful RWA/NAE programs are expensive, and the cost of a quality diet is a major factor in the increased expense. Since high protein levels in particular predispose to NE, nutritionists formulating for RWA/NAE programs may consider limiting total protein levels and targeting high biological value, highly digestible, high quality proteins (26, 30, 41). High inclusion rates of animal proteins, especially fish meal, have been used experimentally to reproduce NE, so it has been posited that rendered animal byproducts per se may contribute to NE, and that all-vegetable diets may therefore be an aid to RWA/NAE programs. Poor quality rendered products, with biogenic amines, rancid fats, indigestible substances, and clostridial spores are a risk in conventional production and probably even more so in RWA/NAE programs, and should be avoided in either case. However, if rendered animal protein sources are of high quality with high digestibility and high biological value, and are used in moderation, they should be safe to use if permitted by the marketing scheme. Since fish meal is especially high in zinc, glycine, and methionine, which appear to be risk factors for clostridial proliferation and toxin production, it should be used with caution (16, 30). Crystalline amino acids to help limit crude protein levels while still attaining ideal amino acid ratios and levels, and enzymes that improve digestibility and therefore promote better protein absorption in the upper gut may be beneficial (5, 26, 28). Animal fat appears to increase *C. perfringens* counts compared with vegetable oil, so there may indeed be some advantage to vegetable fats (25). Small grains (wheat, rye, oats, and barley) with high levels of non-starch polysaccharides are predisposing factors compared to corn theoretically because they increase viscosity of the digesta and slow peristalsis, favoring *C. perfringens* colonization (16, 26, 28, 30, 41). There is evidence that unknown factors in digested corn suppress proliferation *in vitro* while factors in digested wheat and barley may enhance proliferation (2). In one study, barley-derived distillers dried grains with solubles (DDGS) increased the effects of a coccidiosis/*C. perfringens* challenge model in a wheat/barley/sorghum diet (4). With small grains, it appears that a fine grind predisposes to NE compared to a coarser grind (6), but pelleting may negate the effect of grind size (17). Addition of whole small grains to the diet may also be beneficial by increasing gizzard activity, acidity, and digestion in the upper gut (18, 39). Any factor that results in irritation of the

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gut, secretion of mucus, or decreased protein digestion and absorption may predispose to NE and should be avoided. Such factors may include feed outages, sudden changes in diet composition, mycotoxins, under- or overcooked soybean meal, tannins, and so forth (26, 30, 41).

Environmental Risk Factors

Wet litter is strongly associated with NE (22), so management of the diet and ventilation to maintain dry litter is important. Clostridia do not thrive in acidic environments (20) or high salt concentrations, and acidification or salting of the litter has been used to good effect for gangrenous dermatitis and NE. Sodium bisulfite, alum, salt, or Glauber's salt (sodium sulfate) at roughly 0.25 Kg/m^2 (50 pounds per 1000 $feet^2$), and other mineral and organic acids have been used commercially. Multiple applications can be made, such as before placement and again at the anticipated time of risk for NE. Many clinicians believe that they realize benefits from acidification of the water, although controlled research to support this is lacking. Various combinations of short-chain volatile fatty acids (typically acetic and propionic), mineral acids (sodium bisulfite), methionine, and iodine are used. Care must be taken that concentrations do not restrict water intake or result in wet litter.

High stocking density is commonly accepted as a predisposing factor for NE (26). In the one paper to date directly examining the impact of density on NE, increased density failed to increase the effects of challenge at 17 days on mortality, weight gain, and feed conversion at the final 21-day weighing, but did increase lesion scores and cecal *C. perfringens* counts at 24 days, when the birds were necropsied (42). The reduced density in this study (15 birds/m^2, 0.64 $foot^2$ per bird) was approximately half of current National Chicken Council standards and the high density pens would exceed current industry standards only at market weights above 2.9 Kg (6.35 pounds) (31). Profit for the farmer is usually maximized at higher stocking densities (19), and current commercial stocking densities generally reflect the tipping point between health, welfare, performance, and profit. Whether stocking densities can be reduced enough to significantly impact the incidence of NE in RWA/NAE production while remaining commercially feasible is unknown. At present, recommendations for minor reductions in stocking density as a tool to control NE are difficult to support. Breed may influence susceptibility to NE (22, 24). Significant differences in apparent susceptibility to NE between crosses of two different male lines on the same female line were documented in a spontaneous outbreak in the field (38).

Vaccination for Necrotic Enteritis

An alpha toxoid NE vaccine administered to breeder pullets to provide maternal antibody to the progeny is licensed in the United States and has been shown to offer protection under field conditions (12). While this and other studies have demonstrated protection from alpha toxin-based preparations, it should be borne in mind that alpha toxin has been shown not to be critical in the pathogenesis (41). A limitation of any maternal vaccination strategy is that the peak age of occurrence of NE (usually at 2–4 weeks of age) coincides with the decline in maternal antibody. Multiple immunizations are necessary for a good immune response to toxoids and immunogenic proteins of *C. perfringens* and a single vaccination at day of hatch is not sufficient (29). Multiple parenteral boosters are not practical in broilers. Advancing knowledge about the virulence factors of *C. perfringens* (such as NetB and collagen adhesin) and pathogenesis may lead to more successful immunization methods in the future.

Alternative Control Measures

A variety of non-drug substances have been promoted as aids to enhance gut health and to treat or control coccidiosis and NE in antibiotic-free production. These products include probiotics, prebiotics, organic acids, botanical extracts, yolk immunoglobulins, bacteriophages, yeast products, and others (13). Probiotics and competitive exclusion (CE) cultures are available for hatchery application, usually by spray, and most of the alternatives are available for feed or water administration. Because of logistical issues, integrators usually prefer feed-administered products, especially if intended for large-scale application for prevention. Any feed-administered product must be able to survive typical pelleting conditions. Water-administered products are more commonly used for treatment and metaphylaxis. Grower compliance in administration of products in drinking water can be problematic, as can issues with fouling of drinker lines and nipples with some products. Any product used in commercial production should be approved as a food additive by the FDA, or generally recognized as safe by the FDA (GRAS), or listed by the American Association of Feed Control Officials (AAFCO). Most if not all of these alternative products are not formally cleared as new animal drugs by the FDA, and hence are not subject to the same scrutiny and regulation for purity, safety, potency, and efficacy as an approved drug.

Probiotics have been defined as live microbial feed supplements which beneficially affect the host by improving its microbial balance (30). They range from defined single-organism products to complex mixtures of undefined normal gut flora, the latter usually understood as CE cultures. The proposed modes of action include competition with pathogens for nutrients and binding sites, production of inhibitory short-chain volatile fatty acids, alteration of gut pH, production of

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antimicrobial substances such as bacteriocins, and alteration of gut immune responses (30). Competitive exclusion cultures are administered early in life, typically at day one, to promote early colonization with beneficial complex flora to exclude pathogens. Products administered in feed typically are composed of one to several organisms, and are usually spore-formers such as *Bacillus* species so they can survive pelleting. Many are not normal flora, do not permanently colonize, and must be fed continuously; these are often referred to as direct-fed microbials (DFM). Water-administered products may contain these same DFM organisms and/or non-spore-formers such as *Lactobacillus*, *Streptococcus*, and *Enterococcus* species. In a cross-sectional survey of risk factors for NE in the United Kingdom, the use of CE products did not appear to lower the risk of NE (22). Complex, undefined CE cultures generally have fared better in experimental studies than simpler, defined DFMs (13), but the CE cultures are not readily available in the United States and are difficult to mix and administer.

Prebiotics have been defined as indigestible feed ingredients that selectively stimulate the growth or activity of beneficial normal gut flora, to the detriment of pathogens (13, 30). They include carbohydrates such as lactose, lactitol, inulin, pectin, stachyose, raffinose, arabinogalactans, mannan-oligosaccharides (MOS), malto-oligosaccharides, fructo-oligosaccharides (FOS), galacto-oligosaccharides, gluco-oligosaccharides, glycol-oligosaccharides, xylo-oligosaccharides, lactulose, and lactosucrose. MOS is extracted from yeast cell walls, and in addition to its role as a carbohydrate source for beneficial bacteria, it and other yeast products are purported to bind bacterial fimbriae and act as pathogen-associated molecular patterns to stimulate the innate immune system (33). They also are reported to increase villus development (30).

Organic acids such as formic, acetic, propionic, butyric, lactic, malic, tartaric, sorbic, fumaric, caprylic, capric, lauric, myristic, and oleic acids have been suggested to improve growth and feed conversion and reduce pathogen colonization. One proposed mode of action is diffusion of the non-dissociated molecule into the bacterial cell, where it dissociates, reducing intracellular pH and exhausting the proton-ATPase pump (30). Microencapsulation of acids to preserve the non-dissociated form into the lower gut is a recently developed delivery strategy. Two reviews (1, 30) have indicated that these acids have not demonstrated the effectiveness against NE that they have against gram negative enteropathogens such as *Salmonella* and *E. coli*.

Botanical extracts such as essential oils, terpenes, flavonoids, phenolics, and saponins with purported selective antimicrobial and/or anticoccidial properties have been investigated for control of bacteria, coccidiosis, and NE, and include artemisinin, betaine, citric extracts and organic acids, *Echinacea purpurea*, gentian violet, mushrooms and their extracts, oregano (carvacrol), thyme (thymol), cloves (eugenol), mustard (allysothiocyanate), garlic (allicin), curcumin, piperine, turmeric, Persian lilac, bitter melon, green tea, cinnamon (cinnamaldehyde), capsaicin, *Yucca schidigera* extracts, eucalyptus, cabbage tree extracts, golden wattle tree extracts, seaweed extracts, marjoram, rosemary, sage, yarrow, hops, grape pomace, pennyroyal, and others (14, 33).

It is becoming more commonplace to see combinations of products, such as probiotics and prebiotics or essential oils and organic acids, in a so-called "synbiotic" approach (33). A field study showed no significant difference between the essential oils-based alternatives used in RWA flocks (21). In summary, a large volume of literature is accumulating concerning various alternative products, but as observed by at least three reviewers, there is often a publication bias toward positive results, the results overall are frequently variable or conflicting, and the alternatives to date only partially compensate for the loss of antibiotics, with slow adoption in the field (1, 23, 33).

Gut Barrier Function and Associated Issues

In Europe, cholangiohepatitis is a recognized consequence of gut barrier disruptions associated with antibiotic restriction programs. It is hypothesized that bacterial showers from the damaged intestinal epithelium escape the filtering action of the liver and may serve as the source of other systemic problems, such as bacterial arthritis, osteomyelitis (chondronecrosis with osteomyelitis), and vertebral osteoarthritis. Control of coccidiosis and NE should lessen the incidence and severity of these issues, but may not totally eliminate them. The alternative control measures listed above are also promoted to help address these issues.

Litter Moisture Issues

Deep, used litter is more absorbent than new litter and can therefore be an advantage where used litter is permitted and can be successfully managed. As indicated previously, under United States conditions used litter does not appear to be a risk factor for coccidiosis and NE, and in some cases appears protective (38). Its greater absorbency may be one reason for this observation. Control of ammonia is a greater challenge with used litter. The litter acidification amendments discussed under NE are actually used primarily to control ammonia, with any effects on clostridial proliferation in the litter being a secondary advantage. Other than these amendments and the diet, the control of both ammonia and litter moisture, which go hand in hand, is entirely dependent on

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proper ventilation. Removal of caked litter between flocks when litter is reused is important to maintain proper absorptive characteristics, to lessen footpad and skin issues, and to control pathogen pressure. While excellent production and poultry health have been achieved in the United States on used litter, disease carry-over from flock to flock can become an issue. All-in, all-out systems with adequate downtime between flocks are a must with reused litter. Other strategies such as windrowing and composting the litter between flocks or heating the houses at 38 °C for 4 days will help reduce pathogens in used litter. If disease carry-over becomes an issue in used litter systems, then a total clean-out, washing, and disinfection is necessary.

General Disease Prevention Measures

Coccidia and *C. perfringens* are ubiquitous (30, 44) and would be difficult to exclude from production facilities. Nevertheless, in a cross-sectional survey of risk factors for NE in the United Kingdom, a number of variables related to hygiene and biosecurity were associated with lower NE prevalence, including use of dedicated clothing and footwear, hand washing, downtime greater than 14 days, and cleaning and disinfection (22). Additionally, as antibiotic treatment must be minimized, general disease prevention becomes more important than ever. Biosecurity practices should be designed, promoted, enforced, and monitored to minimize not only catastrophic but also local endemic diseases. Refer to the section on biosecurity in this chapter for more guidance. Ventilation, water, litter, light, and feed presentation should be managed to decrease stressors, respiratory challenges, and challenges to the skin barrier. A water sanitation program is highly recommended. Vaccination programs for both the breeders and the broilers, particularly for immunosuppressive diseases (Marek's disease, infectious bursal disease (IBD), and chicken infectious anemia) and respiratory diseases should be robust, and proper administration and immune response carefully monitored. Adequate downtime between flocks is even more critical for RWA/NAE programs than conventional programs. More robust disease monitoring to allow more rapid detection and response is important to minimize the number of houses that must be treated and diverted to conventional markets.

Managing the Impacts of Antibiotic Restrictions in Turkey Production

While the challenges of commercial turkey production without antibiotics are similar to those for broilers, the importance of some of the challenges is different. Control of coccidiosis is very important but NE, while it can develop, occurs much less frequently. Bacterial challenges with agents such as *Bordetella avium* and *Ornithobacterium rhinotracheale* are much more prevalent in turkey production. Success in RWA/NAE turkey production requires close attention to detail, including biosecurity, water sanitation, brooding, poult quality, feed presentation, animal welfare, vaccination, ventilation, supportive care, and coccidiosis control.

Control of Coccidiosis

Coccidiosis control is much more problematic in RWA/NAE turkey production due to the lack of currently available preventative tools. As with RWA/NAE broiler production, ionophores are not permitted, and the list of available chemicals approved for use in turkeys includes only diclazuril and zoalene. Clopidol is also sometimes used for control of coccidiosis but its approval in turkeys is only for prevention of leukocytozoonosis. As in broilers, overuse of any of these compounds rapidly results in development of resistance and loss of efficacy. Because turkeys are slaughtered at older ages than broilers, coccidiosis vaccination is a good choice to control this organism. Unfortunately, there is only one vaccine marketed for turkeys in the United States and its efficacy, due to a variety of factors, has been somewhat variable.

Water Sanitation

Because bacterial infections are more prevalent in turkeys than in broilers, consistent and effective sanitation of closed drinker systems is of paramount importance. A variety of products including various forms of chlorine dioxide, chlorine, hydrogen peroxide, and iodine are available and a variety of application technologies are currently marketed. When selecting a chemical and water sanitation application system an important factor to consider is a frequent and easy monitoring system for the active ingredient. All water sanitation systems should be monitored at least weekly and more frequently in high risk flocks and high risk areas.

Ventilation

In today's modern enclosed poultry houses the role of proper ventilation to maintain proper humidity, temperature, and air quality cannot be overemphasized. The respiratory defense mechanisms of a bird are different than those of mammals and depend heavily on proper mucociliary clearance. This is especially important in turkey production where bacterial challenges are frequent. Noxious gases such as ammonia, excessive dust, and high humidity all can have a significant detrimental effect on the respiratory defense mechanisms. The result

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is bacterial-induced respiratory diseases that are sometimes not amenable to alternative therapies other than antibiotics.

Supportive Care

The ancillary and alternative products discussed under broiler production have also been advocated for use in turkeys.

Animal Welfare

Unfortunately, one discussion point that is frequently overlooked or ignored in discussions of RWA/NAE production is the welfare and comfort of the animals. Even with the best husbandry, animals will occasionally develop disease. Pressures to preserve the RWA/NAE status of a flock create a serious ethical dilemma for the veterinarian. The issue of the welfare of the animals needs to be brought to the forefront and thoroughly discussed by all parties involved. The responsibility of all participants in the food chain is not to preserve an artificial marketing status but rather the welfare and comfort of the animals.

Managing the Impacts of Antibiotic Restrictions in Table Egg Production

The VFD, prescription status for water medications, and marketing campaigns that restrict antibiotic usage have had minimal impacts on table egg production because the egg industry has essentially been working in an antibiotic-free climate for many years due to the gradual removal from the market of approved antibiotics for egg-type chickens. The list of antibiotics affected by the new regulations and labeled for egg-type pullet or layer use is limited to four clearances for pullets in feed (chlortetracycline, oxytetracycline, neomycin and oxytetracycline, and tylosin), five for pullets in water (chlortetracycline, oxytetracycline, sulfadimethoxine, sulfamethazine, and tylosin), one for layers in feed (chlortetracycline), and one for layers in water (sulfamethazine). Antibiotic usage in pullets is minimal due to good control of the most common problem, NE associated with coccidiosis, through vaccination and management. In layers, the main reason for antibiotic usage is *E. coli* infections and to a lesser degree, NE. Both of these diseases are likewise well controlled by vaccination for *E. coli* and management for both diseases. A majority of egg producers use a progressive team approach to layer health management utilizing a team involving veterinarians, a vaccinologist, a nutritionist, and a management specialist in order to accomplish an antibiotic-free program. This team must address nine main components in order to be successful in antibiotic-free (ABF) egg production.

Vaccination Programming and Administration

The veterinarian should formulate a comprehensive vaccination program that addresses all common bacterial and viral diseases for which effective vaccines exist. The second member of the team should be an expert in poultry vaccine administration. This could be a veterinarian or vaccine company representative that has expertise in the proper handling and administration of vaccines to optimize the response. It is critical to reap the maximum benefit from vaccines to avoid even mild outbreaks of disease. This person, through training of staff, observations of vaccination crews, and sampling of vaccinated flocks, should continually work toward the goal of administering the vaccines in the right condition and proper site to 100% of the birds in a flock. The increase in the number of pullet aviaries and floor growing systems has created issues in achieving optimum immunity from vaccinations due to bird movement in those systems.

Biosecurity Program

Eliminating the introduction of disease pathogens for which there are no vaccines (avian influenza or *Gallibacterium anatis* for example) and antigenic variants of existing vaccine strains is the backbone of the ABF program, with the goal of ensuring that a reduced infectious dose of pathogens will reach the flock compared to current conventional programs. Outdoor access required by some welfare programs and organic production increases the risks of disease introduction. Control measures to minimize disease introduction from wild bird or animal sources will need to be developed for each specific site. Table 1.4 lists the minimum components of biosecurity that should be included in the plan (9).

Water Sanitation

Water sanitation is a critical component of ABF programs to preclude pathogens from entering via water. *E. coli* water contamination can lead to high mortality from colibacillosis. Chlorine-based continual sanitizing systems appear to do the best job with chlorine dioxide systems being popular.

Nutrition

The third team member is a qualified nutritionist who can optimize the ingredient mix and use of enzymes to prevent bacterial diseases. The general nutritional principles discussed above to reduce NE in RWA/NAE broiler systems are largely applicable to table egg production.

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Table 1.4 Minimum components of a biosecurity plan for table egg layers (9).

- Designate a biosecurity officer for each farm
- Establish the line of separation (LOS) and perimeter buffer area (PBA) for each facility
- Establish employee parking and entry procedures into facilities; Danish entry system with dedicated farm clothing and footwear and hand sanitation at LOS recommended
- Define employee actions off farm to minimize introduction of disease (outside employment, ownership of private fowl, waterfowl hunting, etc.)
- Control employee and other personnel movement while within the LOS; the LOS may not be crossed without re-entering through the established entryway
- Establish bird movement procedures including crews and bird moving vehicles
- Establish procedures for egg pickup
- Establish procedures for manure removal
- Establish procedures for visitors and pre-visit requirements
- Establish dead bird disposal procedures
- Establish ongoing rodent, free-flying bird, and insect monitoring and control programs
- Clean water supply
- Bird source requirements
- Feed supply and delivery requirements
- Litter supply requirements
- Developing steps for proper cleaning and disinfection (C&D) of equipment, floors, walls, and ceilings between flocks

Preventative Use of Non-Antibiotic Products

The non-antibiotic alternatives discussed under broilers may be useful to decrease the risk of disease that would require antibiotic intervention. Organic acids are not used routinely in layers for either treatment or prevention of enteric disorders since over usage may affect shell quality.

Bird Management, Environment, and Housing

The fourth component in the equation for ABF production is bird management and housing, with the goal of reducing the level of stress on the bird that can be immunosuppressive. Managers should frequently consult the primary breeders' technical manuals and staff for assistance in developing programs suited to each specific breed, as well as equipment suppliers for optimal management of equipment. Optimal feed presentation, light levels and hours, air quality and temperature, water availability, space allocations, and sanitation should be assured at all times.

Non-Antibiotic Treatments for Disease

There are means of managing outbreaks of disease due to bacterial infections without the use of antibiotics. Examples include vaccination in the face of a viral or bacterial disease outbreak, additional iodine or chlorine added to water during an outbreak, increasing heat and ventilation to dry the litter during a NE outbreak, or increasing the dosage of probiotics, fermentation metabolites, prebiotics, and so on during an outbreak.

Disease Surveillance

A good disease surveillance program is needed to assess the efficacy of control programs and identify adjustments.

Training of Employees

Employee training can pay many benefits in improved disease prevention by improving their observation abilities and skills in managing flocks. Regularly scheduled training sessions put on by management and the team members in regard to biosecurity, disease recognition, vaccinations, and bird and environment management are suggested. Investing in sending key people to meetings and layer health management schools is also recommended.

In summary, an increase in management inputs will be needed to be successful in producing eggs without the use of antibiotics. Enlisting the services of a qualified veterinarian, nutritionist, vaccine expert, and management consultant on your ABF consulting team is a must to provide advice in regard to vaccination programs, vaccine administration, biosecurity, nutrition, water sanitation, use of non-antibiotic feed or water additives, bird management, use of non-antibiotic interventions during outbreaks of disease, and disease surveillance. Investments in continual training of personnel on these subjects by ABF consulting team members, industry meetings, or health management schools should be a high priority.

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Antimicrobial Therapy (Including Resistance)

Randall S. Singer, Timothy J. Johnson, and Charles L. Hofacre

Summary

Judicious antimicrobial therapy includes proper diagnosis, knowledge of antibiotic properties, dosage, spectrum, interactions, and early initiation of treatment. It is not as simple as offering the drug to a poultry flock. The limited arsenal of drugs available for poultry makes it imperative to combine an accurate diagnosis with antimicrobial knowledge to result in the most efficacious and cost-effective approach to disease treatment with minimal potential risk of antimicrobial resistance development and selection.

Introduction

Successfully treating a bacterial infection without any adverse effects involves many important factors, including the choice of antimicrobial, the route of administration, and the dose and duration of treatment. One possible side effect from antimicrobial therapy of any food animal is the potential for increasing the level of resistance in the bacterial population of those food animals. Antimicrobial resistance can lead to decreased effectiveness of future antimicrobial therapy in the food animal population, but can also pose a potential risk to human health. This topic will be reviewed later in this section. This section will not discuss the antimicrobials or the dosages to treat particular bacteria—that discussion will be left to the authors of chapters 16–24 (Section III—Bacterial Diseases) that discuss each individual bacterial infection. This section will focus on the many factors that must be taken into consideration to improve the chances of a successful treatment.

Treatment of commercial poultry can be divided into three broad categories: prevention of infection, treatment of subclinical bacterial disease, and treatment of clinically affected birds. A common application of antimicrobials in the prevention category targets clinical enteric disease, commonly referred to as necrotic enteritis (NE), resulting from a *Clostridium perfringens* infection (14). Disease prevention antibiotics are commonly given in the feed of broilers and turkeys. In contrast to disease prevention, treatment of the clinically affected birds is based on the observation of birds in the flock exhibiting clinical signs of a bacterial infection. When some birds are demonstrating clinical illness, there will be many other birds in the flock that are healthy but that have likely been exposed to the infection and are possibly incubating the disease. This entire flock will typically be treated, and thus is often described as a treatment and control administration of antimicrobials. Antimicrobials for treatment and control of disease are most commonly given in the water. The decision of whether to treat should be made by the veterinarian based upon the proportion of birds in each category, the age of the birds (how close to slaughter), the value of the birds (breeders vs. broilers), and many other factors that will be discussed in detail.

Antimicrobial therapy in US poultry production changed dramatically in January 2017 due to key regulation and policy revisions. In 2012 the US Food and Drug Administration (FDA) published *Guidance for Industry* (GFI) #209 which described a broad policy shift regarding antimicrobial drugs used in animal agriculture. This document was intended to limit medically important antimicrobial drugs to uses in food-producing animals that: (1) are only considered necessary for ensuring animal health, and (2) include veterinary oversight or consultation (29). This document was followed in 2013 with GFI #213 (30), which provided more detail on implementing the key principles in GFI #209. Specifically, GFI #213: (1) defined the term "medically important", (2) voluntarily removed claims relating to production uses (growth promotion/feed efficiency), and (3) brought remaining therapeutic uses under veterinary oversight by changing the marketing status from over the counter (OTC) to Veterinary Feed Directive (VFD) or prescription (Rx). Finally, the VFD regulation was updated, and these new regulations went into effect in October 2015 (31). The VFD describes requirements relating to the distribution and use of VFD drugs (feed-use drugs that require supervision of a licensed veterinarian) and is considered a critical step for facilitating the transition of antimicrobial therapy in animal agriculture to veterinary oversight.

A point of confusion for many in the general public was related to the term "voluntary" in GFI #213. The only aspect of this policy that was voluntary was the request by FDA to have the animal pharmaceutical companies remove indications for growth promotion/feed efficiency from all labels of medically important antibiotics. Once these changes were made, the veterinarian would be required to follow the label instructions, as there is no permitted extralabel use of in-feed antibiotics. All of the animal pharmaceutical companies complied with GFI #213, and therefore, as of January 1 2017 there are no longer approved medically-important antibiotics for growth promotion/feed efficiency in the United States. Tables 1.5 and 1.6 show the currently

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Table 1.5 Approved feed medication of US poultry that requires a Veterinary Feed Directive (VFD). Table courtesy of Dr. Steven Clark.

VFD medications	Chicken	Turkey
Albamix (Novobiocin)	√	√
Aureomycin® (Chlortetracycline)	√	√
ChlorMax® (Chlortetracycline)	√	√
Inteprity™ (Avilamycin)	√	—
Lincomix® (Lincomycin)	√	—
Neo-Oxy® (Neomycin + Oxytetracycline)	√	√
Neo-Terramycin® (Neomycin + Oxytetracycline)	√	√
Pennchlor® (Chlortetracycline)	√	√
Pennox® (Oxytetracycline)	√	√
Pharmastatin (Nystatin)	√	√
RofenAid® (Sulfadimethoxine + Ormetoprim)	√	√
Stafac® (Virginiamycin)	√	—
Terramycin® (Oxytetracycline)	√	√

Table 1.6 Approved feed medication of US poultry that does not require a Veterinary Feed Directive (VFD). Table courtesy of Dr. Steven Clark.

NonVFD medications	Chicken	Turkey
Albac® (Bacitracin Zinc)[a,b]	√	√
Amprol® (Amprolium)	√	√
Avatec® (Lasalocid)	√	√
Aviax® (Semduramicin)	√	—
Bio-Cox® (Salinomycin)	√	—
BMD® (Bacitracin Methylene Disalicylate)[b]	√	√
Clinacox® (Diclazuril)	√	√
Coban® (Monensin)	√	√
Coyden® (Clopidol)[a,c]	√	√
Deccox® (Decoquinate)	√	—
Flavomycin® (Bambermycin)[b]	√	√
Hygromix® (Hygromycin B)[a]	√	—
Maxiban® (Narasin + Nicarbazin)	√	—
Monteban® (Narasin)	√	—
Nicarb® (Nicarbazin)	√	—
Robenz® (Robenidine HCL)	√	—
Sacox® (Salinomycin)	√	—
Stenorol® (Halofuginone)[a]	√	√
Topmax™ (Ractopamine)[a]	—	√
Zoamix® (Zoalene)	√	√

[a] Not currently marketed.
[b] Includes label claim for improved weight gain and feed conversion.
[c] As an aid in prevention of leucocytozoonosis caused by *Leucocytozoan smithi*.

Table 1.7 Approved water soluble medication of US poultry that requires a prescription. Table courtesy of Dr. Steven Clark.

Prescription medications	Chicken	Turkey
Aureomycin® Soluble (Chlortetracycline)	√	√
Di-Methox® (Sulfadimethoxine)	√	√
Lincomycin Hydrochloride Soluble (Lincomycin HCL)	√	
Neo-Sol® (Neomycin)	—	√
NeoMed® (Neomycin)	—	√
Oxytet® Soluble (Oxytetracycline)	√	√
Pennchlor 64® (Chlortetracycline)	√	√
Pennox 343® (Oxytetracycline)	√	√
PoultrySulfa® (Sulfamerazine, Sulfamethazine, Sulfaquinoxaline)	√	√
TetraMed® 324 HCA (Tetracycline)	√	√
Tetroxy® HCA Soluble (Oxytetracycline)	√	√
Tet-Sol™ 324 Soluble (Tetracycline)	√	√
Tylan® Soluble (Tylosin Tartrate)	√	√
Tylovet® Soluble (Tylosin Tartrate)	√	√

Table 1.8 Approved water soluble medication of US poultry that does not require a prescription. Table courtesy of Dr. Steven Clark.

Non-prescription medications	Chicken	Turkey
Amprol (Amprolium)	√	√
BMD® Soluble (Bacitracin Methylene-Disalicylate)	√	√
Safe-Guard® AquaSol (Fenbendazole)	√	—

approved feed medications for US poultry, and Tables 1.7 and 1.8 show the currently approved water soluble medications for US poultry.

Routes of Medication

Commercial poultry are raised to provide a safe, wholesome protein source that is economical for the world's human population. To that end, the welfare of the bird and the cost of the meat must be accounted for. In disease prevention, it is generally accepted that feed-grade antimicrobials are less expensive than the same drug in a water-soluble formulation. It must be emphasized that sick birds will have a decline in both feed and water consumption. However, the decline in water consumption is usually less than the decline in feed consumption. Therefore, in choosing a route to administer an antimicrobial to a clinically affected flock, especially early in the infection, water medication may be more effective

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than by the feed. In the event that the course of the disease lasts longer than 5–7 days, as is often the case with some diseases such as *Pasteurella multocida* infection in breeders, the veterinarian may choose to switch the route of administration after initially reducing the signs by water medication to the same drug in the feed.

Another consideration in selecting a water route of administration is the ambient temperature. Because poultry have very limited means to eliminate heat from their bodies, they utilize the cooling effect of increasing water consumption. Therefore, water consumption increases significantly as the ambient temperature increases. This must be taken into account when selecting an antimicrobial and its dosage. This is especially important when considering the use of a sulfonamide, because the therapeutic dose is close to the level that can result in toxicity (12).

Flock treatment is almost always the preferred route, and thus mass methods of administering antimicrobials are generally used. Therefore, parenteral administration of antimicrobials to individual birds in an entire flock is cost prohibitive except when the flock is in the hatchery, that is, *in ovo* at 18–19 days of incubation or 1 day of age. If an antimicrobial is to be administered in the hatchery, be aware of the effects some antimicrobials may have on any live vaccine that may be concurrently administered. For example, the aminoglycoside gentamicin has a highly basic pH and can damage the cells for the cell-associated Marek's disease vaccine if used at too high a dose (greater than 0.2 mg/chick) or if the antibiotic is improperly mixed with the vaccine in the diluent (21).

Feeding, watering, and lighting schedules also must be taken into consideration. Laying hens will begin to eat, and then consume water, when the lights are turned on. In replacement birds that are under feed restriction to control body weight, both feed and water are limited to only a few hours each day. Broiler chickens and turkeys, which have continuous feed and water availability, tend to eat and then drink on intermittent intervals of 3–4 hours.

Administration of Antimicrobials to Commercial Poultry

Antimicrobials administered in the feed must be uniformly mixed and remain stable until consumed. The prescribing veterinarian must take into consideration the length of time to have the feed manufactured and transported to the farm and then the length of time to deliver it through the farm's feeding system (i.e., amount of nonmedicated feed currently in the feed tank).

Administering the antimicrobial in the drinking water allows for a more rapid delivery of the antimicrobial but requires several calculations to be considered:

- Freshly medicated solutions should be prepared every day.
- The volume of water consumed in 24 hours in the house to be treated must be determined.
- Bulk tank medication administration method is achieved by adding the volume of medication for that day into the total volume of water to be consumed by the flock for that day.
- The proportioner administration method is used for farms that do not have a bulk tank. A water proportioner is a device that meters the antimicrobial from a highly concentrated "stock" solution into the drinking water to achieve the appropriate concentration.
- Dosing based on body weight (i.e., mg/kg of body weight) of a representative sample of birds is much preferred to dosing based on water consumption. If the dose is calculated on water consumption, the ambient temperature must be taken into consideration or a toxic overdose may occur if the temperature rises or an under dose may occur below the therapeutic level if the temperature declines. A rule of thumb is for every 1 °F increase in environmental temperature above 70 °F results in a corresponding increase of water consumption by approximately 4%. In addition, younger birds consume more water daily/unit of body weight than older birds. Hens in egg production drink more water/unit of weight than non-laying hens or roosters.
- Pulse dosing can be considered when the birds' water consumption is limited (i.e., broiler breeder pullets) (3). This is a short intensive treatment in which all of the medication to be administered in a 24-hour period is consumed by the flock in a 4- to 6-hour period. Note: This method should only be used with bactericidal antimicrobials that have a wide margin of safety for toxicity.

Pharmacologic Consideration

The primary goal of antimicrobial treatment is to cure the flock from the current illness. Success requires taking many interacting factors into consideration. The activity of an antimicrobial against a bacterial strain is referred to as the targeted strain being either resistant or sensitive. The methods used to determine this sensitivity of a particular isolate are all performed on artificial media in a diagnostic laboratory. They do not consider whether the drug can be absorbed from the birds' intestines (i.e., aminoglycosides) or whether the drug is bound by ingredients in the feed or water (i.e., tetracyclines/calcium level) or whether the drug can reach the site of the infection (i.e., synovial fluid of a joint). It should also be remembered that the *in vitro* susceptibility is usually determined on only one bacterial isolate from the flock and in many infections of poultry the bacterial infection is often secondary to a viral or environmental insult. This

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results in a flock infection of several different bacteria which may have a wide range of antimicrobial susceptibilities. This is especially true with *E. coli* airsacculitis (11).

The immune status of the flock also must be considered when selecting an antimicrobial agent. A bacteriostatic drug, such as oxytetracycline, may be highly effective for treating *E. coli* secondary to an infectious bronchitis virus challenge. For an *E. coli* airsacculitis infection following the immune suppressive virus, infectious bursal disease (IBD), the same oxytetracycline therapy may be ineffective in curing the flock. In cases in which the immune system is compromised, it is recommended to use bactericidal antimicrobials because bacteriostatic drugs inhibit or slow the bacterial growth and require the birds' immune system to kill the bacteria.

Judicious Use Principles in Poultry

Judicious use of antimicrobials in poultry that are being raised for production of meat or eggs for human consumption begins with disease prevention. However, when a flock begins to exhibit the clinical signs of a bacterial disease, the veterinarian must base the decision to treat upon good professional judgment (i.e., experience), laboratory results, medical knowledge, and information about the flock to be treated. The birds should be physically examined, if possible, by the veterinarian or by a skilled paraprofessional (service person) which should include antemortem and postmortem examination. When possible, a bacterial culture can be done to confirm the diagnosis and determine the susceptibility of the isolates. The rapid spread of disease on poultry farms often necessitates beginning treatment prior to the results of the bacterial culture and sensitivity. When laboratory results are completed, the veterinarian must use clinical judgment to decide between continuation or change in therapy. Because a flock will have birds in the three categories of illness (clinically ill, incubating with no outward signs of illness, unaffected susceptible), all of the birds in a house and not just the clinically affected will be treated. This strategic use of antimicrobials in anticipation of a major disease spread is justifiable under good husbandry practices. Finally, responsible antimicrobial therapy allows sufficient withdrawal time for the antimicrobial from the feed or water to ensure no drug residue in the meat or eggs for human consumption. In some instances, the veterinarian may require a longer withdrawal than is written on the drug label because of clinical judgment. For example, some sulfonamides are excreted in the birds' droppings in an altered but active metabolite and because birds are coprophagic, they may have sulfonamide exposure even after the drug is removed from the feed or water.

There are many sets of guidelines globally for the judicious use of antimicrobials. The American Veterinary Medical Association (AVMA) has a general set of principles (1), and these include: "Therapeutic exposure to antimicrobials should be minimized by treating only for as long as needed for the desired clinical response"; "Regimens for antimicrobial treatment, control, or prevention of disease should be based upon current scientific and clinical principles, such as microbiological and pharmacological tenets"; and "Antimicrobial use should be confined to appropriate clinical indications. Inappropriate uses such as for uncomplicated viral infections should be avoided." Judicious use principles specific to the poultry industry have also been developed, for example by the American Association of Avian Pathologists (AAAP) in conjunction with the AVMA (2). In general, veterinarians should strive to optimize therapeutic efficacy and minimize resistance to antimicrobials to protect public and animal health.

Antimicrobial Resistance

Antimicrobial resistance is a growing concern for human health because of the increasing incidence of bacterial infections that are refractory to antimicrobial therapy. Many of the genes encoding antimicrobial resistance are transferable between bacteria, and therefore, resistance genes that are present in bacteria of animals can be transferred to bacteria that cause human disease. Of course, the reverse is also true. All uses of antimicrobials in animal agriculture have the potential to increase the prevalence, distribution, and spread of resistant bacteria and resistance genes, again highlighting the need to observe judicious use principles when antimicrobial therapy is needed. Currently, the degree to which the use of antimicrobials in poultry impacts antimicrobial resistance in human bacterial pathogens remains uncertain but is definitely non-zero (28).

All uses of antimicrobials have the potential to select for bacteria that can survive in the presence of that antimicrobial. Therefore, development of a large number of bacteria that are resistant to an antimicrobial is greatly dependent on the level of that antimicrobial agent that contacts the bacterial population, such as at the site of infection in the bird's body. If the dose of the antimicrobial does not reach a concentration high enough to kill or inhibit the target bacterium, then a selection pressure exists that can shift the bacterial population toward a population that can survive in the presence of that antimicrobial. Even if the dose is high enough to kill or inhibit the target bacterium, those bacteria in the population that possess mechanisms capable of resisting the action of the antimicrobial will survive, and these resistant bacteria can then spread.

There are specific genes that give the bacterium the ability to survive in the presence of an antimicrobial. Some are genes normally present on the bacterial

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genome that mutate to a form that renders the antibiotic ineffective. Other genes are acquired from other bacteria, a process known commonly as horizontal gene transfer. An example of gene mutation to allow the bacteria to grow in the presence of the antimicrobial occurs when there is a mutation in the DNA gyrase gene that results in fluoroquinolone resistance. These resistant bacteria survive to reproduce and the resistance then spreads by multiplication of the resistant bacterial strain.

The rapid dissemination of most antimicrobial resistance in Gram-negative bacteria is primarily achieved through horizontal gene transfer, or the movement of genetic material between two unrelated bacterial cells (10). Genes encoding antimicrobial resistance are most commonly moved between bacteria via their presence on conjugative bacterial plasmids (16). Plasmids are extrachromosomal elements that are self-replicating, not essential to the bacterial host cell, and often capable of self-movement (conjugation) from one bacterium to another. They are also often highly stable once established in a bacterium. Plasmids are associated with resistance to antimicrobial agents because of their propensity to acquire additional genetic material within their genome, and they come in a variety of different types, each with unique replicons and a distinct set of genetic traits (16). Plasmid type determines many phenotypic traits, such as the frequency of conjugative transfer, the range of bacterial hosts in which it can successfully replicate, and the propensity to acquire resistance genes. Among *E. coli* and *Salmonella enterica* alone, there are more than 30 plasmid types identified and this number continues to grow (10). Plasmids associated with multidrug resistance are primarily a concern among *E. coli*, *S. enterica*, and *Klebsiella pneumoniae*, although numerous other Gram-negative bacteria have been shown to possess multidrug resistance-encoding plasmids (6, 9, 32).

Each plasmid type has one or more distinct "genetic load" regions where they are able to acquire accessory genes, such as antimicrobial resistance genes. Some plasmids have more of these regions than others, and some plasmids are able to acquire more genetic load within a region than others. Resistance genes are usually inserted into these genetic load regions via conjugative transposons or mobile units called integrons (23). With integrons, resistance genes are not fixed once acquired; that is, they can be discarded and/or additional genes can be acquired at any time. These flexible elements play a major role in plasmid evolution and the evolution of resistance phenotypes. The primary multidrug resistance-associated plasmid types that are a concern in poultry are known as IncF, IncI1, and IncA/C (18). Each type mentioned has signature sets of resistance genes that they commonly possess. For example, IncA/C plasmids often contain the genes $bla_{\text{CMY-2}}$ and *floR*, encoding resistance to third-generation cephalosporins and phenicols, respectively (6). IncI1 plasmids commonly carry extended spectrum beta lactamase genes belonging to the bla_{TEM} and bla_{CTX} classes. In poultry, *E. coli* and *Salmonella* spp. often harbor these genes and plasmids.

In poultry production, plasmid-associated antimicrobial resistance is of concern for several reasons. First, many plasmids are considered to be highly plastic and capable of acquiring arrays of resistance genes encoding resistance to multiple antimicrobial agents. For example, IncA/C plasmids have recently been identified among *E. coli* and *S. enterica* of poultry and encode resistance to up to 12 different classes of antimicrobial agents (18). In addition to the carriage of multiple resistance genes by a single plasmid, bacteria of poultry commonly carry multiple plasmids (18). Finally, co-carriage of antimicrobial resistance genes with genes conferring other phenotypes routinely occurs. For example, avian pathogenic *E. coli* often carry virulence factors that co-reside with antimicrobial resistance-encoding genes (17). Furthermore, these plasmids also may possess genes encoding resistance to heavy metals and disinfectants (15). Therefore, a scenario emerges in which resistance genes may be selected for in the absence of antimicrobial pressures. This complicates the ability to control the dissemination of multidrug-resistant bacteria once they are established in an environment. Examples of these complex plasmid structures containing antimicrobial resistance and disinfectant and heavy metal resistance as well as virulence factors can be seen in Figure 1.10A, B, and C.

The dissemination of multidrug resistance in poultry has become a major concern in *Salmonella* spp. because of the potential risk that these bacteria pose to human health via foodborne transmission. It seems that certain *Salmonella* serovars have a greater propensity to acquire multidrug resistance than others, and one serovar of particular concern in poultry is *Salmonella* Heidelberg. *S.* Heidelberg isolates harboring multiple resistance genes have been identified in live chickens, live turkeys, humans, and retail meats, and some are identical using pulsed-field gel electrophoresis (13, 20, 26). Of particular concern are the IncA/C plasmids, which have been identified among serovars Heidelberg, Kentucky, and Typhimurium in poultry and humans (7, 8, 24, 25). Isolates harboring this plasmid are typically resistant to ampicillin, amoxicillin/clavulanic acid, cefoxitin, ceftiofur, chloramphenicol, sulfisoxazole, tetracycline, trimethoprim/sulfamethoxazole, and gentamicin. Other plasmid types, such as IncF and IncI1, are also common among *Salmonella* spp. of poultry but do not confer such a wide array of phenotypic resistances. Also, unlike IncI1 and IncF plasmids, IncA/C plasmids have a broad host range and are likely also moved between *Salmonella* spp. and other Proteobacteria within the environment (19).

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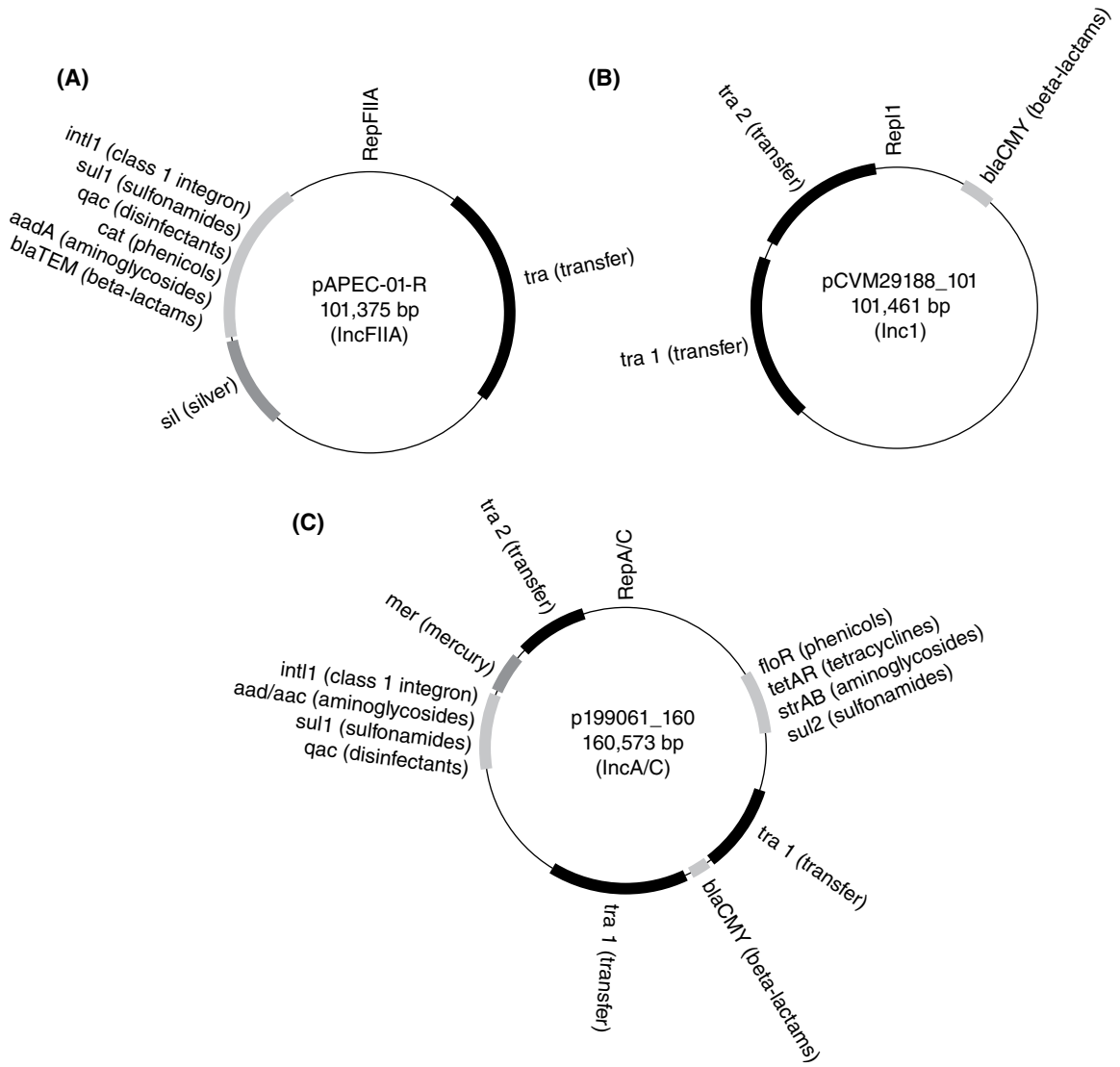


Figure 1.10 Circular maps of transmissible resistance-encoding plasmids isolated from poultry bacteria. Transfer regions are shaded in black, antibiotic resistance genes are shaded in light gray, and heavy metal resistance genes are shaded in dark gray. (A) Map of pAPEC-O1-R, an IncFIIA plasmid isolated from avian pathogenic *E. coli*. (B) Map of pCVM29188_101, an IncI1 plasmid isolated from S. Kentucky. (C) Map of p199061_160, an IncA/C plasmid isolated from avian pathogenic *E. coli*.

Once multidrug-resistant bacterial populations are established within an environment, they are difficult to eliminate. Certainly, plasmid dissemination from one bacterium to another can occur within the avian gastrointestinal tract, within poultry litter, and among beetles (4, 22, 27). There is documented evidence that antimicrobial therapy in response to disease will enhance the dissemination of plasmid-encoded multidrug resistance (5). Cessation or reduction of antibiotic usage has been suggested as a method to reduce the numbers of multidrug resistant organisms. However, the evidence for this effect is contradictory in the scientific literature. The best current practices to limit the spread of multidrug-resistant organisms are likely the same as those used to reduce disease transmission, such as thorough clean-out procedures and good biosecurity practices. In those instances in which a bacterial infection occurs and treatment becomes necessary, follow judicious use guidelines with isolation of the bacteria, determination of antimicrobial sensitivity, and use the appropriate dose and duration of therapy.

Acknowledgement

The authors wish to acknowledge Steven Clark for providing the information in Tables 1–4.

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Public Health Significance of Poultry Diseases

Roy D. Berghaus and Bruce Stewart-Brown

Summary

Several poultry diseases have the potential to impact human health. Some diseases are rarely reported in humans but are of theoretical concern. Others are commonly associated with human illness. Direct transmission of pathogens to poultry workers is uncommon; however, it should not be discounted. Human infections with certain lineages of H5N1 and H7N9 avian influenza viruses are recent high profile examples of bird-to-human transmission. Foodborne pathogens such as *Salmonella* and *Campylobacter* are primarily a concern for consumers, but also have the potential to infect individuals working in production and processing environments. Educating poultry workers with respect to zoonotic pathogens and their modes of transmission is an important step toward disease prevention.

Introduction

Poultry and humans have dramatically different physiologies, and many pathogens are incapable of crossing the species barrier between birds and man. Nonetheless, there are a number of diseases that humans and poultry share (1). These can be zoonoses (poultry to humans), reverse zoonoses (humans to poultry), or diseases acquired from a common environmental reservoir. Most zoonotic diseases can be prevented through an understanding of basic disease transmission principles and the adoption of preventive practices.

Infectious organisms may be transmitted by direct or indirect mechanisms. Direct routes include body-surface to body-surface contact, contact with soil or vegetation harboring infectious organisms, and large droplet transmission over short distances. Indirect routes include airborne transmission by small particles suspended in air, vehicle-borne transmission by objects that passively carry the organism or provide an environment for growth, and vector-borne transmission by an insect or other living carrier via mechanical carriage or biological propagation.

Common biosecurity practices including the use of gloves, eye and respiratory protection, and protective outerwear are all important elements of zoonotic disease prevention (95). Good hand hygiene and routine injury prevention are also essential. Thoroughly washing hands with soap and water after working with poultry is always recommended. When soap and water are not available, the use of alcohol-based hand sanitizers may be an effective alternative. Protection of eyes, nose, and mouth helps reduce the risk of mucous membrane exposure and inhalation. Protective outerwear should include disposable or reusable coveralls that can be sanitized between uses, head covers to help keep the hair and scalp free of contamination, and disposable or washable footwear. Skin lacerations should be kept covered, and injuries resulting from contact with animals or equipment should be promptly cleaned and protected. Eating and drinking should be done away from the poultry house.

All personnel that work around poultry should be trained and educated about zoonotic disease prevention. Persons with weakened immune systems are at increased risk for contracting many zoonotic diseases. Populations with increased susceptibility may include young children, pregnant women, the elderly, and persons who are immunocompromised due to medications or disease. Individuals with immune dysfunction are encouraged to discuss their health status with a health care professional before working around poultry or other animals.

This chapter provides a brief overview of public health issues for several infectious diseases that are common to poultry and humans. It does not include all such diseases and it is not meant to serve as a human medical reference. Rather, it provides a short synopsis of the disease manifestations in humans and may serve as a starting point for further inquiry. Diseases are presented alphabetically within categories defined by the type of infectious agent (i.e., viral, bacterial, fungal, and parasitic). For each organism or disease, there is a brief description of the nature of the disease in humans, its occurrence, and reservoirs and sources of infection.

Viral Diseases

Arboviral Encephalitis

Arbovirus is a generic term referring to viruses transmitted to vertebrates by the bite of arthropod vectors including mosquitos, ticks, and flies. More than two dozen arboviruses are capable of causing neurological disease in humans (40). However, the discussion here will be limited to three that cause disease in both humans and poultry: Eastern equine encephalitis virus (EEEV), Western equine encephalitis virus (WEEV), and West Nile virus (WNV).

Nature of the Disease in Humans

The clinical presentation varies with virus and host characteristics, but most infections are asymptomatic.

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Of those patients that do become ill, most experience a flu-like illness with sudden onset fever, headache, and fatigue. A variable number of patients progress to develop neuroinvasive disease with typical signs of meningitis or encephalitis. Recovery from neuroinvasive disease can take several weeks to months, and sequelae such as weakness and paralysis are common. Case-fatality risks for patients with severe illness caused by EEEV, WEEV, and WNV infections have been estimated at 50–70%, 3–7%, and 1–17%, respectively (3, 66).

Occurrence

The distribution of arboviruses is dependent on their specific reservoir hosts and vectors. EEEV and WEEV are found in both North and South America. In North America, EEEV is found primarily along the Atlantic and Gulf coasts, and WEEV is found primarily in the western United States. WNV has been identified in Europe, Africa, Asia, Australia, and North and South America. Between 1999 and 2007, the number of human WNV neuroinvasive cases reported in the United States was 11,125, compared to 80 cases for EEEV and one case for WEEV (70). In North America, approximately 90% of human cases are identified between July and September. Neuroinvasive disease is more likely in the elderly and in persons infected through organ transplantation (66).

Reservoirs and Sources of Infection

The principal mode of transmission to humans is from the bite of an infected arthropod vector. For all three of the viruses discussed here, the primary enzootic transmission cycles occur between birds and mosquitos. Transmission is not limited to ornithophilic mosquito species, however, as several other genera of mosquitos have the potential to act as bridging vectors between birds and humans. The incubation period is typically 3–14 days (40). Humans do not produce a sufficient viremia to serve as an amplifying host; however, non-vector-borne transmission has been documented via blood transfusion, organ transplantation, breastfeeding, and needlestick injury. Infections have been reported in workers on goose and turkey farms during WNV outbreaks, although it is not clear whether this may have been attributable to contact with infected birds or concurrent transmission from infected mosquitos (9, 21).

Avian Influenza

Avian influenza (AI) is caused by type A influenza viruses which are classified by their hemagglutinin (H1–H16) and neuraminidase (N1–N9) subtypes. Human disease caused by direct infection with poultry-adapted viruses is rare, but has been reported sporadically with some specific genetic virus lineages (i.e., A/goose/Guangdong/1/1996 [H5N1], Gs/GD lineage, and A/Anhui/1/2013 [H7N9], Anhui lineage) in some subtypes: H5N1, H5N6, H6N1, H7N2, H7N3, H7N7, H7N9, H9N2, H10N7, and H10N8 (31). Pathogenicity in poultry is not indicative of the pathogenicity in humans.

Nature of the Disease in Humans

Clinical manifestations of human infections with AI viruses vary from mild to severe and depend on the subtype. H7N7 Eurasian virus has mainly been associated with conjunctivitis and mild influenza-like illness, while H5N1 and H5N6 Gs/GD lineage and H7N9 Anhui lineage viruses have been associated with more severe respiratory disease (13). Common presenting signs include fever, cough, dyspnea, pneumonia, and myalgia. In some cases, gastrointestinal signs have been reported. Acute respiratory distress syndrome is a common complication of H5N1 and H5N6 Gs/GD lineage and H7N9 Anhui lineage virus infections, which have reported case-fatality risks of 60% and 40%, respectively (13, 41).

Occurrence

AI viruses are found worldwide, although the distribution of specific subtypes varies. Most cases of human infection reported in recent years have been caused by the H5N1 Gs/GD lineage and H7N9 Anhui lineage subtypes (31). Between 2003 and March 2017, 858 human illnesses and 453 deaths due to H5N1/H5N6 AI viruses (Guangdong lineage) were reported to the World Health Organization (WHO) from 16 countries across Asia, Africa, and the Middle East (94). Between March 2013 and February 2017, annual epidemics of H7N9 Anhui lineage virus infections in China were associated with 1,258 human cases (41). All other AI virus subtypes combined accounted for approximately 124 documented human infections between 1959 and 2014, with 93 of these cases being attributed to H7N7 Eurasian lineage (31).

Reservoirs and Sources of Infection

Wild aquatic birds belonging to the orders Anseriformes and Charadriiformes are the natural reservoirs for AI viruses. Influenza viruses are shed in the feces and respiratory secretions of infected birds. Direct or indirect exposure to infected poultry or contaminated environments is believed to be the most important route of transmission to humans. Mucous membrane exposures and the inhalation of potentially infectious aerosols should be avoided. Ingestion is of theoretical concern although AI viruses are readily destroyed by cooking and to date there have been no documented cases of human infection due to the consumption of cooked poultry or eggs. The incubation period for H5N1 viruses is typically 2–5 days, but may be as long as 7 days (13). Person-to-person transmission of AI viruses is uncommon, but limited transmission has been reported among relatives having close contact with persons infected with the

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H5N1 Gs/GD and H7N9 Anhui lineage viruses. Viral RNA of H5N1 viruses has been identified in the respiratory secretions of infected persons as long as 3–4 weeks after onset.

Preventive Measures

The US Centers for Disease Control and Prevention (CDC) recommends that persons involved with AI outbreak control and eradication procedures wear appropriate personal protective equipment including disposable gloves, protective outer garments, shoe covers, and a fitted respirator of class N-95 or higher (20). The CDC also recommends that workers receive a seasonal influenza vaccination and prophylactic antiviral drugs while handling potentially infectious materials.

Newcastle Disease

Newcastle disease, also known as Ranikhet disease, avian pneumoencephalitis, and pseudo-fowl pest, is caused by avian paramyxovirus serotype 1 (APMV-1) (25, 84). The virulence of strains varies widely, with the severity of disease in poultry ranging from inapparent to near 100% mortality. However, virulence in birds is not a predictor of the potential for human infection.

Nature of the Disease in Humans

In humans, NDV typically causes a transient, unilateral, acute follicular conjunctivitis with no involvement of the cornea. Swelling of the preauricular lymph nodes is common. Conjunctivitis typically lasts for 3–4 days but may persist for as long as 3 weeks. Mild generalized signs of illness such as low-grade fever, chills, headache, and pharyngitis are uncommon but may be more likely following an aerogenous exposure (38). Patients typically make a complete spontaneous recovery.

Occurrence

Newcastle disease virus has been reported in every poultry-producing region of the world. Vaccination is widely practiced, although periodic outbreaks still occur in countries where virulent strains of the virus are no longer endemic. Human infection following contact with infected live birds is uncommon (84). Most reported cases have been in diagnostic and vaccine laboratory workers, veterinarians, and processing plant workers (25).

Reservoirs and Sources of Infection

Birds are the natural reservoir for NDV; over 240 species have been reported to be susceptible to infection (43). Transmission between birds occurs by inhalation of respiratory droplets or the fecal–oral route. Transmission to humans occurs by splashing contaminated liquids in the eye, or by touching the eyes after contact with contaminated tissues or feces. The incubation period in humans ranges from 1–4 days, but 1–2 days is typical (38). Most references flatly state that secondary transmission has never been documented. Transmission from infected humans to susceptible poultry, however, is a potential concern (84).

Preventive Measures

Eye protection should be worn when working with NDV in the laboratory or when handling live vaccines or infected tissues. Wearing disposable gloves and washing hands with soap and water after handling infectious materials is also advisable. Wearing a respirator or mask reduces the risk of aerosol inhalation, although human infection by this route is believed to be uncommon.

Bacterial Diseases

Botulism

Botulism is a paralytic intoxication caused by botulinum toxin, which prevents acetylcholine release from motor neuron synaptic terminals. Botulinum toxin is produced by *Clostridium botulinum* as well as related species *C. baratii*, *C. butyricum*, and *C. argentinense* (68). Although *C. botulinum* is considered a single species, different strains can be distinguished by the type of toxin they produce. There are seven recognized toxin types (A–G), but only types A, B, E, and rarely F cause human illness. Toxin types C and D are the most common causes of botulism in wild birds and poultry but are not associated with human disease (19). Cattle and sheep are susceptible to type C and D toxins, however, and several outbreaks in these species have been linked to poultry litter exposure (81).

Nature of the Disease in Humans

Four naturally occurring forms of botulism are recognized in humans: foodborne intoxication; wound botulism; infant botulism; and adult intestinal toxemia (toxicoinfection). Regardless of the form, the clinical presentation is characterized by flaccid symmetric descending paralysis that begins with cranial nerve palsies and may progress to respiratory arrest. The availability of antitoxin along with improvements in supportive care and mechanical ventilation have improved the case-fatality risk.

Occurrence

Clostridium botulinum has a worldwide distribution. Most foodborne intoxications result from the consumption of improperly preserved home-canned foods. Wound botulism is typically associated with deep tissue injuries such as open fractures, or in recent years with the nonintravenous injection of black tar heroin (33). Infant botulism is believed to result from a toxicoinfection rather than the ingestion of preformed toxin, and has

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been associated with the consumption of honey. Adult intestinal toxemia is rare, and occurs in patients with a history of abdominal surgery, gastrointestinal abnormalities, or recent disruption of the normal flora because of antibiotic administration.

Reservoirs and Sources of Infection

Clostridium botulinum is found in soils throughout the world. Heat resistant *C. botulinum* spores are capable of surviving many food preparation methods, and germination occurs when they are exposed to a warm anaerobic environment with nonacidic pH (greater than 4.6) and low salt and sugar concentrations. The incubation period for foodborne intoxications is typically 12–36 hours, but may range from 6 hours to 10 days (19). Contact transmission from animal-to-person or person-to-person does not occur.

Clostridium perfringens Infection

Clostridium perfringens causes two different types of foodborne disease as well as gas gangrene in humans (14). Foodborne disease is usually caused by enterotoxin producing strains of *C. perfringens* type A, and rarely by *C. perfringens* type C.

Nature of the Disease in Humans

Clostridium perfringens type A food poisoning results when enterotoxin is produced during sporulation of vegetative cells in the intestine. Typical symptoms include acute abdominal pain and cramping, nausea, and diarrhea. Most cases are self-limiting and resolve without treatment in 24 hours (80). *C. perfringens* type C food poisoning is primarily mediated by beta-toxin and is associated with necrotic enteritis (NE) in humans. Symptoms include acute abdominal pain and distension, bloody diarrhea, and sometimes vomiting (14). The case-fatality risk for type A food poisoning is less than 0.1%, while that for type C food poisoning is 15–25% (14, 74).

Occurrence

Clostridium perfringens type A is one of the most common causes of foodborne disease worldwide. In the United States, it causes an estimated one million illnesses annually (74). *C. perfringens* type C food poisoning is rare, and is usually limited to patients with abnormally low intestinal protease production who are unable to inactivate beta-toxin. Young children and the elderly are at increased risk for severe illness due to type A food poisoning, while malnourished individuals and diabetics are at increased risk for developing NE due to type C food poisoning (14, 80).

Reservoirs and Sources of Infection

Clostridium perfringens is a common inhabitant of soil and intestinal tracts of animals and humans, and is commonly isolated from retail meat products. Between 1998 and 2010, poultry was implicated in 30% of foodborne *C. perfringens* outbreaks in the United States that could be attributed to a single food commodity (36). Improper food handling, especially the inadequate cooling and reheating of meat-containing dishes, is a common contributing factor. Spores survive the initial cooking, and after germination can propagate rapidly under inadequate refrigeration. The incubation period for type A food poisoning ranges from 6–24 hours, but is most commonly 10–12 hours. Direct exposure to infected persons or animals does not constitute a disease risk.

Campylobacteriosis

Campylobacteriosis is an enteric infection caused by members of the genus *Campylobacter* (77). Most human infections are caused by the thermophilic species *C. jejuni* or *C. coli*.

Nature of the Disease in Humans

Campylobacter causes an acute gastroenteritis characterized by fever, abdominal pain, and profuse diarrhea that is frequently bloody (50). Most patients recover within one week without antimicrobial treatment. Bacteremia and other extraintestinal infections are uncommon complications. Sequelae of enteric *Campylobacter* infections may include reactive arthritis in 1–5% of patients, irritable bowel syndrome in 1–10% of patients, and Guillain-Barré syndrome in approximately 0.1% of patients (45).

Occurrence

Campylobacter is one of the most commonly reported causes of bacterial gastroenteritis worldwide and is a common cause of traveler's diarrhea. In the United States, *Campylobacter* causes an estimated 845,000 cases of foodborne illness and 76 deaths each year (74). Most cases are sporadic; outbreaks are uncommon, but have been linked to unpasteurized milk, contaminated water, and the ingestion of undercooked poultry. In developed countries there is a male predisposition, a seasonal peak in cases during the late spring and summer, and an increased incidence in children under 5 years old (50). In developing countries there is less evidence of a seasonal pattern, and infection is common in children younger than 2 years of age but uncommon in adults.

Reservoirs and Sources of Infection

Campylobacter species are normal intestinal inhabitants of wild and domesticated animals and birds. Colonization of broiler chickens is common, and contaminated poultry meat is considered the most important source of human infections. Transmission occurs by ingestion of the organism, and approximately 80% of domestically

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acquired infections in the United States are considered to be foodborne (74). Transmission to poultry processing plant workers has been well documented (29). Contact with colonized animals or drinking untreated water are additional potential sources of exposure. The incubation period ranges from 1–10 days, but is most commonly 2–5 days (50). Person-to-person transmission can occur but is uncommon. The duration of fecal shedding can range from 2–7 weeks, although the median duration of shedding is less than 3 weeks.

Preventive Measures

Cook poultry to a minimum internal temperature of 74°C (165°F) and avoid cross-contamination between raw poultry and other foods. Avoid drinking unpasteurized milk and wash hands after contact with poultry or other animals.

Chlamydiosis (Psittacosis)

Psittacosis, also known as ornithosis or parrot fever, is a respiratory disease of humans caused by *Chlamydophila* (or *Chlamydia*) *psittaci* (73, 79). The corresponding disease in birds is referred to as avian chlamydiosis. *C. psittaci* is an obligate intracellular pathogen.

Nature of the Disease in Humans

Psittacosis is primarily a respiratory disease in humans that ranges from a mild flu-like illness to severe pneumonia with respiratory failure and death (5, 79). Typical symptoms include fever, chills, headache, and myalgia. A nonproductive cough is common and may be accompanied by respiratory difficulty. Potential complications include endocarditis, myocarditis, hepatitis, arthritis, keratoconjunctivitis, and encephalitis. Most cases respond well to antibiotic therapy.

Occurrence

Psittacosis and avian chlamydiosis have a worldwide distribution. Psittacosis is a reportable disease in most countries, but the number of reported cases is likely an underestimate because many cases are mild, the symptoms are nonspecific, and diagnosis can be difficult. Beeckman and Vanrompay summarized reported cases of psittacosis from 24 countries between 1996 and 2007, with Australia, Germany, Japan, The Netherlands, and Great Britain having comparatively high numbers of reported cases (5). Most cases are sporadic, but outbreaks have occurred in people exposed to infected pet birds and poultry. There is no evidence to suggest that immunocompromised individuals are at increased risk for infection.

Reservoirs and Sources of Infection

Birds are the natural reservoir for *C. psittaci*, and *Chlamydophila* spp. have been identified by culture or serology in 467 bird species from 30 different orders, including all of the major domestic poultry species (44). Human infections are most frequently associated with exposure to psittacines, pigeons, turkeys, and ducks. Different serotypes have been identified more frequently in certain bird species, but all serotypes are considered to be potentially infectious to humans (5). Humans become infected by inhalation of aerosolized organisms shed in the feces or respiratory secretions of infected birds, or by direct contact with infected carcasses or tissues. Intermittent shedding by subclinically infected birds is common. The incubation period in humans ranges from 1–30 days, although 5–14 days is typical. Secondary transmission of *C. psittaci* has been reported but is believed to be rare (88).

Preventive Measures

Wearing gloves, protective eyewear, and a properly fitted respirator with an N95 rating or higher are recommended when working with potentially infected birds (79). Loose fitting surgical masks may not provide adequate respiratory protection. Necropsies on potentially infected birds should be performed in a biological safety cabinet and carcasses should be moistened with a disinfectant solution to minimize the generation of aerosols during the procedure.

Erysipelothrix rhusiopathiae Infection

The most common clinical manifestation of infection with *E. rhusiopathiae* in humans is called erysipeloid, which is distinct from human erysipelas; a condition that is usually caused by *Streptococcus pyogenes* (69).

Nature of the Disease in Humans

Three forms of *E. rhusiopathiae* infection are typically recognized in humans (90). Erysipeloid is the most common and is characterized by a localized cutaneous lesion, usually on the hand. Pain and swelling may be severe, although there is no suppuration or pitting edema. Systemic illness is uncommon and the condition usually resolves without treatment in 3–4 weeks, or within 48 hours after beginning antibiotic therapy. Diffuse cutaneous and systemic forms of the infection are much less common, although endocarditis is a frequent complication in systemically infected patients (34, 67).

Occurrence

Erysipelothrix rhusiopathiae has a worldwide distribution although the incidence is unknown. Persons with exposure to animals or animal products, including processing plant workers, butchers, fish handlers, food handlers, farmers, and veterinarians are at increased risk of infection (34). Transmission from infected quail and laying chickens to processing plant employees and animal caretakers has previously been reported (59, 60).

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Reservoirs and Sources of Infection

Erysipelothrix rhusiopathiae is a pathogen or commensal organism in a wide variety of animal species. Swine are the most commonly affected domestic animal and are considered the most important reservoir, although several poultry species including turkeys, chickens, ducks, and emus are also susceptible (90). *Erysipelothrix rhusiopathiae* can survive weeks to months in farm and marine environments, and is commonly found in the mucoid slime coating of fish. Transmission occurs by inoculation of the organism into an abrasion, cut, or puncture wound when working with infected animals or in contaminated environments. The incubation period for erysipeloid is 2–7 days. Person-to-person transmission has not been documented.

Escherichia coli Infection

Most strains of *E. coli* are commensal inhabitants of the lower intestinal tracts of warm-blooded animals, however, some strains possess virulence traits that allow them to cause disease. Strains that cause intestinal pathology are categorized as belonging to one of six pathotypes: enterohemorrhagic (EHEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enteropathogenic (EPEC), enteroaggregative (EAEC), or diffuse-adherence *E. coli* (DAEC) (57). Strains with recognized extraintestinal virulence factors or that demonstrate enhanced virulence in an animal model of extraintestinal infection have been designated as extraintestinal pathogenic *E. coli* (ExPEC). Of the six intestinal pathotypes, only the EHEC strains (e.g., O157:H7) are considered to be zoonotic pathogens. Avian pathogenic *E. coli* (APEC) have not been associated with human intestinal infections, but there may be some overlap between APEC strains and human ExPEC strains (55). The discussion here will be limited to the potentially zoonotic EHEC and ExPEC strains.

Nature of the Disease in Humans

Intestinal infection with EHEC serotypes of Shiga toxin producing *E. coli* (STEC) such as O157:H7 typically causes abdominal cramps with an initially watery diarrhea progressing to bloody diarrhea in 1–4 days (65). Approximately 10–15% of patients develop hemolytic uremic syndrome (HUS) with thrombocytopenia, microangiopathic hemolytic anemia, and acute renal failure within 5–13 days after the onset of diarrhea. Extraintestinal *E. coli* infections are associated with a variety of illnesses including urinary tract infection, newborn meningitis, and septicemia (55).

Occurrence

Enterohemorrhagic *E. coli* strains are recognized as an important problem in North and South America, Europe, Japan, and Australia (57). Extraintestinal *E. coli* infections are an important worldwide problem. Shiga toxin producing *E. coli* cause an estimated 176,000 illnesses and 20 deaths in the United States each year (74). Extraintestinal *E. coli* infections are estimated to account for 70–90% of community-acquired urinary tract infections (55).

Reservoirs and Sources of Infection

EHEC strains of Shiga toxin producing *E. coli* have previously been identified in retail chicken samples, and chickens are also readily colonized with *E. coli* O157:H7 in experimental trials (75). Cattle and other ruminants are the most important source of human EHEC infections, however, and avian species are not considered an important reservoir. Transmission of EHEC strains occurs via contaminated foods, person-to-person contact, or by contact with colonized animals. Human infections with ExPEC strains typically originate from the person's own intestinal tract, although poultry may potentially serve as a reservoir for human colonization (52). The incubation period for EHEC strains ranges from 2–10 days, with a median of 3–4 days (57). Secondary transmission of EHEC strains is common, especially among children in daycare centers. The duration of shedding is typically one week or less in adults, but may be three or more weeks in children.

Listeriosis

Listeriosis is caused by *Listeria monocytogenes*. Thirteen serotypes have been described, although three are most frequently associated with human disease: 1/2a, 1/2b, and 4b (62).

Nature of the Disease in Humans

Listeria monocytogenes can result in a variety of clinical syndromes, ranging from febrile gastroenteritis to severe invasive disease (2). Septicemia and meningoencephalitis are most frequently reported in neonates and immunocompromised adults. Focal infections may include brain and hepatic abscesses, cholecystitis, conjunctivitis, endocarditis, joint infections, skin infections, and osteomyelitis. Infection of pregnant women may result in fetal infection, stillbirth, or abortion. The average case-fatality rate for invasive infections is 20–30% (83).

Occurrence

Listeria monocytogenes has a worldwide distribution. In 2010, it was estimated to cause 23,150 illnesses; 5,463 deaths; and 172,823 disability-adjusted-life years (DALYs) globally (49). Populations at increased risk of systemic infection include pregnant women and neonates, immunocompromised adults, and the elderly. Veterinarians and farm workers are at increased risk for cutaneous infections, particularly following large-animal obstetric procedures (54). Most cases of listeriosis are

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sporadic, but common source outbreaks are frequently identified.

Reservoirs and Sources of Infection

Listeria monocytogenes is widely distributed in nature and can be identified in soil, water, silage, and animal feces. *Listeria* is commonly found on poultry farms. It is an important source of environmental contamination in processing plants because of its ability to grow at refrigeration temperatures and form biofilms that are resistant to routine sanitation procedures. Most cases of human listeriosis result from foodborne transmission. Several outbreaks have been associated with ready-to-eat delicatessen meats and soft cheeses made from unpasteurized milk. The overall incubation period has been reported to range from 1–67 days, with longer median incubation times for pregnancy-associated cases (27.5 days) compared to non-pregnancy associated bacteremia (2 days) or central nervous system disease (9 days) (35). The median incubation period for non-invasive gastroenteritis is approximately 24 hours. Transplacental transmission from pregnant mothers to the fetus is common, but transmission from infected patients to household contacts has not been reported.

Mycobacteriosis

Mycobacterial species other than *M. tuberculosis* and *M. leprae* that are associated with human disease are commonly called nontuberculous mycobacteria (NTM). At the time of writing, 169 NTM species have been recognized, but only around 20 have been associated with human illness (78). The discussion here will be limited to members of the *Mycobacterium avium* complex (MAC), which along with *M. genavense* are responsible for most cases of avian mycobacteriosis (85).

Nature of the Disease in Humans

The most common clinical syndromes associated with MAC infections are pulmonary disease, lymphadenitis, and disseminated infection. Common signs of pulmonary disease include chronic cough, fever, chills, night sweats, dyspnea, and weight loss (87). Lymphadenitis frequently manifests as a painless unilateral swelling of the cervical, submandibular, submaxillary, or preauricular lymph nodes. Disseminated infection is characterized by intermittent fever, sweats, weakness, anorexia, and weight loss.

Occurrence

Members of the *M. avium* complex cause disease in humans worldwide. Exposure is common but disease is rare in immunocompetent persons. Pulmonary MAC infections are typically identified in men with preexisting lung disease, in elderly women with no history of underlying lung disease, and in adolescents with cystic fibrosis (78). Lymphadenitis is most common in children from 1–5 years of age, and disseminated infections are usually recognized in severely immunocompromised persons, especially those with advanced AIDS.

Reservoirs and Sources of Infection

Nontuberculous mycobacteria, including MAC, are ubiquitous in the environment and are commonly isolated from soil and water. Humans become infected by ingestion or inhalation of MAC organisms from the environment. Infected animals and birds commonly shed mycobacteria in their feces, but are not considered to be an important source of human infections (85). While birds may serve as an important reservoir of some *M. avium* strains, molecular studies suggest that bird-type *M. avium* isolates are genetically distinct from those that are typically isolated from humans and swine (86). Person-to-person transmission of MAC has not been documented, but has been reported for *M. abscessus* in cystic fibrosis patients (47).

Salmonellosis

Nontyphoidal *Salmonella* infections may be caused by any of the non-host-specific *Salmonella* serotypes that commonly affect both animals and man. *Salmonella enterica* serotypes Enteritidis and Typhimurium have a broad host range and are two of the most common nontyphoidal serovars isolated from humans. *Salmonella* Gallinarum and *Salmonella* Pullorum are host-restricted to poultry (76).

Nature of the Disease in Humans

Nontyphoidal salmonellosis typically manifests as an acute enterocolitis or gastroenteritis with sudden onset headache, abdominal pain, diarrhea, nausea, and sometimes vomiting. Fever is usually present (4). Most cases are self-limiting, with diarrhea resolving without treatment after 3–7 days. Bacteremia is a potentially serious complication that occurs in 1–5% of cases (15). Possible sequelae of bacteremia include endocarditis and disseminated focal infections.

Occurrence

Salmonella has a worldwide distribution. There were an estimated 153 million human cases of nontyphoidal salmonellosis and 57,000 deaths globally in 2010, with 78.4 million of these cases resulting from foodborne transmission (46). Children, the elderly, and people with compromised immune systems are more likely to develop severe disease. Poultry and eggs are frequently identified as sources of infection in foodborne salmonellosis outbreaks. Of the 224 foodborne salmonellosis outbreaks identified in the United States between 1998 and 2012

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that could be attributed to a single food commodity, 64 (29%) were attributed to poultry (22).

Reservoirs and Sources of Infection

Nontyphoidal *Salmonella* are capable of colonizing the gastrointestinal tracts of a broad range of wild and domesticated animal hosts including poultry, reptiles, and rodents. Transmission occurs by the fecal-oral route. Forty-five US outbreaks between 1996 and 2012 were linked to contact with live poultry from mail-order hatcheries (6). The incubation period is typically 12–36 hours, but can range from 6–72 hours. Secondary transmission can occur but is uncommon with appropriate hygiene. The median duration of fecal shedding is approximately 4–6 weeks after infection, although *Salmonella* can still be identified one year postinfection in 5% of children younger than 5 years of age and in 1% of adults (4, 15).

Staphylococcus aureus Infection and Foodborne Intoxication

Staphylococcus aureus frequently colonizes the skin and mucous membranes of humans and animals, including poultry. It is both a commensal organism and a frequent cause of clinically important infections. Antibiotic resistant strains, especially methicillin-resistant *S. aureus* (MRSA), have become increasingly common in recent years.

Nature of the Disease in Humans

Staphylococcus aureus causes a wide variety of clinical manifestations ranging from minor skin pustules to septicemia and death (39, 91). Common cutaneous infections include impetigo, cellulitis, folliculitis, carbuncles, furuncles, and abscesses. Most superficial infections respond well to cleaning and topical antibiotics. Hematogenous spread of localized infections can lead to serious complications including arthritis, endocarditis, osteomyelitis, pneumonia, meningitis, and sepsis. Staphylococcal foodborne intoxication is mediated by the production of heat-stable enterotoxins in uncooked or inadequately refrigerated foods (8). Signs include acute onset of nausea, abdominal cramps, vomiting, and often diarrhea. Most cases of foodborne intoxication resolve without treatment in 1–2 days. *Staphylococcus aureus* is also the causative agent of toxic shock syndrome in humans.

Occurrence

Staphylococcus aureus has a worldwide distribution and is one of the most common pathogens associated with skin and soft-tissue infections. *S. aureus* is the second most common cause of hospital-acquired bloodstream infections (91), and causes approximately 240,000 foodborne intoxications in the United States each year (74). Newborn infants and the chronically ill are at increased risk for developing *S. aureus* skin infections (39).

Reservoirs and Sources of Infection

The anterior nares are the most common site of human colonization. Approximately 20% of persons are persistent carriers, 30% are intermittent carriers, and 50% are non-carriers (91). Transmission is by direct or indirect contact. Hands are the most important vehicle for transmission, and at least two-thirds of infections are believed to result from autoinfection (39). Airborne transmission is uncommon but may result from sneezing by nasal carriers. Retail chicken meat is frequently contaminated with enterotoxigenic *S. aureus* strains, although colonized food handlers are believed to be responsible for most cases of foodborne intoxication. Signs typically appear within 3–4 hours after ingesting staphylococcal enterotoxins (8). Person-to-person transmission of *S. aureus* is common. Live poultry have been implicated as a potential source of livestock-associated MRSA (32).

Fungal Diseases

Cryptococcosis

Cryptococcosis is a fungal infection caused by members of the genus *Cryptococcus*. There are more than 30 species belonging to this genus, although only *C. neoformans* and *C. gattii* are considered major pathogens. Cryptococcosis is not considered to be a zoonosis; rather humans and animals both acquire infection from environmental sources (10).

Nature of the Disease in Humans

The central nervous system (CNS) and lungs are the most frequently recognized sites of *Cryptococcus* infection. Meningitis is the most common presentation in immunocompromised patients, while pulmonary disease may be more common in immunocompetent patients. Even with appropriate antifungal treatment, the six-month case-fatality risk for cryptococcal meningitis in HIV-infected patients may exceed 35% (28).

Occurrence

Cryptococcus neoformans has a worldwide distribution and occurs most frequently in immunocompromised persons. *Cryptococcus gattii* has historically been limited to tropical and subtropical regions, although it has recently been recognized in British Columbia, Canada, and in the Pacific Northwest region of the United States. In contrast to *C. neoformans*, *C. gattii* causes disease in both immunocompromised and immunocompetent individuals. It has been estimated that there are approximately 720,000 cases of cryptococcal meningitis in HIV-infected persons in Sub-Saharan Africa each year, with approximately 500,000 fatalities (64).

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Reservoirs and Sources of Infection

Humans are infected with *Cryptococcus* by inhalation of desiccated encapsulated yeast cells or basidiospores from the environment. Guano from old pigeon lofts or roosts is an important environmental source of *C. neoformans*, while *C. gattii* is frequently found in the hollows of *Eucalyptus* and other tree species (53, 61). The incubation period is unknown but CNS disease may be preceded by a pulmonary infection acquired months or years previously. Person-to-person transmission has been reported but is believed to be rare (89).

Dermatophytosis (Favus)

Microsporum gallinae is a contagious zoophilic fungus that is responsible for causing dermatophytosis in poultry and in humans (11, 30). This condition is alternatively referred to as favus, dermatomycosis, or ringworm.

Nature of the Disease in Humans

Like other dermatophytes, *M. gallinae* affects keratinized areas of the body including the hair, nails, and skin. Lesions begin as small circumscribed areas of erythema, crusting, and scaling, and subsequently spread peripherally. Skin lesions are not associated with systemic illness, although treatment with topical and/or systemic antifungal medications for 4–8 weeks may be required to eliminate the infection.

Occurrence

M. gallinae possibly has a worldwide distribution, although it is rarely reported as a cause of disease in either poultry or man. Miyasoto et al. identified 44 human cases that had been reported in the literature as of 2010, with 34 of these cases being reported in Nigeria and Iran (56). Young children, the elderly, immunosuppressed persons, and those with diabetes may be at increased risk of infection.

Reservoirs and Sources of Infection

Gallinaceous birds are considered the most important reservoir for *M. gallinae*. Transmission occurs by direct contact with infected animals or humans, or indirect contact via contaminated fomites. The incubation period is unknown, but for other dermatophytes has been reported as 4–14 days (12). Dermatophyte infections are easily transmitted from person-to-person while lesions are present. Infectious materials may remain viable in the environment or on contaminated objects for months to years (26).

Histoplasmosis

Histoplasmosis is caused by the fungus *Histoplasma capsulatum* (17). Two varieties cause disease in humans: *H. capsulatum* var. *capsulatum* and *H. capsulatum* var. *duboisii*. The discussion here will focus on the more widely distributed and well-known *H. capsulatum* var. *capsulatum*. *Histoplasma* is not considered to be either a contagious or zoonotic pathogen.

Nature of the Disease in Humans

Histoplasmosis is associated with a wide spectrum of clinical illness, with as many as 95% of sporadic infections in endemic areas being asymptomatic (17, 92). Acute pulmonary histoplasmosis is characterized by fever, myalgia, non-productive cough, dyspnea, and chest pain. The infection is usually self-limiting, but in immunocompromised patients or in those exposed to a large inoculum may progress to acute respiratory distress syndrome. Chronic pulmonary histoplasmosis is a progressive infection characterized by the formation of cavitary lesions in patients with preexisting emphysema. Progressive disseminated histoplasmosis is a systemic manifestation that typically only occurs in individuals with inadequate T-cell immunity. Complications of histoplasmosis include mediastinal granuloma, fibrosing mediastinitis, pericarditis, and broncholithiasis.

Occurrence

Histoplasma capsulatum var. *capsulatum* causes histoplasmosis across the Americas, parts of Africa, eastern Asia, Australia, and rarely in Europe. In the United States, *H. capsulatum* is endemic in the Ohio and Mississippi River valleys. Young children, the elderly, and immunosuppressed persons are at increased risk for developing histoplasmosis. While most cases are sporadic, large outbreaks have been reported, often in association with construction projects or other activities that involve disturbing soil near bird roosts (7, 17).

Reservoirs and Sources of Infection

Histoplasma grows in the soil, particularly in areas contaminated with bird and bat droppings. In the United States, the presence of birds, bats, or their droppings was noted in 77% of reported outbreaks between 1938 and 2013 (7). Humans become infected by inhalation of airborne microconidia. The typical incubation period is between 4 and 14 days (17). Transplacental transmission has been reported, but contact transmission from animal-to-person or from person-to-person does not occur (17, 93).

Parasitic Diseases

Avian Mite Dermatitis

Avian mite dermatitis is most frequently caused by *Dermanyssus gallinae* (the poultry red mite or chicken mite) or *Ornithonyssus sylviarum* (the northern fowl

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mite). *Ornithonyssus bursa* (the tropical fowl mite) is a less frequent cause (18). Synonyms include gamasoidosis, acariasis, and fowl mite dermatitis.

Nature of the Disease in Humans

Typical clinical signs in humans include pruritic erythematous papules marked by a pinpoint red spot (18). Excoriation of lesions is common. Avian mites have also been associated with occupational asthma and otitis externa in poultry workers (48, 71). Symptoms are alleviated when the source of mites is removed. Mites are not typically observed on human skin as they leave quickly after biting.

Occurrence

Avian mites have a worldwide distribution. Avian mite dermatitis is not reportable and the incidence is unknown. Most published case reports result from proximate exposures to abandoned bird nests in an urban setting (16, 63). Mite infestation of layer and breeder flocks is common, however, and is recognized as a frequent cause of discomfort in poultry workers (82).

Reservoirs and Sources of Infection

A broad variety of avian species serve as the natural hosts for *Dermanyssus* and *Ornithonyssus*, although mites from these genera can survive at least five months and three weeks, respectively, without a host (63). When no birds are available, the mites will seek out alternative food sources, including humans and other mammals. Humans are an accidental host and do not serve as a reservoir of avian mites.

Cryptosporidiosis

Cryptosporidiosis is caused by intracellular protozoan parasites belonging to the genus *Cryptosporidium*. Three avian *Cryptosporidium* species are currently recognized: *C. baileyi*, *C. galli*, and *C. meleagridis*. Of these three, only *C. meleagridis* is considered to be a zoonotic pathogen (72).

Nature of the Disease in Humans

Cryptosporidiosis is primarily associated with enteric disease in humans. Watery diarrhea, abdominal cramping, and increased gas production are the most common clinical signs, and may be accompanied by vomiting, fever, and loss of appetite (23, 27). The median duration of illness in immunocompetent persons is 10–14 days, and signs may persist for up to one month. Immunocompromised persons may experience severe chronic diarrhea, and are at increased risk for complications including pancreatitis, cholangitis, bronchial involvement, and death.

Occurrence

Cryptosporidium species have a worldwide distribution and are one of the most common causes of protozoal diarrhea in humans. More than 95% of human infections are caused by *C. hominis* or *C. parvum*. *Cryptosporidium meleagridis* is the third most commonly identified species in humans (24, 72). Young children, immunocompromised persons, and people with occupational exposure to infected animals are at increased risk of infection.

Reservoirs and Sources of Infection

Cryptosporidium meleagridis has been identified in a wide range of avian and mammalian hosts including turkeys, quail, chickens, partridges, parakeets, deer, mice, dogs, and humans (24). Birds are not considered a major reservoir for *C. parvum*, which is the most common zoonotic species. Infectious oocysts shed in the feces of infected animals and humans can survive for several months in the environment. Transmission to susceptible hosts occurs by the fecal–oral route. The incubation period is 3–12 days (27). Large numbers of oocysts are shed in the feces of infected individuals, and person-to-person transmission is common. Oocyst shedding can continue for several weeks after the resolution of clinical symptoms (23).

Toxoplasmosis

Toxoplasmosis is caused by infection with the obligate intracellular protozoan *Toxoplasma gondii*. Cats are the definitive host for *T. gondii*. Mammals and birds serve as intermediate hosts.

Nature of the Disease in Humans

Postnatal infection with *T. gondii* is asymptomatic in approximately 90% of immunocompetent children and adults (58). In the remaining 10%, mild transient cervical or occipital lymphadenopathy is the most common clinical presentation. Immunocompromised persons frequently experience severe disease including encephalitis, chorioretinitis, and pneumonitis. Infection of pregnant mothers is usually asymptomatic, but may lead to abortion or infection of the fetus. Congenital infections can result in chorioretinitis, hydrocephalus, intracranial calcifications, and mental retardation (51).

Occurrence

Toxoplasma gondii has a worldwide distribution and exposure to the organism is common. The overall seroprevalence in the adolescent and adult US population for 2009–2010 was estimated at 12.4%, and the prevalence among women from 15–44 years of age was estimated at 9.1% (51). The incidence of congenital toxoplasmosis in the United States has been estimated to total approximately 365 cases per year. Immunocompromised persons and infants with congenital infections are at increased risk for severe disease.

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Reservoirs and Sources of Infection

Cats become infected after ingesting an infected intermediate host, and will shed oocysts in their feces for 1–3 weeks following their first infection. Oocysts do not become infective until 1–5 days after excretion, although they can remain viable in the environment for several months. Humans become infected by ingesting oocysts shed by cats or by consuming infected intermediate hosts. Historically, undercooked lamb and pork have been common sources of human infection. Commercial poultry raised in confinement are rarely infected with *T. gondii*, however, infection of free-range chickens is common (37). The incubation period is 5–23 days (42). *Toxoplasma* can be transmitted from mother to fetus, although this typically occurs only when the mother's first exposure to the parasite occurs during gestation. Person-to-person transmission does not occur by direct contact, but transmission by organ transplantation or blood transfusion has been documented.

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2

Host Factors for Disease Resistance

Introduction

Silke Rautenschlein

Domesticated and wild birds are vulnerable to many microorganisms including viruses, bacteria, fungi, and parasites that share the environment in which they live. In confined houses, such as the ones used for intensive rearing of commercial poultry, the concentrations of microorganisms can reach very high levels. Some of these microorganisms are pathogenic and invasive and can cause severe clinical disease or death. The birds manage to survive the microbial challenge primarily because the immune system provides protection against infection and unrestricted replication of microorganisms. Because of the importance of immunity in health and disease, the study of the mechanisms of immunity has received much attention within the last few decades, leading to effective disease control strategies. One of the most important contributions of immunity to human and animal health has been the development of vaccines that have dramatically reduced the incidence of infectious diseases. Today, highly effective vaccines, including genetically engineered recombinant vaccines, are commercially available to protect poultry populations against a multitude of infectious agents.

Although the avian immune system has not been studied as extensively as that of mammals, important advances have been made. Chickens are the most studied avian species in this field. Some of the information can be transferred to other avian species but also differences may be found (as reviewed by 1). In the first subchapter, a broad outline of the basic elements of the avian immune system will be presented. Although there are great similarities between immune mechanisms of birds and mammals, there are also important differences. The recent availability of recombinant avian cytokines and growth factors allows the *in vitro* culture of immune cell populations providing opportunities to study their functions in more detail (3). In addition, the advances made in the development of transgenic chickens will allow a deeper understanding of the immune mechanisms in birds (2).

The second subchapter will address the role of genetics in regulating immune-mediated resistance to disease. The genetic background of a host determines how the immune response to a given microorganism will evolve, and, ultimately, if protective immunity will be generated. This is well demonstrated by the great variation that is often observed between individuals within a population in their response to a common disease agent. Some individuals may succumb to infection and die, whereas others may show no phenotypic signs of infection. This wide variation in response to the same agent is attributed to an intrinsic polymorphism of genes that regulate the expression and interaction of various components of the immune system. Although genetic resistance to a disease is a multigenic trait, resistance or susceptibility to disease is often attributed to the genes that regulate the major histocompatibility complex (MHC). The MHC encodes a set of cell surface proteins that are necessary for antigen recognition by T cells and, consequently, the ability of T cells to generate specific immunity. The MHC proteins are genetically diverse and polymorphic. Association of specific MHC haplotypes with disease resistance has been exploited by designing breeding programs that select for resistance. The entire genomes of the chicken as well as of other avian species have been sequenced and analyzed by different molecular approaches (4). This has provided new opportunities for comparative immunology, and allows identifying and manipulating genes that control immunity and disease resistance. In the era of antibiotic withdrawal as feed additives, growing resistance of microbes, and consumer concerns regarding anti-infectives, this new information on disease resistance is likely to have a major impact on commercial poultry production.

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The Avian Immune System

Silke Rautenschlein

Summary

The Avian Immune System. The organization and mechanisms of immunity in birds are comparable to mammals. Physical barriers, innate as well as acquired immune mechanisms build the basis for protection against invading pathogens.

Measurement Procedures. Most of the commercially available tests for the evaluation of immune parameters are based on antibody detection. The measurement of immune activity on the cellular level by bioassays is difficult and mainly applied to experimental settings. Also molecular methods and high-throughput cell-detection systems are available, which may be used for field investigations in the future.

Practical Applications. Securing a functioning immune system by different means including genetic selection, nutritional and management as well as vaccination strategies is important for the overall health development of poultry and subsequently food safety.

Introduction

The immune system plays a critical role in defending birds against environmental pathogens. The overall organization and mechanisms of immunity in birds are quite similar to those in mammals. Early studies on the cloacal bursa (bursa of Fabricius) and the thymus of chickens provided some of the basic information that led to the identification of the dichotomy of the immune system into B and T cell compartments. The recognition of this dichotomy initiated an era of extensive research on the mechanisms of immunity across species. This influx of research activity that began more than five decades ago continues unabated and is responsible for making immunology one of the fastest growing branches of biology. Emerging concepts of immune mechanisms are constantly being revised by new information.

The immune system of birds, as of mammals, is complex and comprises a number of cells and soluble factors that must work in concert to produce a protective immune response. A properly functioning immune system is of special importance to birds because commercial poultry flocks are raised under intensive rearing conditions. Under such conditions, the flocks are vulnerable to rapid spread of infectious agents and disease outbreaks. A variety of vaccines must be used, often repeatedly, to protect flock health. The protective efficacy of a vaccine depends upon a vigorous immune response against the antigens present in the vaccine. If animals are immunosuppressed and respond poorly to a vaccine, the flock health is placed in jeopardy. The understanding of how the immune response is generated is of interest, as is the knowledge of how to protect flocks from stressors that may induce immunosuppression (22, 64).

This review is intended to provide a broad overview of selected aspects of the avian immune system. For more detailed information, the reader may consult several books and reviews (e.g., 1, 24, 59, 70).

Anatomy of the Immune System

The immune cells reside in primary lymphoid organs (PLO) or secondary lymphoid organs (SLO). The thymus and the bursa of Fabricius (also called *Bursa cloacalis* or cloacal bursa), respectively, are the PLO where T and B cell precursors differentiate and undergo maturation. The thymus is an elongated, multilobular structure located along the length of both sides of the trachea with some lobes extending into the anterior thoracic cavity (Figure 2.1A). Thymic lobes are divided into lobules, and each lobule has a peripheral cortical area where lymphocytes are densely packed and a central medullary area where the lymphocytes are less densely packed (Figure 2.1C). The bursa of Fabricius is a sac-like extension of the hindgut, is located dorsally to the cloaca, and part of the gut-associated lymphoid tissue (GALT) (Figure 2.1B). The bursa of Fabricius is organized into follicles; each follicle is filled with lymphocytes. As in the thymus, the lymphocytes are arranged into a peripheral cortex and a central medulla (Figure 2.1D).

Functional immune cells leave the PLO and populate the SLO, the principal sites of antigen-induced immune response. The SLO, characterized by aggregates of lymphocytes and antigen-presenting cells, are scattered through the body (Figure 2.2A–H). Examples of SLO include the spleen, bone marrow, Harderian gland (located ventral and posteromedial to the eyeball), conjunctival-associated (CALT), bronchial-associated (BALT), and gut-associated lymphoid tissue (GALT). The bursa of Fabricius also may serve as an SLO. Chickens lack the mammalian equivalent of lymph nodes but have lymphoid nodules along the course of lymphatic vessels.

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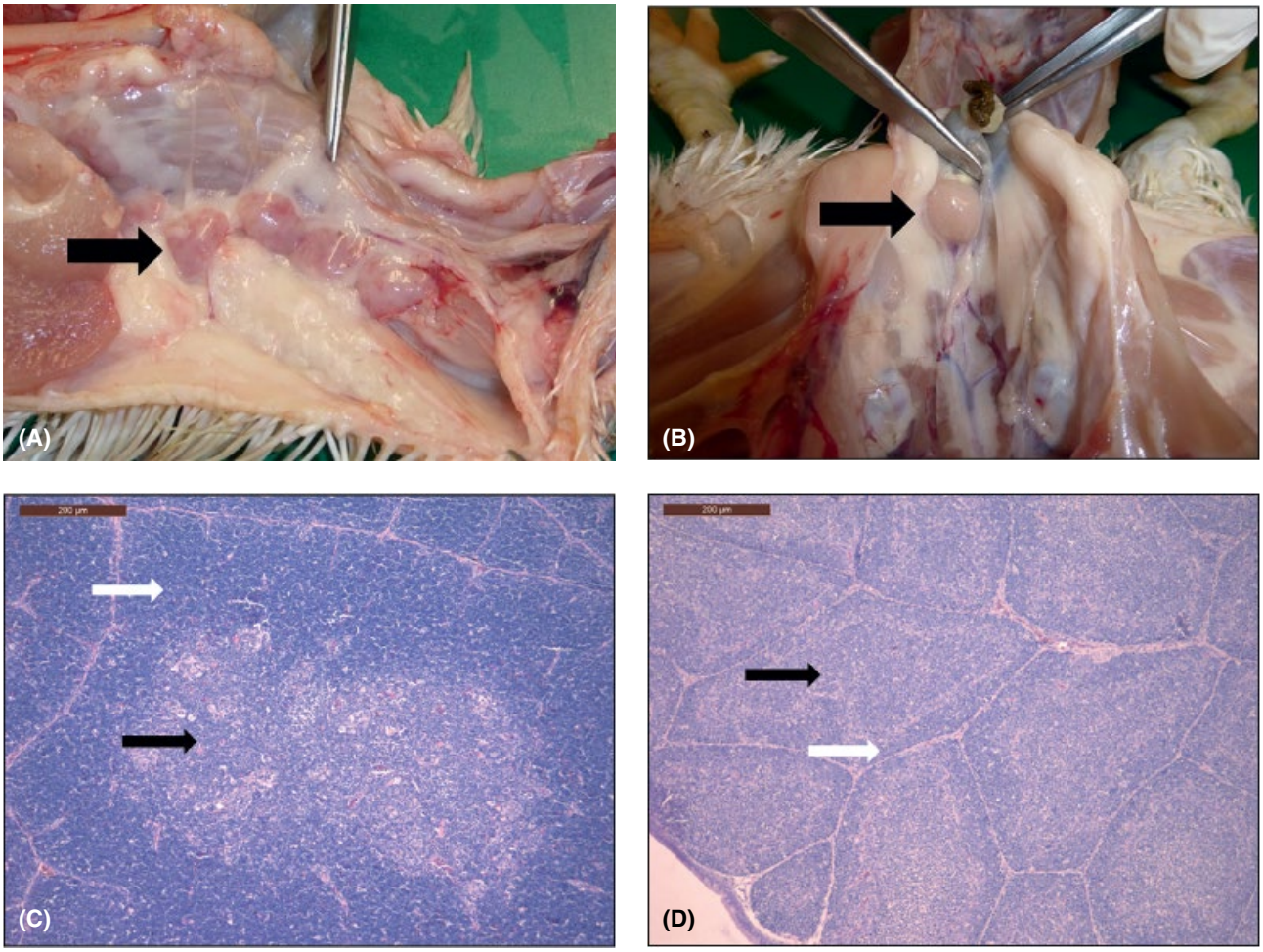


Figure 2.1 (A) Multiple lobes of the thymus lie on each side of the trachea, present is a view of one side, arrow indicates one thymus lobe. (B) The cloacal bursa (bursa of Fabricius, indicated by the arrow) is the sac-like structure extending from the end of the intestine. (C) The lobular histologic structure of the thymus is evident; each lobule is comprised of the dark-staining cortex and the paler medulla. (D) Bursal lymphoid follicles are separated by thin connective tissue septae. Black arrows indicate the medulla and white arrows the cortex area. (*For color detail, please see the color section.*)

General Features of the Avian Immune System

An outline of the mechanisms birds use to defend against pathogens is presented in Figure 2.3. Birds have well-developed innate defense mechanisms. Physical barriers such as the egg shell for the developing embryo, feathers, skin and epithelia, mucus, ciliary movement in the respiratory tract, or normal mucosal microflora prevent pathogens from entering the body. For the pathogens that enter the body, the first line of defense is provided by innate immune mechanisms such as phagocytic cells that include granulocytes (mainly heterophils), macrophages (48), complement (26), thrombocytes (61), and natural killer (NK) cells (18, 68) and other secreted or cell surface associated factors. These include regulatory molecules such as cytokines, chemokines, and host defense peptides. The innate immunity is initially more rapid than the adaptive response, but the adaptive immune system, which consists of B and T cells, has immunological memory as an important feature allowing an enhanced and faster immune response after secondary exposure. Booster vaccinations, used routinely in poultry, take advantage of this memory response. The magnitude of the innate response is not influenced by repeated contact with the same stimulus.

Both the innate but also the adaptive immunity start evolving during embryonic development. But the development of the immune systems is not completed at hatch and will take some weeks before the immune system is fully mature (15, 44). Around hatch, B lymphocytes migrate from the bursa of Fabricius to seed the SLOs. First populations of T lymphocytes leave the thymus around embryonation day 15, and the second and third wave of migration then takes place after hatch. There is clear evidence that the development of the immune system, spe-

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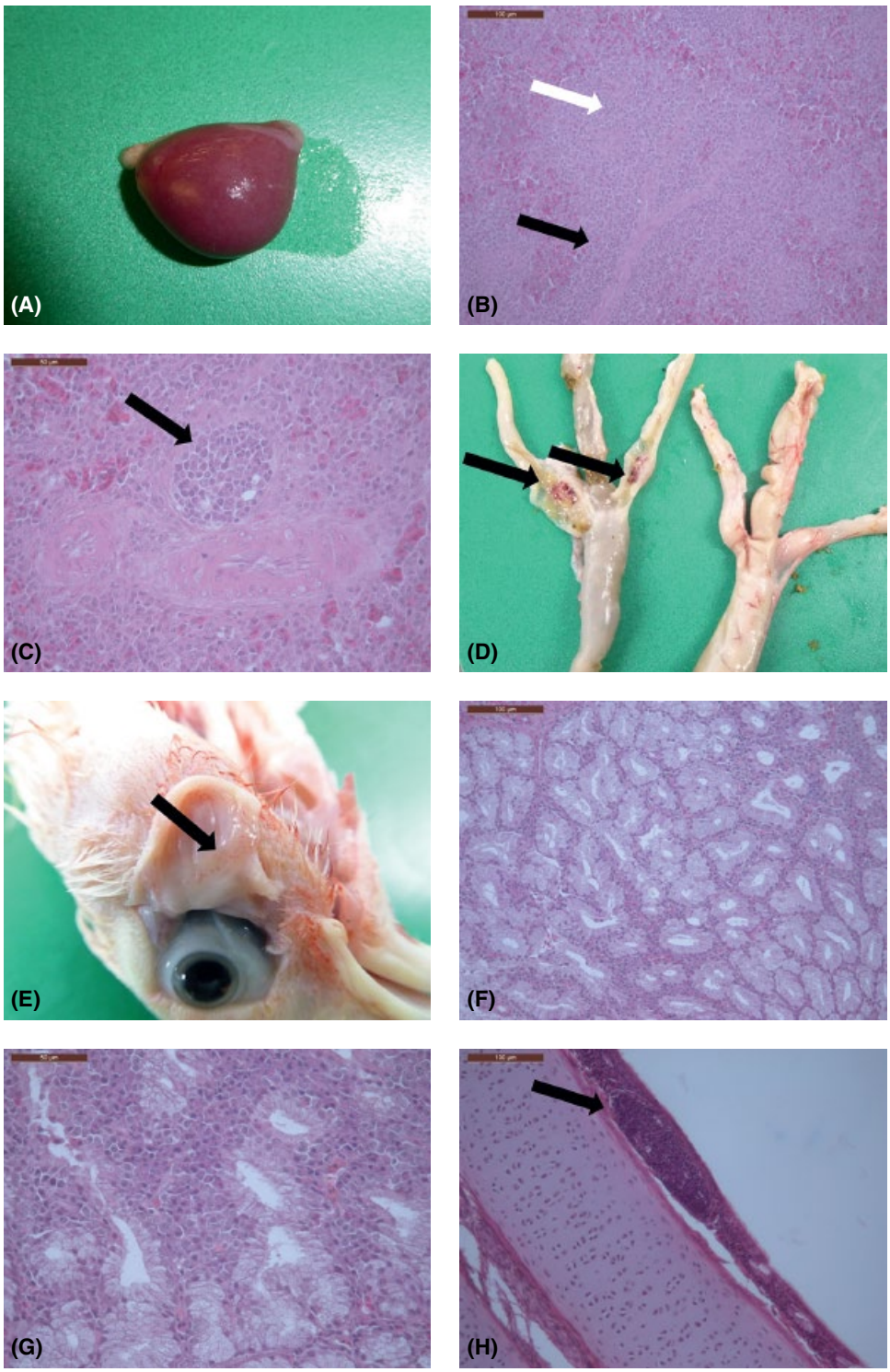


Figure 2.2 (A) The spleen. (B) Periarterial lymphoid sheaths (see black arrow) and periellipsoidal white pulp (white arrow). (C) A bursa-dependent lymphoid follicle (black arrow) is located adjacent to a small artery. (D) Cecal tonsils, opened (left, arrow indicates lymphoid tissue) and unopened (right). (E) Small nodules in the conjunctiva and the conjunctival-associated lymphoid tissue (CALT). (F) The Harderian gland contains lymphoid cells in the connective tissue between the glands. (G) Plasma cells are the predominant cell population in the Harderian gland. (H) Nodular deposits (black arrow) of lymphoid tissue are located in the mucosa of the trachea. (*For color detail, please see the color section.*)

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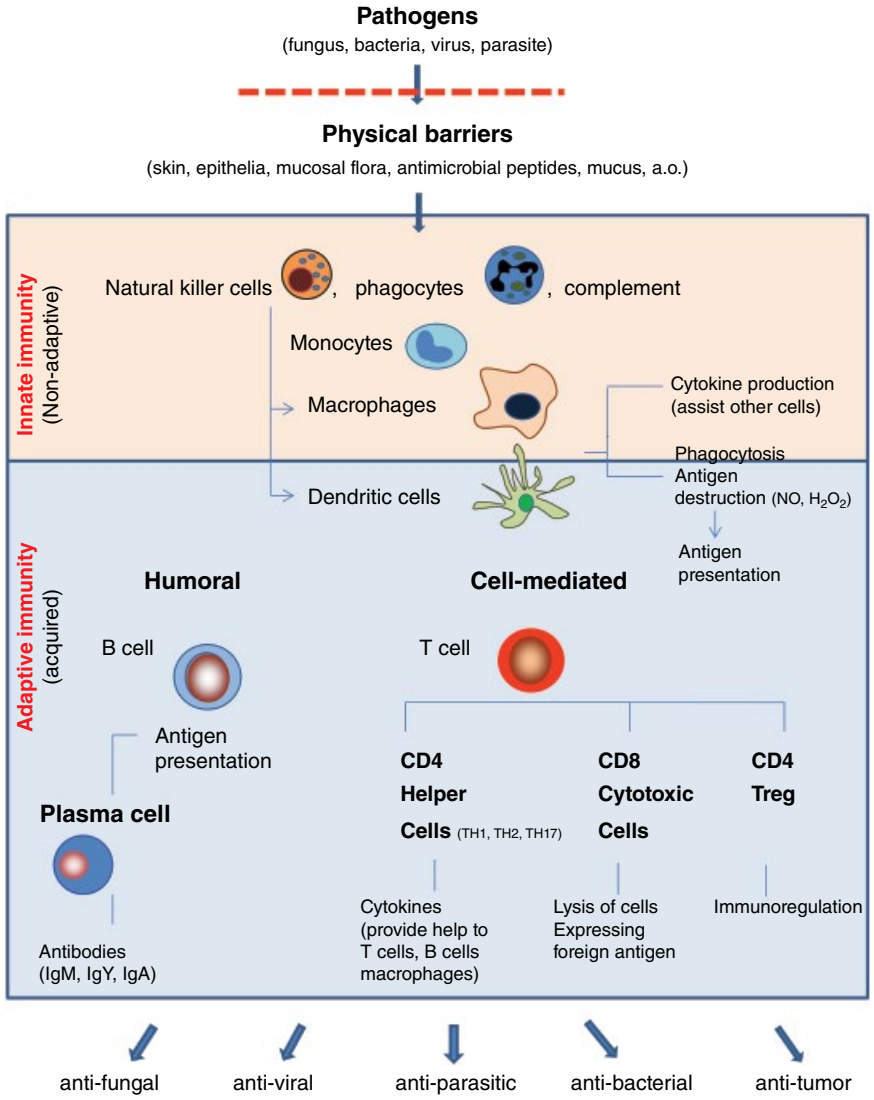


Figure 2.3 Physical and immunological mechanisms of defense against pathogens in birds. Treg = regulatory T cells; a.o. = and others; CD = cluster of differentiation.

cifically the adaptive immunity, depends significantly on the interaction with the colonizing microflora (39, 66). Nutrition can be an important regulator of the immune response (as reviewed by 37), as it is "expensive" in terms of energy and nutrients, with variable impact of innate and adaptive reactions (39, 66). Most susceptible to nutrient deficiencies are developing T cells. Immunomodulatory effects of certain nutrients have been described (37).

Innate Immunity

Host defense peptides (HDP) are a diverse group of small peptides, which were originally called antimicrobial peptides, but have been shown to not only kill bacteria but also to have immunomodulatory functions (as reviewed by 10). These include differentiation, activation, and chemotaxis of leukocytes, as well as enhancement of phagocytosis. The complete β-defensin and cathelicidin cluster has been described for chickens, and more information is also becoming available for other avian species (60). Liver-expressed antimicrobial peptide-2 (LEAP-2) belongs to a third class of HDP (42). These HDP become of interest as anti-infectives with the indication to reduce the use of antibiotics. In addition enzymes, proteins, and peptides such as lysozyme, transferrin, C-reactive protein, and collectins including C-type lectins, may be involved in the innate host defense by killing bacteria for example by opsonization and enhancement of phagocytosis or by restricting their growth conditions (as reviewed by 10). Mannan-binding lectin (MBL) is one of the most studied collectins and is upregulated during infection in chickens (25). Mannan-binding lectin activates the complement system (lectin pathway), acts as an opsonin, and is involved in apoptotic cell clearance and thrombus formation (as reviewed by 26).

Other serum proteins also contribute to the complement system. Together with cell surface complement receptors and regulatory proteins, they contribute to the classical and alternative pathways of the complement

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cascade after activation (26). The activation of the complement system results in the production of a series of proteins. Some of these proteins bind covalently to bacteria, which results in bacterial death or enhanced bacterial phagocytosis and destruction.

Also, natural antibodies are found in chickens, which have been detected without a known antigenic challenge and thought to contribute to the first line of defense linking innate and adaptive immunity (2).

Cytokine and chemokines are important players in the innate immune response but also contribute to the adaptive response as discussed later. They are regulatory molecules, which are generally secreted by immune and non-immune cells but may also act occasionally as cell surface molecules during immunological development and immune responses (28). Genes encoding most major avian cytokines and chemokines and their receptors have been cloned and sequenced and a number of recombinant cytokines are available (27), and some have experimentally been used in therapeutic approaches and as adjuvants in avian species (20, 49). The biological activity of avian cytokines is generally quite similar to their mammalian counterparts, although avian cytokines show little cross-species biological reactivity. There are different cytokine and chemokine families including interleukins, interferons, tumor necrosis factor superfamily members, and colony-stimulating factors. They have partially redundant functions including antiviral, proinflammatory, or anti-inflammatory activities, with the chemokines being specifically involved in leukocyte trafficking. Cytokines bind to specific receptors on the surface of target cells and regulate immune responses by signaling between cells. They may act systemically or locally in an autocrine or paracrine fashion. Receptor-bound cytokines and other membrane-associated molecules often act together to stimulate the effector function in a target cell.

Cells of the Innate Immunity

The cellular components of the innate immune system consist of the activity of specialized epithelial cells and more classically defined immune cell populations. These include macrophages, granulocytes, thrombocytes, and natural killer cells. The recognition of pathogens by cells of the innate defense such as macrophages and heterophils, but also dendritic cells, which are specialized antigen-presenting cells linking the innate and acquired immunity responses, is facilitated by the presence of toll-like receptors (TLRs) that are present on the cell surface or are intracellular (5). Toll-like receptors are proteins that recognize conserved pathogen-associated molecular patterns (PAMPs) expressed by many pathogens. The chicken immune system contains a slightly different TLR repertoire compared to mammals. Chickens were shown to lack TLR9, while TLR15 and TLR21 seem to be unique to chickens, the latter binding CpG-DNA (12, 32, 33). Toll-like receptors may sense PAMPs at the cell surface or intracellularly associated to membrane-bound vesicles, and stimulate cells to produce cytokines and chemokines. Other pathogen recognition receptors (PRR) at the cell surface include selectins, dectin molecules, Fc and complement receptors (26). Additional PRR are found intracellularly and detect products of pathogens that include nucleotide-binding oligomerization domain (NOD)-like receptors and the retinoic acid-inducible gene I (RIG-I)-like receptors. The repertoire may vary between avian species (8).

Macrophages that are scattered extensively in the tissues of the host are the first line of defense against pathogens. These cells, identifiable by cell surface-specific antibodies (45), phagocytose and destroy the invading pathogens and prevent active infection. Macrophages and dendritic cells, another phagocytic cell present in the tissues, respond to the encounter with the pathogen by secreting cytokines and chemokines that initiate a local inflammatory process (64).

Avian species lack neutrophils. Instead heterophils are the predominant granulocytes in the circulating blood of most birds. Eosinophils and basophils can also be found, but it is not clear if functional eosinophils really exist in chickens (28). Heterophils are highly phagocytic and use a vast repertoire of microbial killing mechanisms including the production of an oxidative burst, cellular degranulation, and production of extracellular matrices of DNA and histones (HETs) (as reviewed by 17). Heterophils accumulate at the site of inflammation within 6–12 hours in an attempt to localize and destroy the pathogen.

Natural killer cells are non-T, non-B lymphoid cells that are cytotoxic for virus-infected and tumor cells (as reviewed by 53). Natural killer (NK) cell development is bursa and thymus independent, and they do not need to be induced by immunization. Avian NK cells express surface CD8αα homodimers and are large granular lymphocytes, morphologically similar to their mammalian counterparts. The cytotoxic activity of NK cells is not restricted by the major histocompatibility complex (MHC), and different receptors belonging to the immunoglobulin (Ig) superfamily or C-type lectins are expressed on their cell surface contributing to the recognition of possible NK cell targets (as reviewed by 26). In chickens, the intestinal epithelium is particularly rich in NK cells although these cells are also present in spleen and peripheral circulation (18). A combination of a panel of antibodies and functional assays should facilitate studies on NK cell activity in healthy and diseased chickens (26). Distribution of NK cells in normal chickens suggests that precursors of these cells originate in the bone marrow and migrate to the spleen and intestinal epithelium where they acquire functional maturation.

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The *in vivo* NK cell numbers and activity in chickens vary with age, genetic background, exposure to infectious agents, and presence of tumors (65).

Natural killer cells and certain other effector cells also may induce target cell lysis if the target cells are coated with antibodies. The antibody molecules present on the surface of target cells interact with Fc receptors present on NK cells and this interaction triggers the cytotoxic attack against the target. The destruction of antibody-coated target cells is called antibody-dependent cellular cytotoxicity (ADCC) and has been detected in several avian species (65).

Adaptive Immunity

Pathogens that cannot be denied entry by physical barriers or controlled by innate immune defense mechanisms initiate a specific immune response (adaptive immunity). Adaptive immunity is highly specific to the agent that stimulates its development, whereas nonadaptive or innate immunity is not antigen-specific. Adaptive immunity is mediated by a variety of cells, the most important of which are T cells, B cells, macrophages, and dendritic cells. T cells, the principal cells of cell-mediated immunity (CMI), recognize foreign antigens after the antigens (such as microorganisms) have been processed by antigen-presenting cells (APC). Macrophages, dendritic cells, and B cells are the most important APC. Thrombocytes also may serve as APC (74). The APC generally break down complex antigens and present to T cells small fragments of the antigen in conjunction with the MHC molecules. T cells and the APC must share the same MHC for T cells to recognize and react to the antigen being presented (64).

The MHC molecules are glycoprotein receptors encoded by the genes within the MHC. The chicken MHC, also referred to as the B locus, is much smaller than the mammalian MHC. The organization of the chicken MHC is quite different from that of the mammalian MHC (31). The B locus consists of different loci: BF that encodes class I antigens, BL that encodes class II antigens, and BG that encodes BG antigens on erythrocytes, and additional genes within the tripartite motif-containing (TRIM) region (29). Class I and class II molecules are highly polymorphic and are critical for antigen presentation by the APC. The BF molecules (class I antigens) are present on a wide variety of nucleated cells including erythrocytes. The expression of the BL molecules (class II antigens) is much more restricted. These molecules are expressed on macrophages, dendritic cells, monocytes, B cells, and activated T cells. While class I molecules bind peptides from proteins, which are mainly found in the cytoplasm, classical class II molecules bind peptides derived from processed proteins taken up from the extracellular space (29). Chickens predominantly express class I and most likely also class II molecules that influence the repertoire of bound peptides and, possibly, CD8 T cells, which may be associated with disease resistance (see below, as reviewed by 30). There are also non-classical MHC molecules expressed. These include for example CD1 molecules, which present lipid antigens to various kinds of T cells (52). In chickens, two nonpolymorphic CD1 genes were discovered within the B locus (57).

Whereas T cells require that the antigen be processed before it can be recognized, the recognition of an antigen by B cells is not dependent on prior processing. B cells can recognize the antigen as it interacts with immunoglobulins that project from the cell surface. B cells are responsible for humoral immunity and produce antibodies against the antigen.

Most microorganisms stimulate both CMI and humoral immunity, although the type of immunity most critical for defense may vary with the microorganism. Some of the important features of the CMI and humoral immunity are discussed below.

Cell-Mediated Immunity

T cells are the most important cells of CMI. Many subpopulations of T cells with diverse functions have been identified in chickens. These subpopulations express unique surface antigens that can be detected with monoclonal antibodies (as reviewed by 60, 64). As in mammals, avian T cells have two surface receptors that bind antigens: T cell receptor (TCR)αβ or TCRγδ, which each consist of two chains. Both types of TCRs (αβ and γδ) are closely associated with another molecule called CD3, which is present on all T cells. Only the TCR portion of the TCR-CD3 complex interacts with the antigen. The CD3 molecule, which is comprised of a complex set of proteins, transmits to the cell the signal of antigen/TCR interaction. The TCR molecules are diversified by rearrangement of single V (variable), D (diversity), and J (junctional) segments derived from multiple polymorphic copies of genes (somatic DNA recombination). Additionally, the diversity of the antigen-binding sites is reached by junctional modifications (deletions and additions of nucleotides) of the DNA at the TCR locus and pairing of the TCR chains (as reviewed by 67). The chicken TCRβ locus is different from that of mammals and contains two Vβ families: Vβ1 and Vβ2 (7).

Surface molecules CD4 and CD8 differentiate two important functional subsets of αβ T cells. CD4 is mainly expressed on the surface of helper T (TH) lymphocytes, whereas CD8 is expressed on the surface of cytotoxic T lymphocytes (CTL). There is great interspecies and MHC-haplotype dependent variation in the relative proportions of circulating CD4 and CD8 cells. Double positive cells (CD4+CD8+) are detectable mainly in the thymus and are an indication for premature T cells but

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may also appear in the periphery (76). CD4+ cells co-expressing CD25 (CD4+CD25+) are defined as regulatory T cells (Tregs) (63). These cells downregulate immunity. Disruption in their function may result in autoimmune and other inflammatory diseases. Additional co-stimulatory and co-inhibitory molecules may be present on T cells to optimize and regulate signaling through the TCR.

Helper T Cells

Helper T cells (CD4+ cells) recognize processed exogenous antigens in conjunction with MHC II and other co-stimulatory molecules. When the TCR on the surface of T cells comes in contact with the specific antigenic fragment on the surface of the APC, T cells become activated, proliferate, and initiate an immune response directed against the antigen.

As demonstrated in mammals, recent studies in chickens provide evidence that antigen-induced activation stimulates TH cells to differentiate into two types of effector populations: TH1 and TH2 (13). Differentiation of TH cells into TH1 or TH2 populations is determined by the nature of the stimulating antigen and is mediated by cytokines. Intracellular pathogens that accumulate within macrophages and dendritic and other cells stimulate the differentiation of TH1 cells, whereas extracellular antigens stimulate the differentiation of TH2 cells. TH1 effector cells promote proliferation of CD8+CTL, activate macrophages, and enhance their microbicidal activity. The principal function of TH2 effector cells is to help B cells produce antigen-specific immunoglobulins of various isotypes.

Cytotoxic T Cells

Most CTL express CD8 surface molecules. A small proportion of mammalian CD4 T cells also may have cytotoxic activity, although the presence of avian CD4 T cells with cytotoxic ability has not been documented. CD8+ CTL recognize endogenous antigens in conjunction with MHC I (43). Internalized antigens such as viruses are degraded into small peptides. Small antigen peptides, usually 7–13 amino acids long, are then transported to the endoplasmic reticulum, where the peptides become attached to MHC I. The peptide-MHC I complex is then transported to the cell surface for possible recognition by antigen-specific CTL (64).

One of the most important functions of CTL is the elimination of virus-infected cells. Because most nucleated cells express surface MHC I, virus infection of almost any cell can lead to potential recognition and lysis by CTL. CTL activity has been shown to regulate pathogenesis of avian viral and neoplastic diseases (as reviewed by 64).

γδ T Cells

Chickens have a higher proportion of γδ T cells than mice or humans. They may reach 20–60% of circulating lymphocytes, and in the intestine about 60% of the intraepithelial lymphocytes are γδ T cells (as reviewed by 15). Peripheral γδ T cells in chickens can be divided into three subsets: CD8-, CD8αα+, and CD8αβ+ cells. Their functions are not fully clear yet, but studies have indicated that they exhibit cytokine production in response to innate signals, suggesting their involvement in the innate immune response (19). In addition, subsets of chicken γδ T cells were shown to express spontaneous cytotoxic activity and can lyse target cells in a MHC unrestricted manner (16).

Cytokines in Adaptive Immunity

T cells, B cells, macrophages, and dendritic cells all secrete cytokines. Cytokines produced by TH cells in particular play a key role in modulating an immune response. TH1 cells, which promote a CMI response, produce predominantly IFN-γ, which activates macrophages and enhances destruction of cell-associated pathogens. Another major cytokine produced by TH1 cells includes IL-2. IL-2 is critical for proliferation of a number of immune cells including TH, TH1, and TH2 cells; CTL; NK cells; and B cells. The TH1 cell activity and cytokine secretion is stimulated by IL-12 and IL-18, both produced by macrophages, dendritic cells, and B cells.

Cytokines produced by TH2 cells, which promote B cell activation and antibody production, include IL-4, IL-5, IL-9, IL-13, and IL-19 (as reviewed by 28). Other TH lineages have been described but their existence in chickens has not been formally demonstrated (28). IL-1β, a product of a number of cell types, most notably macrophages, stimulates TH2 cell activity.

Humoral Immunity

Immunoglobulins or antibodies secreted by B cells constitute the principal component of humoral immunity. Antibodies may be present in many body fluids but are most readily detected in the serum or the plasma fractions of blood. Exposure of birds to microorganisms stimulates the production of specific antibodies, which, in turn, react with microorganisms and hasten their destruction. Important mechanisms by which antibodies contribute to defense against pathogens include: (1) neutralization: antibodies bind to and neutralize specific pathogens, particularly viruses. Neutralized viruses are unable to attach to surface receptors of target cells and are thus prevented from replication; (2) opsonization: bacterial pathogens, which can replicate extracellularly, are more readily internalized and destroyed by phagocytes if the pathogens are coated with antibodies; and (3) complement activation: antibodies bound to the surface of pathogens can activate complement and produce new complement proteins. The complement proteins attach to receptors on phagocytes, which facilitate the phagocytosis and destruction of pathogens.

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Table 2.1 Properties of chicken immunoglobulin isotypes.

Isotype	Heavy chain (kDa)	Number of H chain Ig domains	Homology to mammalian (%)	Serum concentration (mg/mL)	Sources	Structure and comments
IgM	70	5	About 30	1–2	Serum	900 kDa consistent with heavily glycosylated $(\mu_2 L_2)_5$ plus a J-chain
—	—	—	78 for TM*	—	Cell surface	$\mu_2 L_2$ monomer of membrane IgM, no J-chain
IgY	67	4	30–35	5–10	Serum egg yolk	175 kDa, $\gamma_2 L_2$ monomeric form $\gamma_2 L_2$, high concentrations (10 mg/mL) of IgY are found in egg yolk (low concentrations in egg white)
IgA	65	4	32–41	~3	Serum	170 kDa, $\alpha_2 L_2$-monomeric form without J-chain
—	—	—	—	—	Bile	350 kDa, consistent with $(\alpha_2 L_2)_2$ plus a J-chain
—	—	—	—	—	Mucosa (tears, saliva)	600–700 kDa, consistent with $(\alpha_2 L_2)_4$ plus a J-chain

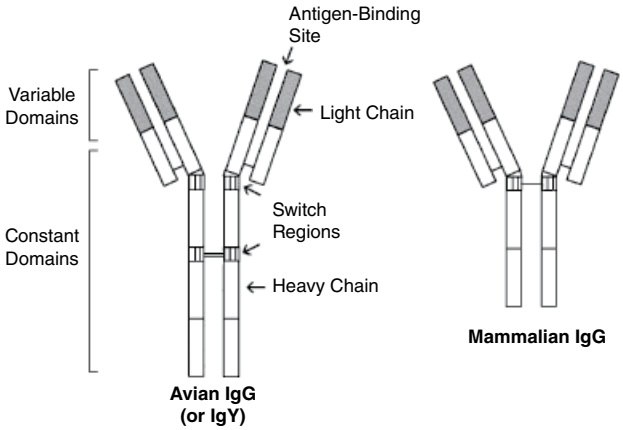


Figure 2.4 This figure shows the typical structure of an Ig molecule. All Ig molecules have two distinct types of polypeptide chains. The smaller polypeptide chain called the "light chain" is common to all classes of Ig, whereas the larger chain called the "heavy chain" is structurally distinct for each class or subclass of Ig. Covalent and noncovalent forces connect the two chains. The structure of the heavy chain determines the biological function of each class of Ig. Genes encoding all three classes of avian Ig have been cloned and sequenced, which has facilitated the generation of recombinant avian and chimeric antibodies *in vitro* and expression of recombinant avian Ig in plants (as reviewed by 64).

Chickens have three main classes of immunoglobulins: IgM, IgY, which is an evolutionary predecessor of both IgG and IgE of mammals (71), and IgA (Table 2.1). But they lack a homologue of mammalian IgD. Figure 2.4 compares the relative structure of mammalian and the larger avian IgY.

IgM is found on the surface of most B cells and is the first antibody produced following primary immunization. As the immune response progresses, the IgM-producing cells switch to the production of IgY or IgA. This phenomenon is called "class switch." The antigen-binding specificity of the antibodies does not alter during or after the switch. The class switch occurs because the antibody-producing B cell begins to splice the variable (V) region genes (V genes) to the constant (C) region genes (C genes) of the heavy chain of a different class of Ig. Cytokines including IL-4, TGF-β and IFN-γ stimulate the B cell to undergo class switch (14).

IgY is the principal antibody produced after secondary immunization and is the predominant Ig class in chicken blood. IgA is the most important Ig involved in mucosal immunity. Chicken secretory IgA (sIgA) exists as a dimer in mucosal secretions, whereas circulating IgA is polymeric or monomeric. IgA complexes with a secretory component present on the surface of mucosal epithelial cells to form sIgA (73). The acquisition of the secretory component protects IgA from proteolytic digestion in the gut. IgA is most concentrated on mucosal surfaces, although small quantities may be found in the circulation. Bile is also a rich reservoir of IgA in birds. IgA protects mucosal surfaces against pathogens, particularly viruses, by neutralizing and preventing their attachment to receptors on target cells (64).

For an antigen to initiate antibody production and clonal expansion, the antigen must interact with a B cell that expresses the homologous Ig receptor. There are potentially thousands of antigens and millions of antigenic shapes in the environment. The immune system maintains an inventory of B cells with such a wide variety of antigenic specificities by a number of genetic mechanisms during the development and maturation of B cells. In mammals, Ig gene rearrangement leads to extensive Ig diversity. In the chicken, because of a relatively small number of Ig genes, the rearranged genes must undergo a process called gene conversion to attain needed diversity (14). In gene conversion, the rearranged light and heavy chain gene complexes acquire clusters of chromosomal pseudogenes. Large segments of highly homologous pseudogenes are present in the vicinity of light and heavy chain genes in the chicken chromosome (51). Repeated exposure will subsequently lead to hypermutations

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contributing to affinity maturation (3) as demonstrated for other species.

Maternal Transfer of Immunity

Transmission of immunity from the hen to the newly hatched chick is critical for protecting the chick against infections during early life. In chickens, Ig are the principal mode of immunity transfer. There is little evidence that the mother's immune cells are passed on to the embryo. Ig from the hen's circulation is deposited in the superficial epithelial and glandular cells of the oviduct (36). From the oviduct, IgY is transferred into the maturing oocyst in the ovarian follicle and accumulates in the yolk sac. The concentration of IgY in yolk is often greater than in the hen. Ig produced locally in the oviduct likely constitutes an insignificant proportion of the transferred Ig. The developing chick acquires maternal IgY from the yolk sac, which then appears in embryonal circulation. Absorption begins at embryonation day seven and accelerates sharply during three days before hatch (38). Absorption continues at least until 24 hours after hatch. Total serum IgY levels in the newly hatched chick increase to their maximum value about two days after hatch. As the egg passes down the oviduct, locally secreted IgM and IgA are deposited in the albumin. During embryonal development, albumin diffuses into the amniotic fluid. The embryo acquires IgM and IgA by swallowing the amniotic fluid. Thus, the newly hatched chick has IgY in the circulation and IgM and IgA in the intestine.

Although maternal antibodies are important for the well-being of the newly hatched chick, the antibodies may interfere with active immunization with live vaccines. Neonatal or *in ovo* vaccination may be necessary to protect newly hatched birds against diseases to which they are exposed shortly after hatch. Besides neutralizing the antigen present in the vaccine, preexisting antibodies also may interfere with the development of active immunity by providing negative feedback to the immune system. In contrast, data in wild birds indicate that presence of maternal antibodies against an antigen may enhance active immunity following immunization with the same antigen (64).

Assays to Measure Immunity

So far, classical serological methods are widely used to evaluate the humoral immune response under field situations. Standardized test systems are commercially available worldwide, which are easy to perform under minimal laboratory requirements. Circulating serum antibodies of the IgY-subtype are mainly detected with these standardized systems, allowing only conclusions regarding the systemic humoral immunity. Local humoral immunity may be assessed in tears collected from live birds (55). If other mucosal tissues need to be sampled, this is normally only possible after sacrificing the birds, and therefore the field application of investigating these samples is more restricted. Advances in molecular methods and the development of high-throughput platforms are opening up new opportunities for the evaluation of other aspects of the innate as well as acquired avian immune response not only under experimental but also under field conditions (4, 9, 69). This is important to ultimately improve disease resistance in poultry flocks and will allow selection for improved immune robustness. Classical cell-based methods and bioassays evaluating the responsiveness of immune cell populations are very well suited for experimental work but, due to the necessity for a specialized laboratory and the relatively low throughput, field application is at this point not practical. Flow cytometry-based tests evaluating absolute or relative immune cell numbers in the circulation in addition to blood smears will provide additional opportunities for larger scale testing of poultry (62). Genotype- and age-related differences have to be considered in the interpretation of the obtained data, and true baseline values for the field are, as yet, rarely available.

Detection of Antibody Levels

Birds exposed to pathogens develop circulating antibodies that generally persist for several weeks after the antigen has been cleared. Some of the commonly used serologic tests include the agar gel precipitation test, virus neutralization test, immunofluorescence test, hemagglutination inhibition test, and enzyme-linked immunosorbent assay (ELISA). Protocols for conducting these tests have been widely described. Antibodies against diverse antigens present in a single serum sample may be quantitated using multiplexed immunoassays (72).

ELISA is by far the most common serologic assay used in commercial settings. Automated technology allows rapid processing of large numbers of serum samples. Computerized data transmission facilitates flock profiling and provides useful information on environmental exposure to pathogens and response to vaccination. ELISA kits that can be used to detect antibodies against most of the common viral and bacterial pathogens of poultry are available commercially. Commercially available ELISA kits from different manufacturers may vary in specificity and sensitivity and give conflicting antibody titers (11).

As noted in Maternal Transfer of Immunity, the transfer of IgY from the yolk sac to the embryo or hatchling occurs by absorption into the recipient's circulation. Maternally derived antibodies decline linearly in the recipient and become undetectable by serological tests after about two to five weeks depending on the titer and genotype of the bird (as reviewed in 64).

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Bioassays to Detect Different Immune Cell Populations

Natural Killer Cells

Natural killer cell assays are mainly based on *in vitro* cytotoxicity against susceptible target cells, but other characteristics such as cytokine release may also be measured by bioassays or molecular methods (46, 75). The target cells (most commonly LSCC-RP9 cells are used) are incubated with effector cells, often spleen cell suspensions, and target cell lysis is measured by radioactivity or flow cytometry (23, 46). In the radioactive assay, target cells are labeled with 51 Cr and incubated *in vitro* with varying concentrations of cell suspension being tested for NK cell activity (effector cells). Two controls are important: (1) adding "neutral" cells such as thymocytes to target cells at the same effector : target ratios as used for the effector cells and (2) the use of NK-resistant target cells. After four hours of incubation at 37 °C, the radioactivity released into the medium is quantitated. Specific cytotoxicity, a measure of NK cell lysis, is calculated as previously described (64).

Macrophages

Because most macrophages adhere to substrates, they can be readily isolated from short-term, *in vitro* cultures of peripheral blood cells (PBL), bone marrow, or single cell suspensions of spleen (34). Peritoneal macrophages may also be induced in birds by intraperitoneal injections of inflammatory stimulants such as Sephadex beads. Some of the assays used to assess macrophage functions include: (1) phagocytosis, (2) cytokine production upon stimulation (e.g., polysaccharides), (3) ability to lyse tumor cells, and (4) production of nitric oxide (NO) upon activation by T cell-produced cytokines, most notably IFN-γ (64). The availability of recombinant cytokines has extended the *in vitro* survival of macrophages in culture (6).

T Cells and Cytokines

Most TH cell assays are based on *in vitro* stimulation of cells with mitogens or specific antigens (as reviewed by 64). Stimulated cells proliferate and secrete cytokines. Concanavalin A (Con A) and phytohemagglutinin (PHA) are often used to induce T cell proliferation as a measure for T cell competence. These mitogens bind to cell surface glycoproteins on T cells and stimulate the cells to proliferate. In the typical assays, spleen cells, PBL, or diluted whole blood are cultured *in vitro* in medium containing Con A or PHA. After 40 hours of incubation at 37–41 °C, proliferating cells are detected by radioactive or colorimetric assays. The incorporation of, for example, radioactive thymidine in cellular DNA or metabolic activities are quantitated (64). Proliferation may also be measured by flow cytometric analysis of dividing cells (54). Results of these mitogenic assays should be viewed with caution because the mitogen-induced proliferation is not antigen-specific, and response to mitogen is an *in vitro* function of T cells. The *in vivo* relevance of this function to other *in vitro* or *in vivo* functions of T cells is not known, and functional T cells may be prevented from proliferating by non-T suppressor cells or suppressor products present in the culture (47).

T cells recovered from immunized animals may proliferate *in vitro* when cocultured with the antigen used for immunization (as reviewed by 64). This antigen-specific proliferation has been shown with several avian pathogens, although the ideal assay conditions are not well established and the test is not widely used.

CTL activity can be measured *in vitro* by flow cytometric-based tests or by the chromium-release assay after coculturing effector cells with radiolabeled target cells. The protocols for the chromium-release assay are quite similar to those described above for NK cell cytotoxicity assays. Because the cytotoxic activity of classical CTL is MHC I-restricted, the CTL assays are difficult to perform in outbred populations of birds. For a successful assay, both effector and target cells must come from the same bird or syngeneic birds. Because of these limitations, the use of CTL assays continues to be restricted to research laboratories (as reviewed by 64). The development of MHC/peptide tetramers to identify specific T cells, for example by flow cytometric analysis, is a method frequently used in the mammalian system to characterize specific T cell responses. This method has not been available for avians so far, but efforts are in progress to push this method forward for chickens (41).

Certain *in vivo* assays may also be used to assess T cell functions. The delayed-type hypersensitivity assay measures antigen-specific response. In this test, an animal immunized against an antigen is intradermally injected with the same antigen. Swelling at the site of the injection comprises a positive response. Local swelling at the site of an intradermal injection of mitogens such as PHA has also been attributed to a nonspecific T cell response.

Cytokines released not only by T cells but also other cell types may be measured by ELISA, ELISpot, or bioassays or using molecular methods including qRT-PCR systems, *in situ* detection methods, microarray, and transcriptomics (e.g., 21, 35, 40, 50, 56).

Stress and Immunity

There is a close association between stress responses and subsequent immunocompetence of poultry (22). The hypothalamo–pituitary–adrenocortical axis is connected to the immune system, while immune cells express receptors for stress hormones such as the adrenal glucocorticoid corticosterone or neuropeptides, and therefore react to stress. Immune cells also may

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release neuropeptides and subsequently connect with the brain axis. While the measurement of glucocorticoids is not well standardized, determination of the heterophil-lymphocyte (H:L) ratio has been frequently used as a parameter to determine stress, as stress evokes marked shifts in the H:L ratio (58).

Genetics of Disease Resistance

Hans H. Cheng and Susan J. Lamont

Summary

Genetic resistance is an attractive and sustainable approach for disease control. The chicken genome assembly and associated tools make it feasible to identify the causative genes and pathways for genetic resistance to pathogens. This knowledge can be applied to better diagnostics, and the breeding of superior commercial birds, which improves animal welfare and yields safe and affordable poultry products. To guide the target audience of animal health professionals, basic genetic concepts and experimental approaches are described. Furthermore, brief summaries of the current status of genetic resistance for key specific diseases are provided.

Introduction

Genetic resistance to infectious diseases is alluring from industrial and academic viewpoints. Losses due to infectious diseases are a significant issue and can be the key factor in determining economic viability of poultry companies. Furthermore, certain pathogens may disrupt trade between countries or undermine public confidence in food product safety. Consequently, genetic resistance can be a powerful approach in combination with other management practices to eliminate or manage infectious diseases.

Modern molecular genetics has provided an arsenal of new tools for identifying disease resistance genes. As a component of genomics, there is great excitement that some of the complexity of biology and, in particular, the immune response, may finally become fully elucidated. Genetic research will continue to identify genes and their associated biological pathways that control complex traits such as disease resistance. Information is expected to emerge on how these genes function and interact, as well as respond to changing environments, to control disease. Ultimately, this information will be transferred to poultry companies to generate elite lines with superior disease resistance. Nonetheless, the field is still limited in the ability to predict and model complex traits.

Studies on genetic resistance and genetics in general underpin the change that will undoubtedly occur throughout biology including veterinary medicine and diagnostics. With the advent of molecular genetic maps and genome sequences, genomics and "discovery-driven research" emerged as the preeminent methods for dissecting and understanding complex traits. Consequently, while genetics has always used a holistic approach to examine the entire organism, with the ability to measure and record millions of data points at the DNA, RNA, protein, and metabolite levels, quickly and economically, the power of existing and upcoming technologies has and will continue to shift the field toward large-scale unbiased screens using and integrating molecular and computational biology.

In this subchapter, we focus on recent advancements in genetic resistance to disease, namely, molecular and quantitative genetics. We aim to convey the high level of excitement (as well as limitations) in these areas, which were enabled by the release of the chicken genome sequence and related technologies. Sections are presented on (1) basic genetic concepts, (2) molecular genetic approaches to identify disease resistance genes, (3) complementary functional genomic approaches, (4) brief summaries of genetic resistance and experimental studies for specific diseases, and (5) future perspectives. The emphasis throughout these sections is on basic concepts; given the short history and dynamic nature of the field, relevant knowledge and state of the art methods will change rapidly.

Review of Quantitative and Molecular Genetics

Classical or Mendelian genetics describes biology in mathematical (quantifiable) terms by using defined phenotypes (measurable traits) that are explained by one or a few genetic loci. "Simple" or qualitative traits certainly exist, as evidenced by the large number of loci and alleles, often with interesting and descriptive names, controlling individual traits. Most traits, however, have natural variation with continuous (quantitative) distribution rather than discrete (qualitative) phenotypic classes within a population. This phenotypic variation can arise from the segregation of multiple alleles (forms of genetic variants) of a single gene, or from numerous genes, combined with modulating effects due to interactions with the environment.

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Genetics strives to understand how the phenotypes of organisms are influenced by their genotypes. More specifically, modern molecular genetics connects genetic variation to specific phenotypic variation. The goal is to determine how variation within a population can be explained at the genetic level, which should facilitate selection to improve the trait in a breeding program. At the simplest level, we wish to know whether different alleles for a given gene give statistically significant differences in a particular trait. With molecular genetic maps and the chicken genome assembly (131), we can extend this same question to the entire genome. Therefore, the goal of genetics is to predict the phenotype of offspring produced by specific parents. More specifically, the major goal of molecular and quantitative genetics is to identify the genes and alleles that account for phenotypic variation within a population.

The basic genetics concepts, described below, have been established for decades and utilized well as evidenced by the great progress made by poultry breeding companies. What has recently changed is the emergence of genomics and related technologies, which have had a dramatic and positive impact, making it possible to identify the underlying genetic basis for complex traits such as disease resistance.

Phenotypic Distributions

Even among individuals with identical genotypes, there may be a range of phenotypes. The phenotypic range is because traits are not simply the outcome of the contributing genes only, but also complex interactions among genes, response to the environment, and other factors. For simple and qualitative traits, the phenotypic distributions of unique genotypes may be very tight and not overlap with others to give discrete classes. This is often not the situation for complex and quantitative traits where a continuum is found.

Disease presents unique issues for quantitative trait analyses, as the definition often varies according to the pathogen. For this subchapter, disease encompasses the unfavorable outcome of the interactions between the host, pathogen, and environments, whereby conditions exist to favor pathogen growth and spread with resulting damage to the host. Disease resistance could be defined as absolute resistance to infection, as limiting pathogen replication or spread, or as tolerance to debilitating symptoms. Regardless of the situation, it is necessary to define what disease means for each pathogen.

Disease is often considered as a simple two-state trait, resistant or susceptible. It is advantageous, however, to deconstruct disease into continuously variable, quantifiable, and/or measurable components. Besides providing additional statistical power, subdividing disease into components has the potential advantage that resistance can be ascribed to one or more components.

Heritability

A prerequisite of breeding for genetic improvement is that the trait of interest is heritable. The extent of similarity from parent to offspring, or heritability, can be quantified. Variation in a trait within a population results from variation in the genotypes (genetic effect), variation due to the environment, and interactions between them. The genetic variation can be further broken down into additive and dominance variance components. Simply, the additive genetic variance accounts for the average effect of each allele of a gene, while the dominance variance component measures deviation from the predicted average of the two alleles. Heritability of a trait is typically reported as the amount of phenotypic variation that can be accounted for by the additive genetic variation, and is represented as h^2. Values range from 0 (no heritability) to 1, where the trait measures of the parents would exactly predict the value of the offspring.

For disease resistance, heritability estimates for specific diseases are generally reported to be low to moderate (0.0–0.3). Importantly, however, heritability estimates are for a particular population (set of birds and genetic composition) in a specific environment. Variation in populations and environments among experiments may be a main reason why there is a wide range of reported h^2 for the same disease traits. This also demonstrates that heritability is not fixed (constant) even for any one population and typically changes following selection (genotypes are altered) or placement in a different environment.

Linkage

Linkage, or the non-random association (co-inheritance) of alleles, is the main tool in the molecular geneticist's toolbox. This genetic phenomenon helps determine if a disease resistance gene is nearby or linked to a specific genetic marker. To illustrate, let's start with the simplest example: a single gene trait. Assume that there is a gene that encodes the cellular receptor to "nasty virus," and virus binding to this receptor is required for cellular infection. The "R" allele of the gene makes a defective receptor and confers resistance to infection and disease, while the other allele "r" makes the normal protein, which confers virus binding, entry, and susceptibility. Because the bird only needs one r allele for disease susceptibility, it would need two R alleles to prevent viral-induced disease and disease resistance is thus a recessive trait. From mating a susceptible bird with the R/r genotype (chickens are diploid, having two alleles for each locus) with a resistant bird (R/R), about half of the progeny should be susceptible with the R/r genotype and the other half should be resistant with the R/R genotype.

To locate the position of the disease resistance gene in the genome, one can test for cosegregation using molecular

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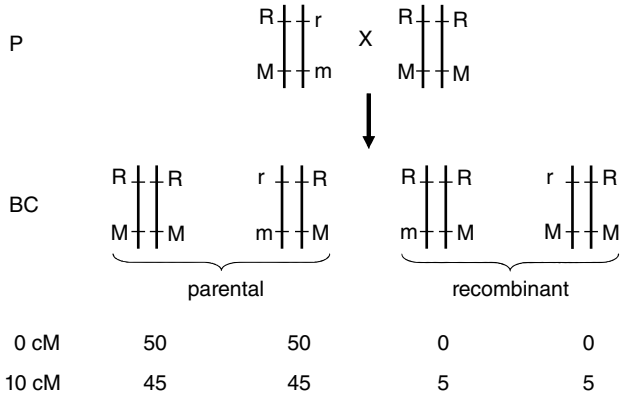


Figure 2.5 Linkage between a hypothetical virus receptor with defective (R) and functional receptor (r) alleles and a molecular marker with alleles M and m. In this example, parental (P) chickens that are susceptible (R/r) and resistant (R/R) to the virus are intermated to produce backcross (BC) progeny. If the loci encoding the viral receptor and the molecular marker are completely linked at the genetic level, then the molecular marker will absolutely predict the virus resistance status of the bird because all resistant birds will have the M/M genotype while all susceptible birds will be M/m. However, if loci are linked but can be separated at the genetic level during meiosis, if the distance between the loci is 10 cM, then the molecular marker will again be predicted but only 90% of the time.

markers (M, m). In Figure 2.5, for 100 backcross (BC) progeny, if all 50 resistant and 50 susceptible birds always are M/M and M/m, respectively, for a marker, then the marker is completely linked with and cannot be separated from the disease resistance gene. This does not prove that marker M is the disease resistance gene itself, but only that the two loci were not separated genetically when the gametes were formed during meiosis.

This example also demonstrates that alleles M and R, and alleles m and r, are each in complete linkage disequilibrium (LD). If the marker locus and disease resistance gene were unlinked, then the alleles should randomly segregate. But this is not the case as M occurs more frequently than is expected by chance in resistant birds and, thus, the marker allele M is predictive of the disease resistance gene allele.

But what if the two loci are not completely linked? Imagine that of the 50 disease resistant birds, 45 had the M/M genotype and 5 the M/m genotype. Likewise, of the 50 susceptible birds, 45 had the M/m genotype and 5 the M/M genotype. Having two copies of the M allele is still a very good indicator of disease resistance but is not 100% accurate. In this case, we can determine the linkage of the two loci by quantifying the percentage of recombinant alleles (non-parental ones, e.g., R and m, or r and M). In this example, there are 10 recombinant progeny out of 100, which means that the disease resistance gene and the marker are 10 centiMorgan (cM) away. The genetic term cM reflects the number of recombination events between two loci detected in 100 progeny.

To appreciate the difficult challenge of finding genes for complex disease resistance, imagine that each typical disease resistance gene accounts for only a very small percent (e.g., 2%) of the total observed variation in the population, as is typical for most diseases. Furthermore, not all R/r birds are susceptible as the r allele may not always be expressed at sufficient levels to allow virus entry. Or not all the birds get challenged with nasty virus as natural exposure is being used. While daunting, these challenges can be partly overcome with properly controlled experiments, the use of dense molecular markers that encompass the entire chicken genome, enough progeny to give sufficient statistical power, accurate phenotypes, and proper biometrical analyses.

Experimental Approaches for Molecular Genetics

The development of genetic maps based on molecular markers defined the birth of genomics. No longer were scientists limited to assaying for genetic effects using markers at undefined locations. With the ability to systematically query the entire genome, the way most experiments were conducted was changed. Rather than using the reductionist approach of formulating a hypothesis about a specific gene and then testing it, genomics became a "discovery-driven" field where the end results help define the next scientific questions and experiments. Genomics approaches dominate modern genetic studies, and are being reinforced and strengthened with whole genome and RNA sequencing, and other high-throughput technologies that quickly deliver thousands to millions of accurate and low-cost data points.

Candidate Genes

Many immune function genes have been confirmed to be, or may be, good candidates for conferring disease resistance. Consequently, prior knowledge to identify candidate genes for specific disease resistance is a valid method and often the best first choice for testing for a genetic effect and, if present, determining its influence. This approach requires a population that is segregating for disease or disease-associated traits, and an informative DNA polymorphism.

Although a gene and its product may be critical components of the immune response and disease resistance, some populations may have no variants in the gene and, therefore, no possibility to detect a genetic effect attributable to the gene. The first requirement, therefore, is to identify DNA polymorphisms (sequence variations) and alleles of the gene that are segregating within the population. Furthermore, evidence for a genetic effect does not prove causation by that gene, but may be a result of

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linkage between the causal gene and the analyzed candidate gene. Finally, only that specific gene and genomic region are being screened for genetic effects, and no information is generated about other genomic regions that may also influence resistance to the disease.

The major histocompatibility complex (MHC) represents a unique situation and opportunity. Due to its importance in many diseases (93), chicken lines have been developed that theoretically vary only in the MHC or B locus (e.g., 7). These lines have near identical genetic background and carry unique and defined MHC haplotypes. So rather than using a genetic marker to the MHC and asking if it is associated with disease resistance, these "B congenic" strains can be used to quickly screen for MHC influences on disease resistance by challenging each line with the pathogen of interest and monitoring disease and disease traits.

Whole Genome Scans

With availability of the chicken molecular genetic map, it is possible to screen the entire chicken genome for disease resistance genes. Briefly, a resource population is identified or produced wherein birds segregate for disease resistance. Some or all animals are measured for disease or disease-associated traits. Likewise, the same animals are genotyped for molecular markers spaced evenly throughout the entire chicken genome. Statistical analyses determine if certain genomic regions or quantitative trait loci (QTL) (genotypes) are associated with disease resistance (phenotypes) and, if so, how much variation each region explains.

Two approaches can be used in genome-wide QTL scans. The difference between the two lies in how the resource populations are produced, which also influences the density and, consequently, number of genetic markers required to detect genetic effects.

Linkage Analysis

In linkage analysis, each marker is tested to determine whether the inheritance of alleles influences disease resistance in a defined population. This type of linkage is known as identity by descent (IBD). Parents that differ markedly in disease resistance are selected and mated to produce progeny, which favors the use of inbred or divergently-selected experimental lines due to their genetic simplicity and defined disease status. In typical QTL scans, the mating structures are often backcross (BC) or F_2 populations. Due to the limited number of generations and recombination events that can erode the amount of LD, marker spacing can be relatively wide at 20–40 cM apart, which reduces the number and cost of markers that need to be genotyped. However, the identified regions may contain many genes.

Although simple in concept, the successful execution of the experiment entails many critical factors. Probably the biggest factor that one can control is the number of progeny. The power to detect QTL with smaller effects increases with more progeny. From a practical standpoint, this means at least 200 birds, if not 1000 or more, for disease traits. Normally, the generation of resource populations and their trait measurements, especially when pathogen challenges are employed, are the rate-limiting step in disease-related studies.

Association

Association or LD mapping, does not require a pedigreed population, which makes it attractive for use in the commercial environment as existing populations can be directly tested. The power of this approach is its reliance on historical recombination events to greatly minimize the extent of LD. And rather than following the inheritance of specific alleles (IBD), association mapping analyzes genetic marker allele frequency or identity by state (IBS). Thus, resistant birds should be highly enriched in frequency for a specific marker allele while the alternative allele(s) should be enriched in the susceptible birds. This method has both advantages and disadvantages. Pro: given the tight linkage required to detect a disease resistance gene, any genetic marker with a significant association with disease will be relatively close to the causal gene, making it almost immediately amenable to further verification and implementation in a breeding program. Con: since LD is small, it requires larger numbers of genetic markers to screen each LD region. Fortunately, "DNA chips" capable of economically scoring tens of thousands of single nucleotide polymorphisms (SNPs) have been developed, which have enabled genome-wide association studies (GWAS) to become very common.

Functional Genomics

As described above, genetics studies rely on statistical association of genotypic variation with phenotypic variation to identify genomic regions and determine how much variation each region contributes. Consequently, the ability to identify a disease resistance gene relies on statistical power and probabilities. For IBD, the best QTL mapping resolution is 5–20 cM intervals, which contains many million bases of DNA and is insufficient for identifying individual genes. For GWAS, the resolution is much better but still lacks the power to definitely identify causative genes.

To complement this genetic approach, several tools exist that query at the RNA, protein, or metabolite level (31). These functional genomic tools strive to identify components that vary between two or more states, for example, gene transcripts that are differentially expressed

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between disease resistant and susceptible chickens. The goal of identifying these molecules is to ascribe gene function and biological pathways to every gene.

Functional genomic assays combined with genome-wide scans can reveal positional candidate genes. Transcript or RNA profiling is the most popular and common approach, largely due to the availability of DNA microarrays and high-throughput sequencers. DNA microarrays monitor the expression of thousands of known genes per sample. Comparing results among samples reveals which genes are differentially expressed. Importantly, because gene expression (RNA) is being measured, unlike DNA, results can vary depending upon tissue, time point, and other influences. Thus, it is critical that several biological replicates are conducted. With the advances in sequencing technologies, it is now feasible, more powerful, and even cheaper to sequence the entire RNA population. Unlike microarrays, sequencing typically results in millions of reads (50 bases to the entire length of the mRNA), which provides an actual count of each transcript, plus more biological information (e.g., alternative splicing). Due to the large amount of data generated, the challenge is to computationally store, handle, analyze, interpret, and share these very large datasets. The list of candidate genes can be further analyzed to provide a higher order understanding. Tools to better analyze and extend RNA profiling datasets are an area of intense study.

Genetic Resistance to Specific Diseases

As discussed above, heritability of resistance to disease is essential to demonstrate a genetic component. Although differences for disease resistance between chicken genetic lines may suggest a genetic basis, this observation is insufficient to prove it. Combined with the need for controlled disease challenges and marker associations, the number of molecular genetics studies conducted to date is limited though growing, especially for genome-wide scans. There is already, however, evidence for genetic control of poultry diseases caused by a wide range of pathogens, including viruses, bacteria, and parasites. Major examples follow.

Avian Leukosis

Avian leukosis viruses (ALV) are retroviruses that can induce tumors. Avian leukosis viruses are classified into various subgroups based on virus-specific cellular receptors and virus envelope glycoproteins. The subgroups that infect chicken are A–E and J with all but subgroup J being exogenous ALVs.

Genetic resistance to ALVs subgroups A–E is well defined and based on specific cellular receptors. Because a single functioning receptor allele is sufficient for virus entry, susceptibility is dominant and the genetics is simple with only one locus involved. Molecular studies utilizing this fact have revealed not only the encoding gene but the basis for differences between resistant and susceptible lines.

Resistance to ALV subgroup A is determined by the *tva* locus on chromosome 28, which encodes a protein member of the low-density lipoprotein receptor (LDLR) family of unknown function (13, 37). There have been two alleles identified that confer resistance. The tva^{r} allele contains a single nucleotide mutation encoding a protein with very low binding affinity to the ALV subgroup A envelope while the other resistance allele, tva^{r2}, has a 4-nucleotide insertion near the beginning of the coding sequencing, which results in an altered protein.

The *tvc* locus, which confers resistance to ALV C, is ~1 cM from *tva* (38). This receptor shows homology to butyrophilins, a member of the immunoglobulin superfamily. The resistant allele contains a premature stop codon and, thus, would not produce a complete and functioning receptor (39).

Resistance to ALV subgroups B, D, and E (endogenous) are all controlled by the *tvb* locus (4). There are several reported alleles for this receptor, which is related to the tumor necrosis factor receptor (TNFR) family. A single nucleotide change that generates a premature stop codon results in a non-functioning receptor and resistance to all three subgroups. The tvb^{s1} or wild type allele is susceptible to all three subgroups while the tvb^{s3} allele confers resistance to subgroup E as the result of a different single nucleotide mutation (61).

The chicken receptor for ALV subgroup J is a Na+/H+ exchanger 1 (NHE1) (30) and although ALV J strains can be highly genetically diverse, all bind NHE1 to infect birds. Interestingly, tryptophan at amino acid position 38 (W38) in the extracellular loop (ECL1) is found in all chickens studied to date and determines ALV J susceptibility (65). However, it is not conserved amongst other avian species that are susceptible to ALV J (e.g., turkeys), which suggests potential methods for engineering ALV J resistant chickens (103).

Marek's Disease

Chickens resistant to Marek's disease (MD) are those that fail to develop characteristic symptoms upon exposure to Marek's disease virus (MDV). Genetic differences in resistance to fowl paralysis, assumed to be MD, have been reported for 70+ years. Resistance to MD is complex and controlled by multiple genes or QTLs (8).

The best characterized genetic resistance to MD involves the MHC. The MHC contains three tightly linked regions known as *B-F* (class I), *B-G* (class IV), and *B-L* (class II), which control cell surface antigens. The *B-G* locus is

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expressed in erythrocytes, which enables convenient typing of blood groups. By measuring the allelic frequency of specific blood groups, certain *B* alleles have been associated with resistance or susceptibility. Chickens with the B^{21} allele are generally more resistant than those with other *B* haplotypes (6, 10). Studies have allowed for the relative ranking of the other *B* alleles: moderate resistance, B^2, B^6, B^{14}; susceptibility, B^1, B^3, B^5, B^{13}, B^{15}, B^{19}, B^{27} (84). The MHC also influences vaccinal immunity as some haplotypes develop better protection with MD vaccines of one serotype than others (9, 11).

In addition to the classical MHC region, chromosome 16 contains another set of class I and class II MHC genes, the *Rfp-Y* locus (92). Data obtained from commercial chickens challenged with MDV suggests an association with MD resistance (129). However, results from matings with experimental lines indicate that the *Rfp-Y* genes do not influence any MD associated trait in these genetic backgrounds (127). These conflicting results further demonstrate the complexity of genetic resistance to MD, and the probable influence of genetic background.

Besides the MHC, other genetic factors have a major influence on resistance to MD. For example, inbred lines 6 and 7 chickens are both homozygous for the B^2 haplotype, yet are MD resistant and susceptible, respectively. Genome-wide QTL scans using these genetically simple and well-characterized lines identified up to 21 QTL (20, 48, 90, 126, 133) that explain MD incidence or MD-associated traits. Of note, all the QTL were of small to moderate effect, however, the various studies were relatively consistent with respect to QTL positions.

As described previously, moving from identification of a QTL to the underlying causative genes is a tremendous challenge. Consequently, additional functional genomic approaches have been incorporated to provide complementary information. Approaches used in combination with genetic mapping include DNA microarrays (78, 111), and two-hybrid assays that have identified nine MDV-chicken protein–protein interactions confirmed by an *in vitro* binding assay (96). As a result, GH1 (growth hormone) (79), *LY6E* (lymphocyte complex 6, locus E) (80) and *BLB* (MHC class II β chain) (95) are viewed as genes that confer MD genetic resistance based on genetic linkage to MD incidence, differential gene expression between MD resistant and susceptible chicks following MDV challenge, and direct protein interaction with an MDV protein. Higher order system analyses have also identified a number of potential candidate genes conferring genetic resistance to MD (111).

Variation in transcriptional regulation, especially *cis*-acting sequences that alter transcription factor binding sites, may account for the majority of phenotypic variation. Thus, genome-wide surveys have identified more than 3,700 genes with alleles that respond differentially to MDV challenge (32, 86). In experimental populations, these allele-specific expression (ASE) SNPs markers account for 83% of the genetic variance and, when used in genomic selection, show a 125% increase in accuracy compared to state of the art pedigree-based methods. This ASE approach shows real promise in commercial populations and provides a model for identifying genetic factors for other infectious agents.

Infectious Bursal Disease

Infectious bursal disease virus (IBDV) causes acute infection and depletion of B cells in the bursa and other organs, often resulting in severe and permanent immunosuppression. Differences between chicken lines indicate a genetic basis (5, 47, 124). Mating of resistant and susceptible lines to form F_1, F_2, and BC populations demonstrated IBDV-induced mortality to be under the control of a fully or partially dominant autosomal resistance gene (21, 22). Multiple studies have shown no association of the MHC with IBDV resistance (21, 22, 52); however, an MHC effect was detected on resistance parameters of specific antibody to IBDV and bursal histopathology (55, 56). The differences among studies may stem from examination of different MHC haplotypes in varied genetic backgrounds. Collectively, studies on genetics of IBDV resistance suggest involvement of the MHC and at least one other, currently unidentified, autosomal gene. Global transcriptional profiling has revealed numerous genes that are differentially regulated between IBDV resistant and susceptible lines, and suggests that resistance may be mediated by more rapid inflammatory response and more extensive p53-related apoptosis of target B cells, thus limiting viral replication in resistant birds (104). Addition of IFNα inhibits IBDV replication *in vitro* but this effect was not influenced by the *Mx* genotype (97).

Avian Influenza

Influenza A viruses are of significant interest to the poultry industry and human health. Infections of gallinaceous poultry species such as chicken and turkey is relatively frequent with highly pathogenic H5 and H7 strains causing extremely high mortality rates. Viral infection triggers the synthesis of type I and III IFN, which in turn activate the expression of numerous IFN-induced genes including *Mx*. Mx proteins block an early step in the influenza virus replication cycle (64). The chicken *Mx* gene has multiple polymorphisms though antiviral activity is dependent only on the protein having Asn at amino acid position 613 (62). There are conflicting reports on the association of *Mx* to genetic resistance in chickens (40, 109, 110, 130), which could be explained in part by the use of viruses that differ in nucleoprotein (NP), the viral protein that determines Mx sensitivity (139).

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Use of B congenic strains has indicated a small role of the MHC region on resistance (53). Results with limited experimental layer lines also demonstrate that horizontal transmission is dependent on host genetics, at least for some low pathogenicity strains, which correlates with cloacal shedding (106). Comparative genomic studies looking to account for the large differences between chickens and ducks identify at least two factors. First, *RIG-I*, a receptor that recognizes viral ssRNA is lacking in the chicken genome (12), and interferon-induced transmembrane protein (IFITM) genes are strongly upregulated in ducks but not chickens in response to pathogenic avian influenza infection (112). Thus, genetic resistance and transmission to avian influenza appears to be complex and controlled by many genes as is the case for mice (16).

Given human health concerns, engineering genetic resistance to avian influenza might be one of the few examples that would make genetically modified chickens acceptable to consumers. Consequently, several groups have explored the use of transgenic technology. The most notable success has been the clever use of expressing a small RNA piece that acts as a decoy to the viral polymerase (85). The transgenic chickens expressing this RNA do not horizontally transmit influenza virus to healthy cagemates. While the transgene does not prevent infection, indicating that further progress still needs to be made, this is an important "proof of concept" that could be applied to combat other avian pathogens.

Salmonellosis

Control of salmonellosis presents special challenges to the poultry industry. Some species of *Salmonella* bacteria are highly pathogenic in chickens, but other species cause little response in the host birds, which can then become asymptomatic carriers that transmit bacteria to flockmates or into the human food chain. Chicks infected with *Salmonella* immediately after hatch can be persistently colonized to adulthood, shedding bacteria vertically to infect table or hatching eggs, or horizontally to infect other chickens (42).

Heritability estimates of various parameters of *Salmonella* response indicate that genetic selection to improve resistance to salmonellosis and carrier state is feasible. Many genes have been associated with genetic control of response to *Salmonella*, with the individual effect of most genes on the phenotypic variation being relatively small (see reviews 25, 66). With astute choices, genetic selection can proceed based on variation in genomic structure or expression (14, 24, 70, 118).

In a comparative genomics approach, chicken homologues of major loci controlling natural resistance of mice to *S.* Typhimurium infection were examined as candidate genes. Both *NRAMP1* (natural resistance-associated macrophage protein 1, now named *SLC11A1*, solute carrier family 11 member 1) and *TNC* (a locus closely linked to *TLR4*, which binds lipopolysaccharide, a major component of Gram-negative bacteria membranes) were associated with *Salmonella*-induced mortality in a backcross of inbred lines (51). The *NRAMP1* and *TLR4* associations were confirmed for several additional response traits to *Salmonella* in other chicken populations (15, 63, 71, 81, 87). Positional candidate genes, based upon their genomic location near *NRAMP1*, the *CD28* gene was associated with enteric *Salmonella* infection (87), and *VIL1* with visceral infection (44).

Biological candidate genes are selected based upon involvement in functions or pathways that are hypothesized to be important in host response. The *MD2* gene product interacts with the TLR4 receptor, and *MD2* SNPs are associated with persistence of *Salmonella* cecal colonization (87). The MHC is associated with resistance to *Salmonella* colonization, morbidity and mortality (35, 83). Several cytokine SNPs have been associated with response to *Salmonella* (43, 63, 88). Genes in apoptotic pathways, specifically *CASP1* and *IAP1* are associated with various *Salmonella* resistance traits (63, 82). Several *avian beta-defensins* (*AvBD*) have SNPs associated with *Salmonella* response (36, 45).

Genome scans have identified many QTL regions associated with resistance to *Salmonella* colonization or salmonellosis, some with effects as large as 37% of the phenotypic variance of the trait (1, 23, 26, 41, 46, 123). Locations of QTLs have also been verified from experimental to commercial populations, which demonstrates the effectiveness of using experimental populations to identify QTLs of value in commercial application (23). One of the major *Salmonella* resistance QTL with greatest effect, *SAL1*, is on chromosome 5. Fine-mapping the *SAL1* QTL region identified two functional candidate genes, CD27-binding protein (*SIVA*) and *AKT1* (protein kinase B, PKB) (41). Fine-mapping of heterophil functional response to *Salmonella* in a highly advanced intercross also strongly supported the QTL location containing *SIVA* and *AKT1* and suggested heterophil function as a major mechanism to explain the host-resistance properties that map to this region (101).

Studies of differential expression in response to *Salmonella* revealed genes that may be active in pathways controlling resistance. Targeted expression studies of specific genes for which SNPs had been associated with *Salmonella* response generally supported these genes' role in *Salmonella* response, including *TLR* genes (2, 3, 94), cytokines (33, 57, 102, 119, 132), and beta-defensins (36, 107). The ability to select poultry on the disease-associated phenotype of mRNA expression of proinflammatory mediators was successful in producing progeny more resistant to organ invasion by *Salmonella* (120).

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Use of microarrays provided a more comprehensive picture of host genes and pathways involved in resistance to *Salmonella* (34, 76, 128, 137). Some observations of highly represented gene families and pathways are supported across multiple studies, suggesting that they have a robust role in response to *Salmonella*. Among these are: *TLR*s, cytokines and cytokine receptors, apoptotic pathways, antimicrobial peptides, T cell receptors and signaling pathways, and CD antigens. A meta-analysis combined data from several studies to increase the power of detection and expand knowledge on biological functional mechanisms, and the results highlighted several biological mechanisms related to energy metabolism, apoptosis, specific protein domains indicating groups of involved proteins, and several cellular morphological structures in which responses occur (121).

Escherichia coli

Escherichia coli is both a source of colibacillosis in production flocks and a potential food-safety pathogen. Genetic control of resistance to *E. coli* has been studied in chicken lines that were divergently selected for circulating antibody level to *E. coli* vaccine (67). Divergent selection for *E. coli* vaccine antibody response modulated mortality rate, and the immune response to pathogenic *E. coli* (49). Using resource populations generated from crossing the divergently selected lines, molecular markers for three genes in the MHC region (*B-F, B-G,* and *TAP2)* were associated with antibody traits (135). Cavero et al. (29) also reported an association of the MHC with mortality after *E. coli* challenge of laying hens.

Genome scans of resource populations produced from the antibody-selected lines revealed QTL for antibody response and mortality. A low-density scan identified markers associated with antibody response to *E. coli*, Newcastle disease virus, and/or sheep red blood cell (SRBC) and with mortality (134). Genotyping individuals with extreme phenotypes (highest and lowest antibody levels) after *E. coli* and *Salmonella* Enteritidis vaccine revealed 12 markers associated with *E. coli* antibody, of which six were also associated with *S.* Enteritidis antibody, indicating that some genetic control was shared across humoral response to multiple bacteria (136).

In gene expression studies, hundreds of genes on microarrays were differentially expressed between broilers that were infected or non-infected with avian pathogenic *E. coli* (108). These genes highly represented several immune pathways, including *TLR*, *Jak-STAT,* and cytokine signaling, which suggests an important role of these immune pathways in host response to *E. coli* infection. Using smaller, immune-focused microarrays and isolated macrophage cells, the cytokines and cytokine receptor genes again were identified as strongly associated with response to *E. coli* (68). Use of RNA-sequencing and multiple immune tissues of *E. coli*-infected broilers helped to elucidate pathways potentially associated with resistance, including TLR, NOD-like, and T cell receptor signaling pathways; cell growth and death pathways; lymphocyte differentiation, proliferation, and maturation; T helper cells, and cytokines (114–116).

Campylobacter jejuni

Campylobacter spp. do not typically cause pathology in chickens, but are a leading cause of foodborne bacterial gastroenteritis in humans. Host variability exists in *Campylobacter* colonization of the chicken intestine and cecum, with marked differences among inbred lines of chickens but without evidence for an MHC effect (17, 113). A GWAS of naturally colonized broilers, using the *Campylobacter* load in the cecal content as the disease response trait and a high-density (580 K) SNP genotyping panel, identified two genome-wide significant QTL on chromosomes 16 and 26, and one suggestive QTL on chromosome 14. The QTLs on chromosome 14 and 16 mapped in the same location as QTL previously identified in advanced intercross populations of two inbred lines with known differences in resistance to *Campylobacter* colonization of the gut, and the chromosome 16 markers were located in the MHC region (100).

Whole-genome expression microarrays were used to compare responses to *Campylobacter* infection between two chicken lines with different resistance to *Campylobacter* colonization (72, 73). Genes for lymphocyte activation and differentiation and for humoral response were upregulated in the resistant line. Genes for erythrocyte differentiation, hemopoiesis and RNA biosynthesis were downregulated in the susceptible line. Interaction analysis between infection status and genetic line revealed distinct mechanisms, with the resistant line responding to infection by upregulating genes involved in apoptosis and cytochrome c release from mitochondria and the susceptible line downregulating both types of genes. Similar to the results with *Salmonella* challenge, a line of chickens bred for high levels of proinflammatory gene mRNA was more resistant to *Campylobacter* colonization than a line with low levels, illustrating the ability to improve resistance to another bacterial pathogen by genetic selection (117).

Coccidiosis

Coccidiosis is caused by several species of parasites of the genus *Eimeria,* each having tropism for a different area of the gastrointestinal tract. Population

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differences, and the ability to genetically select lines for divergent resistance/susceptibility, illustrate the feasibility of improvement in genetic resistance to coccidiosis (19, 54, 99).

Studies comparing B congenic lines (27, 69, 74, 105) or populations in which MHC haplotypes segregate within line or differ among lines (18, 91, 125) provide strong support for the MHC being a coccidiosis resistance locus. However, effects of specific MHC haplotypes greatly vary, dependent upon factors such as *Eimeria* challenge strain and the specific trait used to characterize resistance (e.g., antibody, oocyst shedding, weight gain, severity of intestinal lesions). Both MHC and non-MHC genes control resistance to coccidiosis (75, 77). Erythrocyte loci in addition to the *B* locus have been associated with resistance to coccidiosis, including *Ea-AE*, *Ea-C* (54), and *Ea-I* (89).

Multiple genome scan studies have identified genomic regions or genes associated with response to *Eimeria*. A strong QTL for *E. maxima* oocyst shedding in broilers was located on chromosome 1 (138), and subsequent fine-mapping confirmed this QTL (58). Association analysis of candidate gene SNPs in this QTL region supported the *xyzin* gene as a coccidiosis-resistance gene (50). Using a diverse cross of Fayoumi (resistant) and White Leghorn (susceptible) lines, a genome scan identified 21 QTL regions, and suggested candidate genes in the cytokine and tumor-necrosis families, and pathways related to innate immune and inflammatory responses (98).

Functional genomics revealed a more comprehensive picture of the genetic control of coccidiosis resistance (76). Genes involved in metabolism of lipids and carbohydrates, as well as innate immunity genes, change in expression in *Eimeria*-infected birds (59), suggesting a mechanism for the growth depression that typically accompanies coccidiosis. Both local and systemic expressions of cytokines are modulated during infection (60). Expression changes in liver-expressed antimicrobial peptide (LEAP-2) are associated with *E. maxima*-induced lesion scores (28).

Future Perspectives

Poultry breeding companies have achieved enhanced disease resistance to many pathogens in their flocks using traditional selection methods. However, this is a laborious process that requires the intentional exposure of individuals from elite lines to pathogens, or one that progresses rather slowly due to indirect selection on overall livability. With the emergence of molecular genetics and the field of genomics, there is optimism that studies will be able to identify genes and alleles that confer superior disease resistance. When applied to breeding, the information will provide for the rapid and accurate improvement of commercial lines. In application, the poultry breeders may use markers in LD to increase the frequency of favorable alleles. But as science has shown many times, increasing knowledge gives increasing power that can often be applied in novel ways. So the ultimate goal should be to identify disease resistance genes and their pathways to reveal biological function and pathways.

What will hasten the advancement of knowledge? Efforts to decipher the genome sequence, especially to identify regulatory elements, will aid in identifying candidate causative polymorphisms (122). With high-throughput platforms to determine genotypes, the rate-limiting step is in producing and measuring resource populations. Similarly, accurate trait measurements will be critical. As genomics have already shown, decomposing a trait into specific components provides critical information on biological processes that cannot be or are difficult to obtain by traditional phenotypic measurements. Thus, veterinary medicine can make key contributions by refining trait measurements associated with disease and disease progression.

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Section II

Viral Diseases

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3

Newcastle Disease, Other Avian Paramyxoviruses, and Avian Metapneumovirus Infections

Introduction

David L. Suarez

The virus families Paramyxoviridae, Pneumoviridae, Filoviridae, Bornaviridae, and Rhabdoviridae form the virus order Mononegavirales that includes negative sense, single-stranded RNA viruses with helical capsid symmetry. The Paramyxoviridae family currently has seven genera that include important human and veterinary pathogens such as Rubulavirus, which includes human mumps virus; Respiroviruses, containing mammalian parainfluenza 1 and 3; Morbillivirus, which includes canine distemper, rinderpest, and measles; Henipavirus, containing Nipah and Hendra virus; and the Avulavirus genus that contains Newcastle disease virus (NDV) and other avian paramyxoviruses (APMV). The Pneumoviridae family was recently separated from the Paramyxoviridae family and was recognized to contain two genera that include metapneumovirus and orthopneumovirus (https://talk.ictvonline.org/taxonomy/).

The International Committee on Taxonomy of Viruses (ICTV) officially renamed NDV to avian avulavirus 1 in 2016, and other avian paramyxoviruses to avulavirus 2–13. A more recent revision in 2018 has created an Avulavirinae subfamily with three genera, *Metaavulavirus, Orthoavulavirus*, and *Paraavulavirus* with a total of 20 unique members (https://talk.ictvonline.org/taxonomy/). Newcastle disease virus is now officially Avian orthoavulavirus 1. Because the new avian avulavirus terminology is still not widely adopted and the World Organisation for Animal Health (OIE) still uses NDV to refer to virulent APMV-1 viruses (http://www.oie.int), this chapter will continue to preferentially use the avian paramyxovirus subtypes (1–20) terminology as being synonymous with avian avulavirus 1–20. Twenty serotypes of avian paramyxoviruses have been officially recognized: APMV-1 to APMV-20 (Table 3.3). Of these, APMV-1 remains the most important pathogen for poultry with the virulent forms of the serotype defined as NDV. Newcastle disease virus remains a serious impediment for poultry production in Asia and Africa, and remains a control problem throughout the world. Several of the other APMV serotypes including APMV-2, APMV-3, APMV-6, and APMV-7 may also cause disease in poultry. All of the APMV serotypes are known or likely to have wild bird reservoirs that can spill over to poultry, but the wild bird ecology of these serotypes is largely unknown. The virulent NDVs seem to be the major exception of a wild bird reservoir, with the virulent viruses being endemic in poultry and domestic and feral pigeons. Virulent NDV is also endemic in cormorants, a coastal seabird, in North America, where periodic outbreaks with mortality regularly occurs (1).

Avian metapneumovirus (AMPV) infections continue to emerge as a disease threat with four defined subtypes, A–D, being recognized and producing clinical disease in both turkeys and chickens. The serotype C viruses are genetically closely related to the recently recognized human metapneumovirus (3). The AMPVs also have a wild bird reservoir, but it is unclear what role wild birds play in poultry outbreaks (2).

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Newcastle Disease

Patti J. Miller and Guus Koch

Summary

Agent, Infection, and Disease. Virulent strains of avian paramyxovirus 1 (APMV-1), from the genus *Orthoavulavirus* and species avian orthoavulavirus 1, infect at least 236 species of wild birds and poultry species leading to Newcastle disease. Infections of poultry species are reportable to the World Organisation for Animal Health (OIE). The disease affects the respiratory, gastrointestinal, nervous, and reproductive systems and causes up to 100% mortality in nonvaccinated chickens. The virus is transmitted through infected saliva and feces, and is found worldwide.

Diagnosis. Virus isolation is the gold standard, however, molecular diagnostic techniques, such as real-time reverse transcription PCR are often used to facilitate a faster diagnosis. The OIE definition accepts the finding of multiple basic amino acids in the fusion cleavage site sequence or an intracerebral pathogenicity index assay value of 0.7 or greater as confirmation of a virulent APMV-1.

Intervention. Vaccination prevents morbidity and mortality, but not infection. Vaccination with strict biosecurity is key for Newcastle disease (ND) control. Culling infected birds is often necessary to contain ND outbreaks in countries normally free of the virulent virus.

Introduction

There are twenty accepted species of the subfamily: Avulavirinae still commonly abbreviated as *avian paramyxovirus 1–20 (*APMV-1–20*)*, of the *Paramyxoviridae* family (https://talk.ictvonline.org/taxonomy/) (125, 129, 162, 276, 280). The International Committee on Taxonomy of Viruses (ICTV) officially renamed Newcastle disease virus (NDV) to avian avulavirus 1 in 2016 (5) and further renamed it in 2018 as avian orthoavulavirus 1, but this chapter will use the historical abbreviation of the virus, NDV, to be consistent with the OIE 2014 chapter on infection with NDV in the Terrestrial Animal Health Code (http://www.oie.int) (208).

Newcastle disease viruses are contained within one serotype and infections of poultry with NDV are the focus of this chapter (5). Newcastle disease (ND) is caused only by infections with NDV defined as virulent (vNDV). While infections with NDV of low virulence (loNDV) do not cause ND, they can still lead to clinical respiratory disease when other infections or suboptimal environmental conditions are present. Historically, the abbreviation APMV-1 was occasionally used when discussing loNDV, and NDV was used when referring to the virulent forms of NDV (15). However, to avoid confusion, especially with the recent renaming of the species, in this chapter vNDV will be used to denote strains that cause ND.

Of the 20 identified avian avulavirus species, avian orthoavulavirus 1 (NDV) is the most important for poultry (16, 125, 129, 276, 280, 313). From 2006–2009, between 56–68 counties reported ND outbreaks in domestic poultry to the OIE, and this number is likely an under-reporting of the countries with the disease (309). Because clinical signs of infected birds vary depending on the host species, the virulence and dose of the infecting virus, and the immune status and age of the host, it can be especially difficult to recognize the disease. Clinical signs may range from a drop in feed and water intake, and/or in egg production among seemingly healthy, well-vaccinated layers to 100% mortality among nonvaccinated chickens (180).

Pathotypes of disease, defined by clinical signs in chickens after experimental inoculation, were created to describe the virulence of the NDV strains and are listed in decreasing order of virulence: (1) velogenic, (2) mesogenic, (3) lentogenic, and (4) asymptomatic enteric (12). Velogens are able to produce high mortality rates in naive chickens at any age and are further divided into viscerotropic velogenic NDV (vvNDV), consisting of hemorrhagic lesions in the gastrointestinal tract and often neurologic signs, and neurotropic velogenic NDV (nvNDV), which produces neurological signs with some respiratory involvement. Birds infected with nvNDV may have hypermetric stepping, and appear clumsy before torticollis, body tremors, and paralysis become obvious. Mesogens are less pathogenic, producing neurological disease with deaths in only young birds. Lentogens cause primarily respiratory infections that in specific pathogen free (SPF) chickens cause asymptomatic infections, and are commonly used as live ND vaccines in commercial poultry. Viruses of the asymptomatic enteric pathotype have a tropism for the gastrointestinal tract, and are not thought to cause or contribute to clinical disease and are also used as live vaccines.

Definitions and Synonyms

Newcastle disease is defined by the OIE as an infection of birds caused by a virus of avian paramyxovirus serotype 1 (APMV-1) that meets one of the following criteria for virulence (207):

1) The virus has an intracerebral pathogenicity index (ICPI) in day-old chicks (*Gallus gallus*) of 0.7 or greater; or

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2) Multiple basic amino acids have been demonstrated in the virus (either directly or by deduction) at the C-terminus of the F2 protein and phenylalanine at residue 117, which is the N-terminus of the F1 protein. The term multiple basic amino acids refers to at least 3 arginine or lysine residues between residues 113 and 116. Failure to demonstrate the characteristic pattern of amino acid residues as described above would require characterization of the isolated virus by an ICPI test.

Newcastle disease also has been referred to as exotic Newcastle disease (END), pseudo-fowl pest, pseudovogel-pest, atypische Geflugelpest, pseudo-poultry plague, avian pest, avian distemper, Ranikhet disease, Tetelo disease, Korean fowl plague, and avian pneumoencephalitis. In addition, variant APMV-1 isolates from pigeons are often referred to as pigeon paramyxovirus type 1 (PPMV-1). Based on the OIE definition, by containing multiple amino acids in the fusion cleavage site, PPMV-1 isolates are vNDV, and upon infection of poultry are defined as ND according to the OIE terrestrial code. The European Union control guideline definition is based solely on the ICPI value of 0.7 or greater (28). However, despite them falling into the virulent category due to their virulent cleavage sites, upon infection only some PPMV-1 cause clinical disease in chickens, even with ICPI values of 0.7 or greater, but may increase in virulence after multiple passages in chickens (79, 181). Mesogens and velogens that fit the OIE definition will be referred to as vNDV and asymptomatic or NDV of low virulence will be referred as loNDV. It is unclear if the OIE definition will be modified to adapt to the new renamed species of avian orthoavulavirus 1 rather than continue with the historical language of avian paramyxovirus 1 (APMV-1) or NDV acronyms.

Economic Significance

Poultry diseases have a significant impact on human welfare, especially in rural areas where village or backyard chickens are a source of income and a crucial food source (7). Countries with industrialized poultry production also expend large amounts of money to prevent ND or prevent losses from ND, to maintain a ND-free status, or eradicate ND after an outbreak (163).

Public Health Significance

The isolation of NDV from humans is rare; however, when it does occur it is most often isolated from those who work closely with poultry species, have laboratory-acquired infections, or have infection from live NDV vaccines (33). A self-limiting conjunctivitis without involvement of the cornea is the most common presentation after direct inoculation of the eye with infective fluids or aerosol transmission (62). The rare reports of flu-like symptoms in humans after ocular inoculation (42) increase to 88% upon intravenous inoculation (IV) of virus used for cancer therapy (161). Casual contact with vaccinated or infected poultry represents a low risk of human infection. However, immunosuppression may increase the possibility of infection and severe pneumonia (150). There are no reports of human-to-human spread.

History

The first documented outbreaks of ND occurred in 1926 in Java, Indonesia, and Newcastle upon Tyne, England (85). Even though Doyle initially suggested "Newcastle disease" as a temporary moniker for outbreaks caused by these viruses, over time it has become the *de facto* designation (86). The synonym, avian paramyxovirus type 1 (APMV-1), was suggested by Tumova years later to distinguish NDV from other serotypes of avian paramyxoviruses (284). Previous editions of this chapter have more information about the origins of ND and the initial characterization of the virus (17).

Etiology

Classification

See the introduction at the beginning of this chapter for more information regarding classification.

Morphology

Members of the Paramyxoviridae family have a lipid bilayer envelope that originates from budding of the plasma membrane of the host cells in which the virus replicates (155). Newcastle disease virions are pleomorphic, but may appear round with a diameter between 100 and 500 nm or filamentous in shape with a diameter of about 100 nm and variation in length (17). The fusion (F) and hemagglutinin-neuraminidase (HN) glycoproteins, 17 nm in length, are densely packed onto the surface of the virion (181). The nucleocapsid protein appears to be in a herringbone type of pattern and is associated with the phosphoprotein (P) and large polymerase protein (L) (173) (Figure 3.1).

Chemical Composition

The molecular mass (Mr) of a single Paramyxovirus genome is approximately 500×10^6, the buoyant density in sucrose is 1.18–1.20 g/cm^3, and the sedimentation coefficient $S_{20,W}$ is about 1000 S (124). Virus particles are

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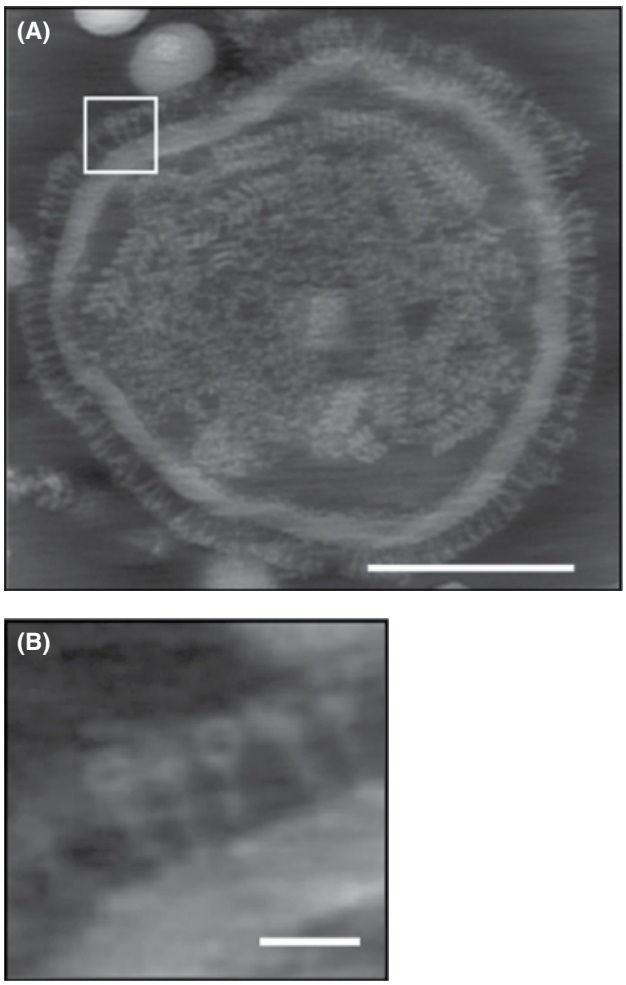


Figure 3.1 Negative staining and electron tomographic analysis of Newcastle disease virus. (A) 44 nm thick digital section taken at the level of the envelope. Bar = 100 nm. (B) Glycoprotein protruding from the envelope. Bar = 10 nm. Reproduced with permission (173).

approximately 20–25% weight for weight (w/w) lipid derived from the host cell and about 6% w/w carbohydrate. The NDV genome is composed of six structural proteins, listed from 3′ to 5′: nucleocapsid protein (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and the RNA-dependent RNA polymerase (RNAP), designated the large polymerase (L) (155). RNA editing of the P protein produces an additional protein, the V protein, when one guanine residue is added to the conserved editing site of the mRNA by the RNAP (54). The V protein is rich in cysteine and binds zinc (267), and the carboxy-terminus portion has an anti-interferon activity, allowing the virus to reduce the innate immune response of the host (217). The host actin protein is also incorporated into NDV and is used for virus entry, replication, and transport across the cell (198).

Biologic Properties

Hemagglutination Activity

The ability of NDV and other avian paramyxoviruses to agglutinate red blood cells (RBCs) is due to the binding of the HN protein to the sialic acid receptors on the surface of the RBCs (34). The agglutination property of the virus and its inhibition by antisera (17) have proven to be powerful tools in the diagnosis of the disease. Chicken RBCs usually are used in hemagglutination (HA) tests, but NDV causes agglutination of RBCs from other species (127). Ito et al. (127) showed that the ability to agglutinate RBCs varied with the strain of NDV, on the species it was isolated from, and on the species the RBCs were collected from. This suggests that the receptor differences between species may affect the transmissibility and replication between species.

Neuraminidase Activity

The HN protein also has neuraminidase enzyme activity (mucopolysaccharide N-acetyl neuraminyl hydrolase) that degrades the sialic acid receptor, preventing self-attachment of viral particles and the resultant clumping of progeny virus. The enzyme activity also allows RBCs to eventually elute from the virus (156).

Cell Fusion and Hemolysis

Paramyxovirus fusion proteins are able to induce the hemolysis or cell fusion of other cells and this fusion is pH independent and occurs at a neutral pH (157). The fusing activity and infectivity of the virus depends on the proteolytic cleavage by a host protease of the precursor F0 protein into the heterodimer (F1 and F2) linked by a disulfide bond (121). Cleavage also may be performed by bacterial proteases (200). Attachment of the virus to the host receptor site during replication is followed by fusion of the virus membrane with the host cell membrane, which may result in the fusion of neighboring cells, and the formation of multi-nucleated cells or syncytia. The rigid membrane of the RBCs usually ruptures when the virus membrane fuses, causing hemolysis. The F0 is cleaved, as discussed previously, and relates to the pathogenicity of the NDV strain, which is discussed later in this chapter.

Virus Replication

The HN attaches the virus to the host cell receptor and triggers the F protein to fuse the virus to the host cell membranes, allowing the viral nucleocapsid complex to enter the host cell. Entry of the virus is possibly dependent on the presence of N-linked glycoproteins and cholesterol, and may occur through the process of endocytosis (45, 172, 252). Paramyxoviruses replicate entirely in the cytoplasm of the host cell. Replication starts when the RNA-dependent RNA polymerase (L) carried in the virus

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particle is released into the cytoplasm, beginning the transcribing of the negative-sense RNA genome into positive sense, 5′ capped and 3′ polyadenylated messenger RNAs (mRNA). The genes are transcribed in a sequential and polar manner, 5′ to 3′ of the coding sequence, leading to a decreased level of transcription of downstream genes (155). Therefore, the 5′ structural proteins are produced in much higher amounts than the 3′ polymerase protein. After the mRNA is translated into viral proteins, the negative sense genome is replicated, producing a full-length antigenomic RNA that serves as a template for the synthesis of full-length genomic RNA (224).

The HN protein of some strains of NDV is also produced as a precursor, HN0. Posttranslational cleavage is needed to remove a 45 amino acid region of the carboxy terminus (109). The HN0 can be cleaved by trypsin and other proteases (chymotrypsin, thermolysin, elastase) that do not cleave the fusion protein of these low virulence viruses (199). The viral HN proteins synthesized in an infected cell are transported to and are incorporated into the cell membrane. Following the alignment of the nucleocapsid and viral RNA close to the regions of the cell membrane containing the viral glycoproteins, virus particles bud from the cell surface (90).

Susceptibility to Physical and Chemical Agents

After soaps and detergents are used to remove organic matter, oxidizing agents (sodium hypochlorite, VirkonS®), alkalides (sodium hydroxide), and acids (glutaraldehydes, formalin, formaldehyde gas) are able to destroy the infectivity of NDV and these are reviewed elsewhere (101). Information on the stability of NDV to heat, hydrogen ion concentrations, chemicals, and solar and gamma radiation can be also be found elsewhere (35, 113, 273).

Virulent NDV has been found in eggs, and poultry meat produced from infected poultry (14, 24, 46, 249). The Terrestrial Animal Health Code of the OIE (http://www.oie.int) allows international trade in processed poultry products from countries with enzootic ND if these products have been processed to ensure the destruction of the ND virus (210). In addition, thermal inactivation curves and Dt values for virulent California/2002 strain and a loNDV (Northern Ireland/Ulster/1967) strain resulted in their inactivation when chicken meat was cooked following the United States Department of Agriculture (USDA) guidelines to reduce salmonella (70°C or 73.9°C for less than 1 second) (279, 287).

Strain Classification

The term strain is used to indicate a well-characterized isolate of a virus or bacteria. The suggested nomenclature for APMV-1 isolates is similar to avian influenza isolates. The isolate should be listed in full the first time it is mentioned in a document: APMV-1/Species/Country (state, city or more specific location)/isolate number, name, or other unique identifier/year of isolation (e.g., APMV-1/Chicken/USA (CA)/S0212676/2002), and then subsequently may be abbreviated as appropriate (CA/2002). Certain isolates have been so well characterized that they often are listed by one name (LaSota, B1, Ulster, Herts33, etc.), even though viruses with the same name may differ (65).

Antigenicity

While all isolates of APMV-1 are of one serotype, antigenic variations between different isolates of NDV have been demonstrated by virus neutralization (VN), agar gel diffusion assays, monoclonal antibodies (mAb), and cross hemagglutination inhibition assays (2, 23, 159, 186). Genetic analysis has become the primary method for characterization and has replaced the use of mAbs for typing NDV isolates (73).

Immunogenicity

All vaccine studies evaluate immunogenicity or the ability of one vaccine to provide significantly better protection over other vaccines for that specific situation. This evaluation may involve the amount of antibodies produced, the reduction in number of sick or dead birds after challenge, and the decreased amount of virulent challenge virus shed. Vaccines formulated with the same NDV strain, or even NDV clones, may not have the same immunogenicity (306). The immunogenicity of a virus also depends on the route of administration of the vaccine (175, 304).

Genetic or Molecular

Phylogenetic analysis of genome sequences is now the standard procedure adopted by most laboratories to characterize NDV strains. APMV-1 isolates have at least three genome sizes; 15,186; 15,192; and 15,198 (65) in keeping with the rule of six of paramyxoviruses (224). While the first analyses focused on partial sequences of the F gene (9, 146, 169, 255), due to its importance in virulence determination, more recent efforts compare the full F or full genome sequences (72, 261, 290). For molecular determination of virulence the partial sequence of the fusion protein of 374 base pairs (bp) in length, surrounding the site where F0 precursor is cleaved to F1 and F2 fragments, is sufficient (209).

With low rates of recombination, but considerable antigenic drift over time, the genetic diversity is represented by groups of viruses denoted by lineages or genotypes (9, 73, 75, 169, 187, 189). Strains of APMV-1 can be grouped into two main classes, class I and class II (64). Class I viruses are mostly loNDV found in wild birds and class II viruses originally were broken into multiple genotypes representing loNDV and vNDV (75, 76) (Figure 3.2). With the addition of more complete genomes available for analysis, a new system to

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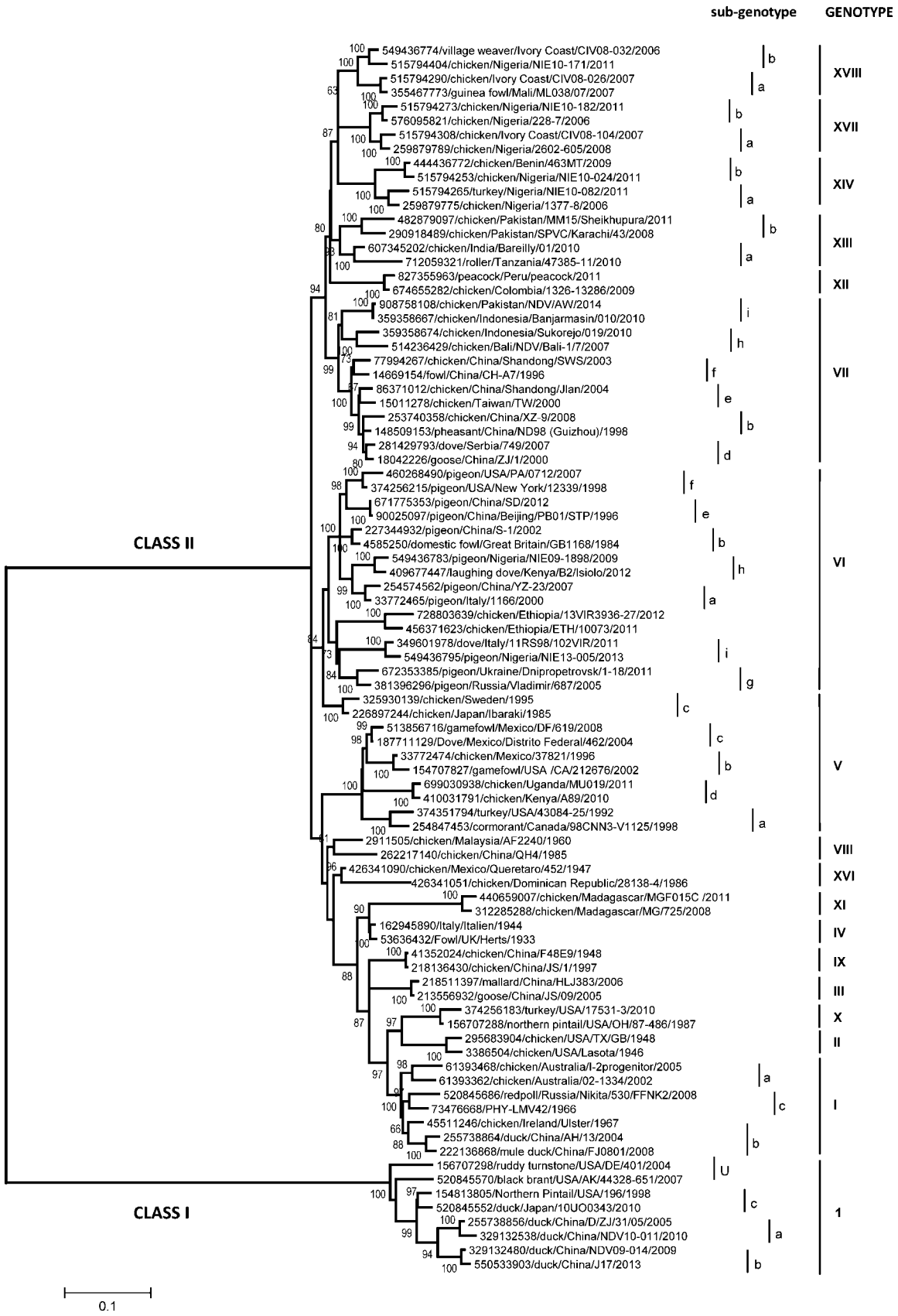


Figure 3.2 Phylogenetic tree constructed using the complete nucleotide sequences of the fusion gene of representative isolates of avian paramyxovirus 1 class I and class II. Evolutionary analyses were conducted in MEGA6 (274). U = unclassified. Reproduced with permission (75).

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define genotypes based on a mean evolutionary distance between genetic groups of 10% has been suggested to define the current genotypes and to identify new genotypes as they arise (73). While most of the sequence data available is for the class II viruses that contain both vaccine viruses and virulent outbreak viruses, more full genome sequence data on loNDV isolated from wild birds is becoming available (95, 236). There are eight predicted lengths of the HN protein (571, 572, 577, 578, 580, 582, 585, and 616 amino acid residues) (318), and some strains are known to produce an HN0 precursor that is cleaved at the carboxy terminus to an active form (95, 236). There is only one documented instance of a vNDV, APMV-1/domestic fowl/Ireland/IECK90187/1990, from class I (9).

Pathogenicity Tests

Before molecular assays and sequencing were readily available, pathotypes were created based on results from pathogenicity assays. The terms "velogenic," "mesogenic," and "lentogenic" were defined as the mean death times (MDT) in chicken embryos, after allantoic sac inoculation, of less than 60 hours, 60–90 hours, and greater than 90 hours, respectively (12). These terms are now also applied to mean high-virulence, moderate-virulence, and low-virulence viruses regardless of the pathogenicity test employed.

The ICPI in day-old chicks differentiates lentogenic viruses with ICPI values of less than 0.7 from virulent mesogenic strains with ICPI values equal to or greater than 0.7 and less than 1.5, and velogenic viruses with ICPI values greater than 1.5 (12). For the ICPI, ten 24- to 40-hour-old SPF chicks are inoculated with 0.05 mL of a 1:10 dilution of bacteria-free, isotonic NDV containing allantoic fluid with a HA titer greater than 16 and watched daily for 8 days. Birds are rated 0 = normal, 1 = sick, and 2 = dead. The ICPI is the mean score per bird observations. The ICPI also is used as one determinant of NDV that requires reporting to OIE.

The intravenous pathogenicity index (IVPI) in 6-week-old chickens also has been used to distinguish velogenic NDV from mesogenic and lentogenic NDV (12), but now is not often used because mesogenic viruses, like the velogens, are defined as vNDV by the OIE. With PPMV-1 strains it is common to have virulent fusion cleavage sites, but mesogenic ICPI values with lentogenic MDT values (220). However, pathogenicity indices may increase in virulence with multiple passages in chickens (18).

While sequencing of the fusion cleavage site is acceptable to demonstrate virulence, if the cleavage site data reveal an NDV of low virulence, the ICPI test is required by the OIE for the *in vivo* determination of virus virulence (see below) (206). Even though infections with some vNDV may result in minimal clinical disease, infections of poultry with any vNDV are reportable (36).

Laboratory Host Systems

Animals

The virus is known to infect more than 236 avian species (133). Newcastle disease infections of birds from 20 of the 26 Orders in the Clements classification system for modern birds have been documented. In addition to poultry species, pigeons, cormorants, and psittacines are commonly infected with vNDV (74, 75, 218, 244). The virus can cause a localized infection in animals other than birds, which allows for the use of NDV as a vector vaccine for many diseases, such as rabies, avian influenza, respiratory syncytial virus, and severe acute respiratory syndrome (SARS) in mammals. Use of NDV as vaccine vectors has been reviewed elsewhere (76, 192).

Chicken Embryos

Most avian paramyxoviruses replicate to high titers with allantoic sac inoculation into SPF embryonating chicken eggs (ECE). However, there are rare reports that a strain, likely well-adapted to a host other than the chicken, may need to be propagated first in chicken embryo kidney cells before being propagated in ECE (149, 178).

Cell Cultures

Newcastle disease virus strains can replicate in primary cells and cell lines of multiple species (145, 148, 193). Not all NDV isolates will replicate to a high titer in cell culture unless they are passed multiple times to adapt them to the system, and some NDV strains may not adapt to some cell types as is seen with the Madin-Darby canine kidney (MDCK) system (193). Cell systems, like chicken embryo fibroblasts, require an exogenous protease to be added for loNDV to undergo more than one round of viral replication (241). The most commonly used cells are primary chicken embryo fibroblasts (CEF) and kidney cells (CEK), chicken fibroblast cell line (DF1), African green monkey kidney epithelial cell line (VERO), baby hamster kidney fibroblast cell line (BHK), and human epithelial type-2 cell line (Hep-2). The use of intestinal epithelial cells (132) and a chicken-induced pluripotent cell line, BA3, for studying host–virus interactions and for vaccine production, respectively, are recent developments in cell culture (262). Cytopathic effects (CPE) consist of cytoplasmic inclusions, giant cells (syncytia), or plaques if an overlay media is used. In general, there can be a 1–2 log decrease in the amount of virus produced in a cell culture system compared to NDV grown in ECE.

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Pathogenicity

Virulence Determinants

F Gene. Entry of NDV into host cells requires the activation of viral envelope fusion glycoprotein through cleavage of the precursor glycoprotein F0 into F1 and F2. Only virus with a cleaved F0 can mediate the fusion of the viral and cellular membrane, which is required for infection of a cell (100, 199) The posttranslational cleavage is mediated by nonviral proteases and the types of proteases capable of cleaving F0 depends on the amino acid sequence motif around the cleavage site. The amino acid sequence of the F0 precursor (Table 3.1) of loNDV are characterized by a monobasic amino acid sequence motif at the C-terminus of the F2 protein and a leucine at the N-terminus of the F1 protein, ^{112}G-R/K-Q-G-R↓L^{117} (14, 59, 138). F0 of loNDV are cleaved only extracellularly by trypsin-like proteases present in the respiratory and intestinal tract. Virulent strains have a multibasic amino acid sequence motif at the C-terminus of the F2 protein, 112R/G/K-R-Q/K-K/R-R↓F^{117}, and a phenylalanine at the N-terminus of the F1 protein that are cleaved intracellularly by ubiquitous furin-like proteases found in most host tissues (105, 199, 200). This difference in protease activation is the major determinant of systemic replication associated with severe disease of vNDV.

The importance of the cleavage site motif is confirmed by using reverse genetics to convert the sequence motif of loNDV isolates into those of a vNDV, which increases the ICPI values from 0 or 0.01 to 1.12–1.28 (see Table 1 in [81]) (213, 223, 239). The opposite is also true that when the virulent strain ZJ1 cleavage is modified to a loNDV strain the ICPI is decreased from 1.89 to 0.13 (122). The ICPI does not always correlate with virulence observed after infection of adult chickens via a more natural route of infection. Thus, LaSota virus that was manipulated via reverse genetics to comprise a virulent cleavage site with a virulent ICPI value only slightly increased its pathogenicity, defined by the clinical disease and the viral tissue distribution observed compared to its parent virus (295). Changing the amino acid at position 114 from a glutamine to an arginine and/or an isoleucine at position 118 into a valine also reduced virulence as measured by ICPI and retarded death after infection via

Table 3.1 Amino acid sequence at the F0 cleavage site.

Virus strain	Virulence[a]	ICPI	Cleavage site AA 111-118[b]	Reference
Herts33	High	1.88	G-**R**-**R**-Q-**R**-**R**↓F-I	(282)
Essex '70	High	1.86	G-**R**-**R**-Q-**K**-**R**↓F-V	(59)
135/93	High	1.30	V-**R**-**R**-**K**-**K**-**R**↓F-I	(203)
617/83	High	1.46	G-G-**R**-Q-**K**-**R**↓F-I	(58)
34/90	High	1.81	G-**K**-**R**-Q-**K**-**R**↓F-I	(59)
Beaudette	High	1.46	G-**R**-**R**-Q-**K**-**R**↓F-I	(59)
Karachi/SPV/33	High	1.85	G-**R**-**R**-Q-**R**-**R**↓F-I	(214)
Kvuzat-Yavne/50-826	High	1.89	G-**R**-**R**-Q-**K**-**R**↓F-I	(214)
Australian isolates				
Peats Ridge	Low	0.41	G-**R**-**R**-Q-G-**R**↓L-I	(303)
QV4	Low	0.39	G-**K**-**R**-Q-G-**R**↓L-I	(255, 303)
Somersby 98	Low	0.51	G-**R**-**R**-Q-**R**-**R**↓L-I	(138, 303)
Dean Park	High	1.60–1.70	G-**R**-**R**-Q-**R**-**R**↓F-I	(303)
PR-32	Low	0.64	G-**K**-**R**-Q-G-**R**↓F-I	(138, 303)
African isolates				
Chicken/MG/'92	High	—[c]	G-**R**-**R**-**R**-**R**-**R**↓F-V	(257)
Niger/1377-7/06	High	1.84	G-**R**-**R**-**Q**-**K**-**R**↓F-I	(50, 271)
Nigeria/228-7/06	High	1.90	G-R-R-Q-R-R↓F-I	(271)
Chicken/Mali/'07	High	—[c]	G-R-R-R-K-R↓F-V	(257)
Burkina Faso/2415-580/08	High	1.69	G-R-R-R-K-R↓F-I	(50)
South Africa/08100426/08	High	1.91	G-R-R-R-K-R↓F-I	(271)

[a] Virulence for chickens.
[b] ↓ = cleavage point. Basic amino acids in bold. Note that all virulent viruses have phenylalanine (F) at position 117 (the F1 N terminus).
[c] Unknown ICPI.

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the respiratory route (251). These mutations were recently detected in viruses isolated from vaccinated birds with neurological and other signs in Africa and Madagascar (257, 265). Unfortunately, the virulence of these isolates was not confirmed *in vivo* in the laboratory.

Altogether, these studies show that the cleavage site motif of F0 is a reliable indicator of the *potential* virulence of NDV isolates, but other factors are clearly involved for the virus to have a virulent phenotype. For example, many PPMV-1 have a multiple basic amino acid sequence motif yet have a low virulence phenotype on the ICPI. These viruses can acquire virulence after multiple passages in chickens (18, 82, 147, 174). A PPMV-1 strain, AV324, rescued from an infectious clone, confirmed that the low virulence is an intrinsic property of the particular strain and not due to mixtures of low- and highly-virulent isolates (80). Furthermore, the exchange of the F gene of the PPMV-1 and of a virulent strain in both directions had no effect on the virulence measured by ICPI (80). All observations on the role of the cleavage site suggest that there must be other factors that contribute to the virulence, such as HN amino acid substitutions.

HN Gene. The basic function of HN has been described in the Biologic Properties section. The close association of HN and F, which may require changes to be made to both HN and F to maintain the interaction, complicates studies on the contribution of HN to virulence. The different HN lengths appear to have no effect on virulence (239). Other reverse genetics studies were not conclusive on the contribution of HN to virulence (reviewed by Dortmans [81]).

V Gene. The V protein allows for NDV to evade the host response by interrupting the type I and II interferon response and apoptosis induction by NDV infection. Prior expression of the V protein rescues NDV replication in primary CEF treated with IFNα/β, and this activity resides in the C-terminal portion of the protein (217). Moreover, V protein-deficient NDV produced by reverse genetics is highly attenuated in CEF and ECE compared to wild-type virus (216). The attenuated phenotype could be restored by the insertion of an influenza A NS1 protein also known to interfere with the IFN response.

V protein-deficient NDV also are less virulent in chickens as revealed by a decrease of ICPI and IVPI (81, 296). Viruses, which are deficient in expressing the V protein, are unable to target the STAT1 protein for degradation (123), and therefore, unable to block the host's interferon-signaling pathway (226). Moreover, cells infected with these NDV mutants have increased levels of apoptosis (217) and *in vivo* the viruses infected fewer tissues and induced milder clinical signs than infections observed with the reverse genetic parent virus (116). The overexpression of the V protein results in a reduction of the host innate immune response and the acceleration of NDV replication (128). Altogether, all studies indicate that the V protein is essential for replication and virus survival in the host.

Replication Complex (NP/P/L Genes). A correlation between virulence and level of viral replication has been observed in many viruses, and can be difficult to interpret as some difference may be specifically due to the particular NDV strain tested (317). The NP, P, and L proteins form an active ribonucleoprotein (RNP) complex (82) and its involvement in NDV virulence also has been studied by reverse genetics (82, 242). Whereas in one study the exchange of L protein alone had a small effect on virulence (242), a different study, in which genes of a vNDV and PPMV-1 strain were exchanged, indicated that all three proteins (NP, P, and L) had a significant effect on the virulence of NDV (82). Similarly, the effects of exchanging the NP gene have been inconsistent (211, 242, 317). Moreover, passing the PPMV-1 isolate in chicken brain revealed three mutations—2 two in the L and one in the P protein—that correlated with increased replication going along with an increased virulence for chickens (83).

In summary, studies indicate that NDV virulence is a complex trait due to the effect of multiple genes, with the multiple basic amino acid cleavage motif of F_0 being the minimum requisite of virulence.

Emergence of Virulent Viruses

Outbreaks of vNDV could arise from transmission of virulent viruses that are enzootic in other bird species. The best-characterized endemic virulent wild bird genotype is in cormorants in North America (244). These strains have ICPI in chickens greater than 0.7, and cause periodic outbreaks with mortality in cormorant populations and may spill over to gull species (74). However, cormorant viruses from subgenotype Va are rarely reported in poultry (73). Apart from cormorants, vNDV strains are sporadically isolated from healthy wild ducks, herons, gulls, swans, Guinea Fowl, and Japanese Quail (66, 266, 294). However, loNDV strains have been isolated frequently from wild birds and appear to be endemic in some duck species (31, 47, 227, 290). Whether loNDV strains circulating in waterfowl may form a reservoir of emerging vNDV is currently unknown.

Four observations indicate that loNDV may be a true threat. First, reverse genetics have demonstrated that only three mutations are required for a LaSota strain to acquire virulence (222). Second, vNDV may evolve from loNDV isolates from waterfowl by passage in chickens (20, 110, 260). Third, the Australian epizootic of 1998–2000 demonstrated that it is possible that some loNDV strains could evolve into a virulent phenotype by similar

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mutations (303) because virulent strains were closely related to a loNDV circulating in the same region. These viruses had only two nucleotide differences at the site coding for the F0 cleavage site (110). A similar observation was made in Ireland in 1990 (60).

The movement of exotic birds, especially psittacine species, started the 1970s panzootic (219). Although there are reports of captive non-gallinaceous birds being infected (17, 293), it is not certain if the virus is endemic in these captive birds or if infection resulted from the spillover from infected poultry. A more common occurrence is vNDV isolated from gallinaceous species (peafowl, quail, pheasants) (8, 70) kept in close contact with chickens, possibility acting as a "bridge species" facilitating additional outbreaks (294).

The emergence of PPMV-1 is another example of how a panzootic begins. It was concluded from phylogenetic studies that pigeon-type APMV-1 strains most likely emerged as a result of chicken-to-pigeon transmission of NDV strains (16). Over time, PPMV-1 isolates appear to have increased its adaptation and virulence in pigeons with a corresponding decrease in adaptation and virulence for chickens (301, 235). There is also evidence of PPMV-1 infecting multiple wild bird species and gallinaceous birds other than turkeys and chickens (16).

Pathobiology and Epidemiology

Incidence and Distribution

Newcastle disease is a global problem. From January of 2013 through December 2016, 71 of the 181 OIE member countries reported ND in domestic poultry (http://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home). The disease is particularly problematic for poultry producers in countries in the Middle East, Africa, and Asia. Confusing the situation, some countries with endemic vNDV still use mesogenic NDV strains as live vaccines that are defined as virulent due to their cleavage sites and high ICPI values (71). Because differentiating infected from vaccinated animal (DIVA) strategies are described but not used for ND, it is impossible to discern infected from vaccinated animals (215, 223).

The spread of vNDV between countries of Asia, the Middle East, Europe, and Africa has been documented (3, 96, 190). Potential transmission from wild pigeons and cormorants continues to be a concern for poultry producers (74, 87). Sporadic outbreaks also occur in countries that do not have vNDV endemic in poultry and where vaccination for ND is routine (135). Underreporting in certain areas confounds the issue on what NDV genotypes are currently circulating among different bird species around the world (76).

The four defined panzootics of ND proposed by Alexander since the disease was recognized in 1926 have been reviewed extensively and this information can be found elsewhere (19, 75, 190). A fifth potential panzootic may be underway as vNDV of a new subgenotype VIIi have been isolated from poultry and pet bird species in Indonesia, Israel, Pakistan, Eastern Europe, and India (96, 190, 194).

Natural and Experimental Hosts

While some species may require a higher infectious dose of NDV than other species to become infected, as with turkeys compared to chickens (10), infection with NDV has been documented in at least 236 species (133). Likely all avian species are susceptible to NDV infection; however, infectious dose and clinical signs depend not only on the species of the host, but also vary with individual NDV isolates (136).

Transmission, Carriers, Vectors, Spread

Horizontal transmission of NDV has been documented many times. Infected birds shed NDV in oropharyngeal secretions and fecal matter (4, 185). Susceptible birds may become infected by inhaling contaminated dust or aerosolized virus (165), or by ingesting such material. Infection by inhaling aerosolized virus is illustrated by the success of applying live NDV vaccines using nebulizers (175). The ingestion of contaminated feces or contaminated carcasses can cause infection in chickens (21) and raptors (253). Applying vaccine to chickens via the drinking water is another demonstration of oral infection (153). While immunized chickens may shed vNDV for 6–9 days after challenge or until they succumb (135), unvaccinated wild or captive exotic birds like parrots, cormorants, and pigeons may have prolonged shedding of vNDV, usually in feces, without clinical signs (93, 134, 151, 296). Signs of clinical disease in Japanese Quail (*Coturnix coturnix japonica*) infected with vNDV vary independently of the dose of virus administered, and appear to be more related to the tropism of the strain of vNDV and also result in prolonged shedding (166, 176).

Cases of vertical transmission of NDV from parent to offspring are difficult to prove beyond doubt due to the possibility of hatchlings being infected by contaminated feces through eggshell cracks or by exposure to a contaminated environment. At least two reports of virulent NDV isolated from embryonating chicken eggs (46), day-old hatchlings, and dead-in-shell birds (243) have been documented. Some NDV may have a tropism for the oviduct, which can be observed indirectly through microscopic lesions and directly using immunohistochemistry (44). Reports of embryos infected with virulent NDV from naturally infected layer hens have been reported, but as expected, the embryos usually die before hatching (160).

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Vaccine virus has been found in reproductive organs after vaccination (230). A small number of ECE experimentally inoculated with one mean egg lethal dose of virulent NDV survived and shed virus in feces (55). Nonvaccinated chickens infected with vNDV resulted in vNDV isolated from the albumen, yolk and eggshell of eggs, and from the ovary and all parts of the oviduct, which only supports vertical transmission if embryos are viable after incubation and hatching (249). Until eggs from naturally infected birds are cleaned and hatched under experimental conditions in a negative pressure isolator and shown to be shedding virulent NDV identical to the outbreak virus, doubt will exist as to the importance of vertical transmission. While this topic is especially critical for endemic countries, it is also important for countries free of ND.

Evidence that humans, mammals (56, 259, 315, 319), or insects (53) are effective biological vectors of spread of ND is lacking, although humans can be infected and develop conjunctivitis. However, spread by humans is more likely mediated by transport of contaminated fomites (94). NDV-contaminated equipment, clothing, shoes, feed, water, vaccines, and poultry products, and NDV-infected birds (wild or domestic) moved or placed into contact with susceptible birds where virus can be inhaled or ingested can lead to the spread of ND (43, 131, 289). Migratory birds and illegally imported birds have been implicated in the introduction of ND in certain regions (22).

Biosecurity with strict importation and quarantine procedures preventing the movement of infected birds, bird products, and equipment from outbreak areas is critical to contain ND and to keep the disease from entering a poultry facility or country free of disease (76).

Incubation Period

The incubation period for natural exposures to NDV varies between 2 and 15 days, averaging around 5–6 days, but may take 3–4 weeks in some circumstances. Experimental infection with virulent viscerotropic NDV in chickens usually results in a 1- to 4-day incubation period depending on the challenge dose. Aerosol transmission may have a shorter incubation time than an infection started through ingestion of the virus.

Clinical Signs

Neither the clinical signs nor the gross lesions are pathognomonic for ND. Nonvaccinated chickens infected with virulent viscerotropic isolates become listless and depressed two days after infection (Figure 3.3A), ending with 100% mortality by the third or fourth day (272). With an oculonasal route of infection, bilateral conjunctivitis with some facial swelling may be present. Often clear mucus will pour from the mouths of infected birds if their heads droop toward the ground, leaving the bird gasping for air as it tries to clear the oral cavity of fluid. This fluid may appear to be nasal secretions but is more likely crop fluid related to stasis of the gastrointestinal tract, and it pours from the mouth when the crop is compressed as the bird is handled (Figure 3.3B). The feces of infected birds may be green and watery. The comb may become blue as the bird becomes cyanotic, but hemorrhages are only seen with some isolates.

The clinical presentations of chickens infected with virulent neurotropic NDV strains that were commonly isolated in the United States during the 1940s and 1950s were like a completely different disease compared to present day outbreaks with velogenic strains. Birds may seem excitable and hypermetric three or four days after infection. Approximately five days after infection, head or muscular tremors, torticollis, and paralysis of one wing or one leg may occur. Typically these birds are bright and alert and die from the inability to reach food and water nine or more days after infection. Mortality is usually around 50%, with greater mortality in younger birds. Mesogenic strains usually result in low or no mortality in 4-week-old chickens, and will present with neurological signs such as head tremors, torticollis, and paralysis (272). Pigeon isolates in chickens may lead to no clinical signs (147).

Well-vaccinated layers infected with vNDV may present with only a decrease in egg production one week after infection, with the fewest eggs produced two to three weeks postinfection, after which the number of eggs produced will start to increase (37). The percentage decrease in egg production depends on the strain of NDV, and the HI antibody titer present during the time of infection. The HI antibody titer affects the mortality rates (61), the amount of virus shed from infected birds (136), and the percent drop in egg production (114). At one month after infection, misshapen and/or bleached eggs may appear for the life of the chicken (Figure 3.3D).

Strains of low virulence usually do not cause disease in adult birds. However, young naïve birds may present with serious respiratory distress following the application of the LaSota vaccine strain. Respiratory disease also may be observed if there are secondary infections or high ammonia levels, or if aerosolized droplets of vaccine are so small that they are delivered deep into lung tissue during vaccination (292). Caution should be used when interpreting signs of clinical disease in field situations where secondary infections may be the primary insult (41).

Clinical signs in other poultry species can be different and depend not only on the species, but also on the specific breed of bird. For example, clinical disease in nonvaccinated commercial turkeys may be less severe than what is observed in SPF turkeys (297). Generally, the order of most susceptible, showing the most clinical signs, to the least susceptible, showing the fewest clinical

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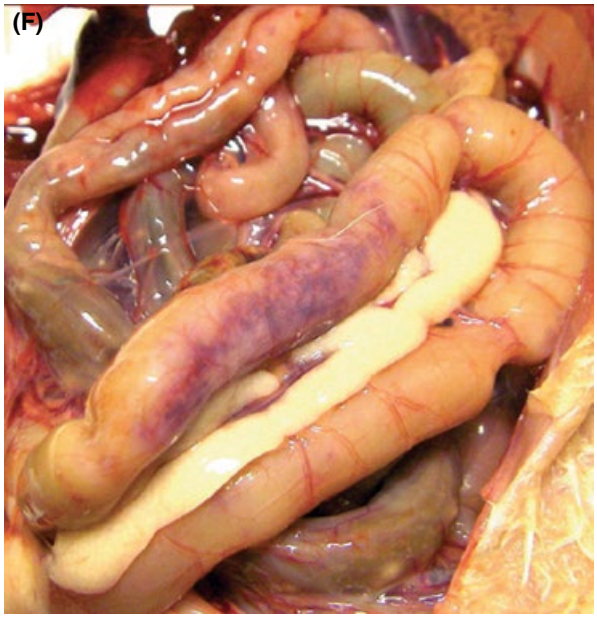












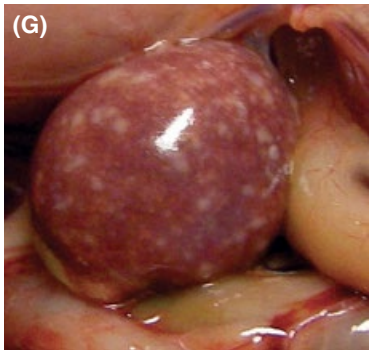




Figure 3.3 Signs of clinical disease and gross lesions upon infection with vNDV. (A) Birds appear depressed and lethargic with ruffled feathers. (B) Crop fluid visible from oral cavity. (C) Hemorrhage of the lymphoid tissue of the lower eyelid. (D) Misshapen eggs produced by commercial layers previously vaccinated with four live B1 vaccines. Eggs were produced 30 days after challenge with vNDV as the only clinical sign of ND. (E) Necrosis and hemorrhage of the cecal tonsils. (F) Necrosis and hemorrhage of small intestinal lymphoid patches (Peyer's patches). (G) Enlarged, mottled spleen with pinpoint areas of necrosis. (H) Hemorrhage and necrosis of proventriculus. (*For color detail, please see the color section*.)

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signs, are chickens, turkeys, pigeons, and ducks (10, 297). Geese are usually grouped with ducks in terms of susceptibility, but there are reports of clinical disease in geese from Asia from infections with genotype VII isolates or with particular strains of NDV (171, 299). Nonvaccinated pheasants are highly susceptible with clinical signs similar to those observed in chickens (8). Ostriches usually present with nervous signs and mortality present only in young birds (13). Chicken isolates in pigeons may cause body tremors (297).

Pathology

Gross Lesions

As with clinical signs, gross lesions and the organs affected depend on many factors and are not pathognomonic for ND. Lesions will likely be absent in vaccinated birds and possibly in nonvaccinated birds. Necrosis and hemorrhagic lesions in the lymphoid-dependent areas of the intestines (lymphoid tissue of lower eyelid, cecal tonsils, Peyer's patches) of infected chickens and turkeys are common with virulent viscerotropic isolates (Figure 3.3C, E, F) (272, 297). Enlarged spleens, with hemorrhagic thymic and proventricular tissues, are also common (Figure 3.3G, H). Chickens will occasionally present with moderate splenic congestion and mild thymic atrophy after infection with a virulent neurotropic NDV, such as a cormorant virus (272). Gross lesions are not seen in the central nervous system of birds infected with any vNDV. Lesions in the respiratory tract are rare (52), except for thickening of the air sacs due to secondary infection (180). In chickens, necrosis and hemorrhage of the laryngeal tonsils in the cranial portion of the trachea is consistently observed with the virulent viscerotropic NDV that caused the last outbreak in the United States during 2002 (52, 297). Egg yolk peritonitis with atrophied follicles and degenerated oviduct may appear during birds in lay (44). Cormorants infected with NDV often present with neurological signs, along with intestinal hemorrhages (41).

Microscopic Lesions

The same variables previously mentioned affect the microscopic lesions found with NDV infection. More detailed information on histological lesions has been published (52). Some of the more common lesions in chickens and turkeys for virulent NDV included: (1) necrosis with lymphoid depletion of cecal tonsils, spleen, thymus, eyelid, and bursa; (2) histiocytic airsacculitis; (3) lymphohistiocytic airsacculitis; (4) gliosis with perivascular cuffing; medulla, cerebellum, and brainstem; (5) yolk peritonitis with foamy macrophages of subserosal space of oviduct; (6) necrotic myocarditis; (7) bone marrow necrosis; (8) pancreatic, thymic, hepatic necrosis; (9) necrosis and ulceration of epithelium of intestine; and (10) hemorrhagic, ulcerative tracheitis. For loNDV, infections in chickens and turkeys may cause lymphoplasmacytic tracheitis with deciliation (154), but lesions are rare (41).

Immunity

Active

As soon as six hours after exposure, splenic cells produce alpha and beta interferon, and interleukin 6 (IL-6) when exposed to vNDV, but not to loNDV (245). The response after exposure may differ not only due to the virulence of the strain infecting the cells, but also may depend on the amount of the virus infecting the cells (136). Interferon gamma (IFN-γ) secreted from NK cells and T lymphocytes activates macrophages and promotes cell-mediated immunity (CMI) one day after infection (69, 170). Whole chicken genome arrays of the host immune response to NDV at 1–2 days after infection demonstrate the induction of type I and II interferons, cytokines, chemokines, and inducible nitric oxide synthase (263, 264). Chemokines MIP-3α and MIP-1β support CMI by recruiting neutrophils (245). The innate immune response alone is not enough to ensure survival of the host after an exposure to a vNDV. However, the activity of NK cells, which are able to destroy virus-infected cells, improves as chickens age and this improvement could provide some explanation for decreased susceptibility with older birds (164). Once the virus overcomes the innate immune response, cellular (158, 237) and humoral responses are initiated by the host (136).

T-lymphocyte differentiation occurs in the thymus gland, producing cells able to rapidly expand after being exposed to the antigen a second time, providing CMI that is measured by lymphocyte proliferation or cytokine secretion (158). Cytotoxic T lymphocytes (CD8) are found in splenic cells of chickens twice infected with NDV (130). Cell-mediated immunity of a T helper cell type 1 (Th1) response, characterized by the production of IFN-γ by macrophages, can be detected three days after infection with a live NDV vaccine strains (281), thus providing signals for a humoral response to develop by the host.

Macrophages also induce nitric oxide (NO) production (254) and the amount of NO produced is elevated in birds with higher antibody levels, demonstrating how CMI and humoral immunity affect each other (111). Similar to innate immunity, CMI alone is not enough to ensure survival of the host after an exposure to a vNDV (237). However, IFN-γ produced in large quantities at the same time as the vNDV is replicating in the host significantly reduces mortality and moderately reduced morbidity, providing evidence of the role of CMI in NDV infection (270). Some NDV isolates produce a V protein that limits the infected host's cells from producing interferon, effectively decreasing the initial innate and CMI response

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after infection (179) through targeting the phosphorylated STAT1 protein for degradation (226).

After exposure to NDV, B-lymphocytes differentiate into plasma cells that secrete three types of neutralizing antibodies specific to the antigen (158). These antibodies are necessary to protect birds from morbidity and mortality after infection with vNDV (61, 238). First, IgM is produced and can be detected as early as four days after vaccination (6). After seven days, IgG (referred to as IgY in birds) and IgA, which is important to the local immunity of the Harderian gland and cecal tonsils, can be detected (6). Local immunity is evaluated by the amount of antigen-specific IgA antibodies that can be detected in tears, tracheal washings, and bile after intranasal or intraocular exposure to live NDV vaccines. Local immunity (IgA) is important in neutralizing free virions in the respiratory, gastrointestinal, and portions of the reproductive tracts (225, 232, 248).

Neutralizing antibodies against the HN and/or the F glycoproteins bind to the virions and prevent their attachment to the host cells, which reduces virus replication (38, 168, 275). Commercial or experimental viral vectored vaccines for NDV generally include only the F gene product, which produces neutralizing antibodies that do not inhibit NDV hemagglutination, providing the possibility to use the concept of differentiation between infected and vaccinated animals (DIVA) (212).

Environment, nutrition, stress, bacterial infections, and immunosuppressive viruses can lead to the suppression of the immune system (102, 119). If the bursa of Fabricius (cloacal bursa) has atrophied from infectious bursal disease virus (IBDV), a decreased immune response will be induced for any vaccine. Ironically, infections with NDV also have the ability to immunosuppress the host and prevent a proper immune response to other vaccines, such as to IBDV (231).

Passive

Maternal antibodies passed to embryos through the egg yolk may be protective depending on the amount of antibody transferred, and the dose and virulence of the challenge virus (316). If present at the time of vaccination with a live NDV vaccine, maternal antibodies can neutralize the live vaccine and lead to reduced protection or a vaccine failure (202, 292). Levels of antibody in day-old chicks are directly related to titers in the parent. On average, the amount of HI antibodies decreases by half every six days (104). While maternal antibodies in broilers are usually detectable only until 7–10 days of age, they may be found up to 30 days of age (104).

Diagnosis

For effective disease management, it is important to be able to identify birds that are infected with NDV and distinguish vaccine viruses from virulent viruses. As discussed earlier, the clinical signs and lesions observed with vNDV infections are not pathognomonic, and therefore, are not specific enough for a diagnosis. In many countries infections with loNDV strains may be due to lentogenic and/or vaccine virus, and thus, are not reportable. In addition, loNDV are commonly found in apparently healthy wild birds (31, 290).

Isolation and Identification of the Causative Agent

Tracheal, oropharyngeal, and/or cloacal swabs from live birds, or organ tissue samples (brain, liver, spleen, kidney, or organs with lesions) from dead birds suspended in an antibiotic/antifungal media should be kept at 4°C for 1–2 hours or frozen on dry ice or liquid nitrogen until they are able to be thawed and processed. Swabs should be fully submerged in media and for tissue samples a 20% w/v suspension is required for transport. Once centrifuged at 1,000 g for 10 minutes, 100–200 µL of a sample is inoculated into the chorioallantoic sac of a 9- to 11-day-old SPF ECE. If SPF eggs cannot be obtained, eggs from a flock free of NDV antibodies should be used for the assay. The eggs are incubated at 37°C and examined daily for 4–7 days (12).

Allantoic fluid from any eggs containing embryos that died after 24 hours and all eggs at the end of the 7-day incubation period should be collected after the eggs are chilled at 4°C to be tested for hemagglutinating activity (HA). This fluid also may undergo RNA extraction for use in molecular techniques. All HA-positive samples should be tested for specific inhibition with an antiserum to NDV (APMV-1), referred to as a hemagglutination-inhibition (HI) assay. All HA-negative samples should be passaged again in ECE and tested for HA activity. APMV-1 isolates may cross react with APMV-3 and with APMV-7, and on rare occasions with APMV-2 and APMV-4. However, the cross-reacting titers to these other APMVs are usually much lower than they are to APMV-1. Molecular diagnostic tests, such as real-time RT-PCR (RRT-PCR) are also routinely used for confirmation. Any diagnostic sample causing embryo death 24–72 hours after inoculation should be further investigated even if there is no HA activity.

If the HA is positive, but the HI is negative for APMV-1, consideration of several other viruses as well as bacteria must be considered. The allantoic fluid can be passed through a 45 µm filter to remove most bacteria before repeating for HA activity. Strains from cormorants isolated in the United States after 2002 rarely demonstrate HA activity with chicken RBCs, and therefore HA activity is not a universal property of NDV isolates (74, 245). As an alternative to using ECE, cell culture may be used for detecting NDV. However, loNDV will not replicate more than one round without the addition of trypsin to the media, except in some kidney cells, such as CEKs.

Direct detection of viral RNA isolated from swab samples or tissues may be tested using RT-PCR (106) or

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RRT-PCR. Real-time RT-PCR assays are not only able to distinguish APMV-1, but some tests can distinguish vNDV from loNDV (97, 98, 108, 152, 183, 201, 244, 250, 311). Reference laboratories can inform researchers which specific assays should be used for different areas of the world depending on the currently circulating strains. In addition, cDNA from RT-PCR fusion gene product can be used to sequence the F cleavage site to assess the number of basic amino acids and the presence or absence of the phenylalanine at the 117 position, which is an important predictor of virulence (59). Next generation sequencing allows for the sequencing of multiple microbial agents from viral stocks (77) and from formalin-fixed paraffin-embedded tissues (1, 310). Immunohistochemistry, immunocytochemistry, and immunofluorescent techniques allow the detection of the specific locations of the viral antigens in tissues or cells (44, 52, 195, 214, 271).

Serology

Serology usually is not a useful tool for the diagnosis of ND, because current serologic methods cannot differentiate antibodies induced from an infection with vNDV, loNDV from wild birds, or those induced by vaccination with live or inactivated vaccines. Diagnostically, serology is most often used to measure the effectiveness of a vaccination program (277). In countries that do not vaccinate, serology can confirm exposure to NDV, but few countries are in a position to do so. In well-managed flocks that take periodic serum samples, rising HI titers accompanied with clinical signs that cannot be attributed to revaccination suggests that an exposure occurred.

Hemagglutination-inhibition assays and enzyme-linked immunosorbent assays (ELISAs) are used most commonly to detect and quantify antibodies to NDV (12). Hemagglutination inhibition assays are commonly performed to evaluate antibody response post vaccination because they correlate well with the more laborious virus neutralization (VN) assays (278). Commercial ELISA kits are also available and are sometimes used to evaluate the uniformity of vaccination for a poultry flock; however, because they do not specifically measure neutralizing antibodies they may not correlate well with protection (278). Although HI assays detect antibodies to only the HN gene, and the ELISA platform typically detects antibodies against all NDV proteins, there is some correlation between the assays. ELISAs are usually specific for the host species because they depend on the use of one monoclonal antibody (mAb) to NDV, and another species-specific secondary mAb. Caution should be taken when using sera from species other than chickens in the HI test because they may cause false positives (less than or equal to 1:8) from nonspecific agglutination of chicken RBCs (67). Such agglutination may be removed by heat inactivation at 56°C for 30 min prior to adsorption with chicken RBCs before testing (207). Attention to using the OIE approved protocol is suggested to avoid discrepancies in results (207). When using 4 HA units of antigen, titers 1:16 or higher are considered positive. The cutoff is equal or greater than 1:8 when using 8 HA units (12).

Differential Diagnosis

The clinical presentation of other diseases, such as aspergillosis, mycoplasmosis, infectious laryngotracheitis, fowl cholera, infectious bronchitis, and highly pathogenic avian influenza, can be confused with ND. A limited number of avian viruses have the ability to hemagglutinate chicken RBCs: virus from the avian paramyxovirus serotypes, the avian influenza virus, and the etiological agent of egg drop syndrome, an adenovirus. Confirmation of APMV-1 can be quickly performed with NDV polyclonal antisera. Appropriate positive and negative controls should be used in the HI assays to prevent a misdiagnosis from cross-reactions with other APMV serotypes.

Molecular Techniques in the Diagnosis of ND

A quick and prompt diagnosis of the disease in the laboratory is essential to the control of ND. The application of new molecular techniques means that results can be obtained within a few hours after receiving the samples in the laboratory. Conventional techniques can take 2–14 days to isolate the virus, and characterize the virus to a level that an outbreak can be confirmed. Rapid confirmation is essential to reduce the economic effects caused by the standstill that likely is imposed on a farm pending the outcome of an investigation. Preparedness to report suspicions and submit samples to the laboratory is directly related to measures imposed by regulatory agencies (91). While molecular techniques can produce more rapid results if the lack of clinical signs does not allow for the proper selection of diseased birds, which might be, for instance, the case in vaccinated flocks or the case of infections with loNDV, virus isolation is necessary. Keep in mind that sampling clinically ill or dead birds from vaccinated flocks is more effective than random testing of healthy birds.

Molecular technologies to detect NDV have been reviewed by Hoffmann et al. (120) and Miller et al. (187, 188). Molecular assays should not only detect NDV, but also differentiate vaccine or loNDV strains from vNDV isolates, according to the OIE definition of ND. Problems with any molecular assays are often related to the genetic variation of the fusion gene of different genotypes of NDV and particularly the cleavage site in F0. Currently, there is no single test that has been fully validated to detect and simultaneously pathotype all class I and II viruses worldwide. Most molecular techniques involve reverse transcription (RT) followed by a polymerase chain reaction (PCR) to produce a DNA copy of the RNA genome as an essential initial step.

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Conventional RT-PCR assays have mostly been replaced by RRT-PCR protocols that use fluorogenic probes to detect amplification in real time. Real-time RT-PCRs can be automated, and thus are better suited for high throughput. While primers and probes targeting the M gene were shown to detect most class II genotype viruses, later it was determined that they often failed to detect class I viruses (141). To accommodate the detection of class I viruses, primers and probes were targeted at the L gene such that primer/probe sequences and assay conditions were compatible with the M gene RT-PCR, allowing for a multiplex RRT-PCR (143). Another laboratory determined that the same M gene RRT-PCR protocol failed to detect some of their isolates and subsequently used a shortened, degenerate probe using locked nucleic acids to maintain probe stability and improve efficiency (51).

The multiplex RRT-PCR allows the detection of most NDVs, but not their virulence. Therefore, it can only be used to exclude ND as a possible cause of an outbreak, but it is not suited to confirm a vNDV infection. Real-time RT-PCR that targets the fusion gene at the cleavage site could be used to detect and pathotype the NDV isolate in a single assay (98). The assay designed by Wise et al. was validated with clinical samples and successfully applied during an ND outbreak in California during 2002, and this assay is authorized for use in laboratories of the National Animal Health Network as a surveillance tool in the United States (307). The primers and probes were targeted to the California outbreak strain, and sequence mismatches have to be considered when detecting vNDV worldwide.

Fuller et al. (98) used a similar strategy, but modified the forward primer and the probe by using the universal base inosine to correct for mismatches and locked nucleic acids to increase binding activity. Unfortunately, the detection limits of all tests varied between 10^1 and 10^3 EID_{50}. Moreover, detection limits may vary with viruses belonging to other genotypes depending on how probe and primer sequences match the target sequences. In addition, the probe specific for loNDV used in this assay does not detect all class I viruses.

In summary, virus isolation should still be used in parallel to molecular techniques when making an initial diagnosis of an NDV outbreak because the presence of embryo mortality ensures further investigation, either if a mismatch in primer or probes is present. As more information on the sequence of an outbreak virus is determined, greater reliance on molecular diagnostic tests can be employed.

Intervention Strategies

Regardless of whether ND control is applied at the international, national, or farm level, the objective is either to prevent susceptible birds from becoming infected or to reduce the number of susceptible birds by vaccination. For the former strategy, each method of disease spread must be considered in prevention policies.

Management Procedures

International Control Policies

A good definition is indispensable for defining the disease status for ND. The OIE is responsible to the World Trade Organization for the standardization of matters relating to animal health that affect trade and has formulated a definition of ND (208). Newcastle disease is defined as an infection of birds caused by a virus (NDV) of avian paramyxovirus serotype 1 (APMV-1), that meets the criteria previously discussed in the section entitled Definitions and Synonyms. The strict definition is required because of the enormous variation in clinical manifestation of the disease and possible masking of infection by vaccination practices to control the disease.

National Control Policies

After the international movement of infected psittacine birds was found to be the cause of the panzootic during the 1970s, many countries increased regulations on the importation of live birds, eggs, and poultry products (115). The importance of biosecurity and preventing domestic poultry from contacting other birds was reinforced. Quarantine stations were designed to be able to hold imported psittacine birds for long lengths of time to ensure they were not infected and shedding vNDV (92).

Recommendations for ND control can be found on the OIE website (http://www.oie.int) (207). Since 1933 NDV strains have been known to infect pigeons (86) and they have continued to infect them worldwide (250). It is known to be the cause of the third ND panzootic that continues today (16, 75, 142, 218). The role of pigeons in the spread of ND has led to strict regulations concerning racing pigeons and mandatory vaccination, even in some countries where poultry are not regularly vaccinated.

In many countries identifying and culling infected animals while simultaneously restricting the movement of birds and bird products within a defined area surrounding the infected birds often controls ND. Disposing of infected carcasses and litter without further disseminating the virus is problematic. Emergency vaccination within a defined area around an outbreak area also may be employed. While each outbreak situation has the same goal of containing and preventing the spread of NDV to other areas, there are variations for each country concerning control policies and measures must depend on variables such as poultry density, vaccination status, and so on. However, it is clear that all countries will need to address not only the control of ND in poultry species, but also in wild bird species that continue to harbor vNDV worldwide (16, 87). In addition,

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the concept of evaluating vaccines for their ability to decrease, if not prevent, the transmission of vNDV among vaccinated birds, as seen with avian influenza vaccine development (228), should be required, especially for countries where vNDV is endemic, as it evaluates the effects of lower shedding and increased resistance of vaccinated birds. Currently, NDV vaccines are evaluated only for their ability to prevent morbidity and mortality (285, 286).

Vaccination policies vary with each country. Preventative or prophylactic vaccination is allowed in most countries of the world. Estonia, Finland, Switzerland, and Sweden currently do not allow preventative vaccination of chickens (30). Finland also prohibits the rearing of poultry outside for portions of the year when they are more likely to mingle with wild birds and requires the administration of inactivated ND vaccines for racing pigeons.

Biosecurity at the farm level is critical to preventing the introduction of diseases to domestic poultry and must be designed taking into account requirements for each location (258). Water and feed quality and pest and litter management are areas that need to be tightly controlled. Routine disinfection and cleaning procedures (40) and post-outbreak protocols should be in place prior to an ND outbreak (27, 177). Biosecurity measures also should be employed in backyard bird situations (196).

Vaccination

The role of vaccination in the control of ND has been to prevent losses from morbidity and mortality because vaccines are not able to completely prevent vaccinated birds from being infected with vNDV. Vaccination may increase the resistance to infection and reduce the quantity of vNDV shed resulting at the end in fewer birds being infected (25, 135, 184, 186, 249). Comprehensive reviews (76) and historical aspects of ND vaccination have been reviewed in other publications, including previous editions of this chapter (17, 182, 256). Vaccination is to be used along with good management and biosecurity practices. In some areas of the world where vNDV is endemic, vaccination is necessary for the survival of backyard or village poultry.

The chapter Principles of Veterinary Vaccine Production in *The Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* reviews guidelines and procedures for vaccine production (205). Regulations from the European Union directive 93/152/EEC states that viruses used as live NDV vaccines should have an ICPI of less than 0.4 or 0.5, depending on the dose (anonymous 1993 commission of the European communities [29]). The OIE Biological Standards Commission states that the virus should have an ICPI of less than 0.7, but that to increase the safety margin the master seed ICPI should not be greater than 0.4 (204). Commonly used live vaccines and their pathogenicity indices for chickens are listed in Table 3.2 (11, 140, 167, 221, 255).

The US Code of Federal Regulations, Title 9 (9CFR), sections 113.329 and 113.205, reviews parameters for the production of the master seed virus and the experimental testing of vaccines for live and killed NDV vaccines, respectively (285, 286).

Types of Vaccines

Inactivated and live NDV vaccines formulated with NDV strains of low virulence, such as B1, LaSota, and more recently Ulster and VG/GA, are the most commonly administered vaccines. Some countries, usually those with endemic vNDV, continue to use live mesogenic NDV vaccines that are considered by the OIE to be reportable for the purposes of trade if isolated from poultry (140, 234). Live B1 and LaSota, developed in the 1940s, continue to be used worldwide (107, 118). More recent strains, Ulster (247), QV4 (39), and VG/GA (225) are also commonly used. QV4 (also called V4) and the I-2 strains have some ability to withstand heat and are often used in areas where a "cold-chain" is not reliable (112).

Mass application of live NDV vaccines in drinking water, sprays, or aerosols is less labor intensive than administering inactivated vaccines to individual birds. Unfortunately, with mass application of live vaccines it is difficult to produce protective antibodies in high percentages of birds in a flock. Ocular delivery provides the best response (93%), while vaccine delivery in water or spray may produce protective antibodies in only 53–60% of the birds (68). The inability to control the amount of

Table 3.2 Commonly used live lentogenic (loNDV) vaccine strains, rarely used mesogenic vaccine strains, and virulent challenge virus strains and their genotype and pathogenicity indices for chickens (11, 140, 167, 221, 255).

Virus strain	Pathotype	Genotype	ICPI[a]
Ulster 2C	Asymptomatic	I	0.0
QV4	Asymptomatic	I	0.0
VG/GA	Asymptomatic/ lentogenic	II	0.0
Hitchner B1	Lentogenic	II	0.2
LaSota	Lentogenic	II	0.4
Mukteswar	Mesogenic	IV	1.4
Roakin	Mesogenic	II	1.5
Beaudette C	Mesogenic	II	1.6
TXGB	Velogenic	II	1.8
Herts 33/1956	Velogenic	III	1.9
CA/2002	Velogenic	Vb	1.8
ZJ1	Velogenic	VIId	1.9

[a] ICPI, intracerebral pathogencity index in day-old chickens.

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water consumed per bird, inactivation of vaccine by heat, or impurities make water delivery problematic. The addition of dried skim milk may increase the stability of the vaccine virus for water application (103).

If applying the ND vaccine in a spray or aerosol, the correct particle size is critical to ensure a proper immune response. If the particles are too small vaccine reactions may develop in the form of respiratory disease because the virus is deposited deep in the lungs, and if the particles are too big, the immune response may not be optimal because the virus drops out of the air before the birds can become infected (312). For day-old chickens with maternal antibodies, aerosol delivery produced a better immune response than water delivery (89). Some live vaccine viruses with ICPI equal to or greater than 0.7 are injected intradermally into the wing-web to decrease the severity of respiratory disease from the vaccine (207).

While live vaccines are relatively inexpensive, easy to administer, and provide mucosal immunity, there are some disadvantages to their use. The value of vaccination may change slightly depending on each situation, and flock immunity may only be reached if more than 85% of the flock has a HI titer above the cutoff level (291, 308). In naive flocks the risk exists that after primary live vaccine application, vaccine virus is shed from immunized birds to their naive flockmates, extending the amount of time that live virus circulates in the flock. This may lead to vaccine reactions demonstrated by respiratory disease, especially if secondary infections or high ammonia levels are present. In addition in such flocks some decrease in body weight gain and feed efficiency may occur in early stages of growth for broilers (300). Therefore, timely primary vaccination at an age that maternal immunity is waning in flocks is recommended. Most importantly, live vaccines are inactivated by high temperatures and require refrigeration. Maternal antibodies might interfere with vaccine virus replication, which can be problematic for countries with stringent vaccine protocols for breeders, which subsequently produce chicks with high maternal antibody levels. In particular, in endemic countries backyard birds need to be well vaccinated to be able to survive the vNDV they are exposed to in the environment. Suboptimally vaccinated backyard birds may get subclinical infections contributing to unnoticed circulation of vNDV (197). Despite these concerns, because of their ease of application, their relatively inexpensive cost, and the level of immunity they induce, particularly in young birds, live NDV vaccines are the most commonly used method to protect chickens against clinical disease.

Oil emulsions with inactivated antigen are the most common killed vaccine (269). Formalin, beta-Propiolactone (BPL), and binary ethylenimine (BEI) are used to inactivate NDV. Whereas BEI is not known to affect the HA potential of the virus after inactivation, formalin and BPL both decrease the HA titers after inactivation (144). The same strains used as live vaccines are also used in inactivated vaccines.

Inactivated vaccines are more expensive to produce and more laborious to administer because they are given intramuscularly or subcutaneously, usually to older birds or layers. Immune responses induced by inactivated vaccines are less affected by maternal antibodies than by live vaccines. Inactivated vaccines produce high neutralizing antibody levels (184, 186). They are often formulated with other avian pathogens and delivered as a multivalent product to decrease the cost of administration. There is a 42-day minimum holding period between the time of injection and slaughter for consumption using oil emulsion vaccines in the United States, which restricts their use in broilers (268). Special attention to avoid to accidental human injection is required when working with oil emulsion vaccines because they can cause severe inflammatory responses in humans (302).

Recombinant technology has allowed for the commercial production of recombinant fowl pox or Marek's disease virus serotype-3, also designated as herpesvirus of turkeys (HVT), as live-vector vaccines to express the fusion glycoprotein of NDV (117, 212, 233). These vaccines may be delivered at the hatchery *in ovo* or in day-old chicks, produce no respiratory vaccination reactions, and can be administered in the presence of maternal immunity. They must be reconstituted as directed by the manufacturers, and require more time, typically 3–4 weeks, before effective immunity is reached. Other experimental vaccines (e.g., virosomes, virus-like particles, immune stimulating complexes [ISCOMS], and NDV antigen expression in transgenic plants) also have been shown to provide effective immunity and have been reviewed in Dimitrov et al. (76).

Although all APMV-1 are of one serotype, there has been more discussion since 2007 (186), concerning the use of recombinant NDV (rNDV) strains engineered (191) to either contain attenuated glycoproteins of circulating vNDV or the attenuated glycoproteins inserted into the backbone of commonly used vaccine strains, such as LaSota. Furthermore, rNDV are now more routinely constructed (48). These vaccines have not improved protection by reducing morbidity or mortality unless early challenges after vaccination or doses of vaccine less than normal are used (49). However, there is evidence that the amount of virulent virus shed from the vaccinated birds can be decreased and that there is some abatement of losses in egg production (57, 187, 240, 314). Others have stated that suboptimal mass application methods rather than antigenic differences are the cause of reported vaccine failures (78, 84).

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Field Vaccination Protocols and Regimens

Vaccination programs may be controlled by government policies depending on the ND status of a country. These programs should take into account the amount of NDV exposure a flock will likely be faced with, the quality of biosecurity available, available vaccines, maternal immunity in hatchlings, the use of other vaccines that could interfere with vaccination (305), available labor, ambient temperature, previous vaccination history, time until slaughter, type of chicken (broiler, breeder, layer), and cost. Many countries have regulations for the restricted use of any genetically modified organisms (GMO), plants, or vaccines. The vaccination protocol used depends on the type of production, the amount of maternal antibodies present, and the frequency and severity of the expected challenge virus (256). In the United States, broiler breeders receive at least three live vaccines over their life span and broilers get one live vaccine at day of age with one or two live booster vaccines later in the field. Applying live attenuated strains as sprays rather than gels in the hatchery along with optimal brooding conditions are essential for optimal control for broilers and broiler breeders in the United States. Layers will likely receive one inactivated and three live vaccines, with additional live vaccine boosts, over their life span. Turkeys require at least three vaccines to maintain sufficient antibody levels (99).

The protective immune response is usually evaluated by examination of HI antibody titers. Single vaccination of a 3-week-old SPF white leghorn with a live lentogenic strain will produce an HI response of around 8 to 16, whereas a single inactivated vaccine will produce between 64 to 256. An HI titer equal or greater than 16 is considered positive when using 4 HAU (≥8 when using 8 HAU) (206).

Vaccines developed for chickens are given to other avian species, but the immune responses differ depending on the species. The genetic lineage of turkeys is an important factor in quantity of HI antibodies produced and the amount of antibody may not correlate directly with the prevention of mortality (283). These genetic differences could account for the discrepancies in results from similar vaccine experiments in turkeys (139). Pheasants are highly susceptible to vNDV and vaccination decreases mortality, but does not prevent shedding of vNDV (200). Guinea fowl and partridges are highly susceptible to vNDV (88, 126), but both are able to mount an effective immune response to NDV following vaccination (229, 298). The quality and size of eggs produced from partridges may be negatively affected by vaccination (63). Ostriches mount good immune responses to NDV vaccines but need to receive booster vaccines to maintain the immune response up to the time of slaughter at 1 to 1.5 years of age (246). Pigeons are commonly vaccinated with inactivated vaccines produced with PPMV-1 seed strains that greatly reduce the amount of vNDV shed from infected animals (137).

Treatments

There are no treatments for vNDV and in most instances all infected birds are culled to contain an outbreak. In special instances exotic or endangered species in an outbreak area may, for a fee, be allowed to move to a USDA quarantine facility for 30 days to be tested for vNDV (288). Treatment for infections with NDV of low virulence consists of addressing secondary bacterial infections with particular attention to Gram-negative bacteria.

Avian Paramyxoviruses 2–15 and 17

Egbert Mundt

Summary

Agent, Infection, and Disease. Avian paramyxovirus (APMV) can be differentiated either by serology or by comparison of the viral genome sequence. In a recent update to the virus taxonomy, 20 different serotypes have been described in the subfamily Avulavirinae, with member species in three genera *Metaavulavirus, Orthoavulavirus, and Paraavulavirus* (https://data.ictvonline.org/virusTaxonomy.asp). Because the change in taxonomy has not been widely adopted, the older nomenclature will continue to be used for this subchapter. The isolates for APMV 2–15 and 17 were mainly obtained from wild bird samples. Clinical signs are usually not observed in domestic poultry. Transmission may occur by the aerosol or fecal–oral spread. Avian paramyxovirus of the different serotypes have been described worldwide.

Diagnosis. Avian paramyxovirus can typically be isolated in 9- to 11-day-old embryonating chicken eggs, preferably from a SPF source. Avian paramyxovirus will typically hemagglutinate chicken red blood cells (RBCs), and hemagglutination-inhibition (HI) tests are often used to identify the serotype. A panel of APMV-subtype specific antisera is needed for differentiation. With the availability of genomic sequences from all 17 APMV subtypes, reverse transcriptase polymerase chain reaction (RT-PCR) can be performed and/or sequence analysis to determine subtype.

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Intervention. Vaccination is possible but is rarely performed. Autogenous vaccines based on inactivated virus propagated in embryonated eggs can be protective.

Introduction

Definition and Synonyms

Avian paramyxoviruses belonging to the genus *Avulavirus* were named based on their historical order of isolation, identification, and characterization as an avian paramyxovirus with 17 serotypes (APMV 1–15, and 17) currently identified (Table 3.3). There are two descriptions for a serotype 15 (46, 77) for which Jeong et al. (34) proposed that one of them should be annotated as serotype 16 based on phylogenic analysis.

Economic Significance

The main economic significance among the members of the genus *Avulavirus* is from the APMV-1 serotype viruses. Usually APMV other than APMV-1 were isolated from wild birds, but occasionally they were also isolated from poultry. For example, APMV-2 and APMV-3, and APMV-6 infections have been associated with clinical symptoms in turkeys (3, 6, 9, 11, 31, 47, 49). APMV-3 was also isolated from chickens (69) and farmed ostriches in South Africa (36). An APMV-7 isolate obtained from turkeys induced clinical disease which was characterized by rhinitis and airsacculitis (63), whereas another APMV-7 was isolated from ostriches although it was considered an incidental finding with unknown contribution to disease (82). In conclusion, there is no primary economic significance for APMV 2–15 and 17, but the viruses should be considered as a possible cause of respiratory diseases and problems in egg production.

Public Health Significance

Viruses of the APMV serotypes 2–15 and 17 are likely not a threat for public health, and no clinical disease has been reported for these subtypes. Serological evidence of APMV-2 in humans has been reported and four virus isolates obtained from cynomolgus monkeys showing respiratory signs of disease provide the only evidence of zoonotic concern (22, 44, 59).

History

The first isolation of APMV from chickens other than APMV-1 was described in 1960 (12). All first descriptions of isolates of the single APMV serotypes 2–15 and 17 are shown in Table 3.3. A more detailed description about the history of APMV 2–11 isolates can be obtained from the 13th edition of *Diseases of Poultry* (53).

Etiology

Classification

All avian paramyxoviruses belong to the family Paramyxoviridae, subfamily Paramyxovirinae, genus *Avulavirus* (79), which are enveloped viruses containing a single-stranded negative-sense RNA genome. Avian paramyxoviruses are grouped primarily based on their reactivity in the HI assay using APMV serotype-specific sera. Due to an increasing availability of sequence capabilities the latest APMV isolates were defined based on phylogenetic sequence similarities.

Morphology

Avian paramyxoviruses are observed as pleomorphic virus particles. The diameter of the viral particles can vary between 100 and 500 nm. Besides being pleomorphic, filamentous forms of variable length also were observed. The viral glycoproteins cover the viral surface and were observed as projections of about 8 nm in length.

Chemical Composition

The viral particle of APMV consists of single-stranded RNA surrounded by a viral envelope containing glycosylated proteins. Nonglycosylated proteins together with the viral RNA mainly build the inner ribonucleoprotein complex. In recent years the full-length genome sequences or the coding part of the viral genome of at least one strain of each APMV 2–15 and 17 has been determined (14, 18, 33, 34, 42, 43, 46, 52, 57, 61, 64, 65, 73–77, 83–85).

Virus Replication

The virus replication of APMV occurs in the cytoplasm of infected cells and is assumed to be similar among all the different serotypes.

Susceptibility to Chemical and Physical Agents

Due to their similar chemical composition, viruses of APMV are likely susceptible to chemical agents as has been described for APMV-1.

Strain Classification

Antigenicity

Discrimination between the APMV serotypes occurs routinely by the HI assay using either serotype-specific defined APMV antigens for the detection of antibodies

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Table 3.3 Examples of descriptions of isolation of APMV 2–15 and 17.[a]

Serotype	Sample origin	Country	Reference
APMV-2	Chicken	USA	(12)
	Gouldian finch	China	(86)
	Orange collared sparrow and house wren	Costa Rica	(25)
	Ruddy turnstone	USA	(20)
	Different species (chicken, robin, canary, parrot, eagle, finch, pheasant)	Saudi Arabia, England, Singapore, Italy, South Africa, Kuwait, Denmark, Slovenia	(50)
APMV-3	Turkeys	USA, Canada	(78)
	Turkeys	England	(49)
	Chicken	Israel	(69)
	Ostrich	Namibia	(36)
	Psittaciformes and Passeriformes	Israel	(67)
	Parakeet	Netherlands	(4)
APMV-4	Duck	Hong Kong	(70)
	Mallard	USA	(24)
	Mallard	South Korea	(33)
	Mallard	Israel	(68)
	Serology in chickens	USA	(80)
APMV-5	Budgerigars	Japan	(58)
	Budgerigars	Australia	(55)
	Budgerigars	England	(29)
APMV-6	Domestic duck	Hong Kong	(71)
	Mallard	USA	(81)
	Domestic duck	Taiwan	(18)
	Mallard	New Zealand	(73)
	Serology in chicken	USA	(80)
APMV-7	Hunter-killed dove	USA	(7)
	Turkeys	USA	(63)
	Ostrich	USA	(82)
	Collard doves	USA, England	(27)
APMV-8	Canada goose	USA	(19)
	Canada goose	USA	(62)
	Serology in wild fowl	Spain	(51)
	Serology in mallards	New Zealand	(73)
	Wild ducks	USA	(72)
APMV-9	Domestic duck	USA	(8)
	Migratory waterfowl	Italy	(17)
APMV-10	Rockhopper penguins	Falkland Islands	(52)
	Magellanic penguins	Brazil	(23)
APMV-11	Common snipe	France	(14)
APMV-12	Eurasian wigeon	Italy	(75)
APMV-13	Goose	Japan	(85)
	White fronted goose	Kazakhstan	(37)
APMV-14	Duck fecal sample	Japan	(76)
APMV-15a	White-rumped sandpiper	Brazil	(77)
APMV-15b	Wild birds	South Korea	(46)
APMV-17	Wild birds	South Korea	(34)

[a] Underlined text highlights the first description in literature.

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or well-characterized APMV serotype-specific antisera usually obtained after repeated vaccination of specific pathogen free (SPF) chickens. The tests can be performed as recommended by the World Organisation for Animal Health (OIE) in chapter 2.3.14 of the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2011* for the determination of APMV-1 antigen or antibodies (60). Analysis of sera from species other than chicken or chickens with antibodies against a variety of pathogens might pose a problem for a clear diagnosis (80). It was also found that high-antibody titers against one APMV serotype might result in cross-reactivity with other serotypes (80). Based on HI and neuraminidase inhibition (NI), APMV 1–9 isolates were divided into two subgroups (48). The first subgroup contained APMV-2 and 6 and the second subgroup contained APMV 1, 3, 4, 7, 8, and 9. Alternative serological tests including the NI assay (32, 38, 58, 78), serum neutralization (57, 78), and agar gel diffusion tests (1, 8, 32, 40) have shown similar antigenic groups to the HI test. It has been suggested that with the availability of sequences for the APMV serotypes, new classification guidelines should be established that use genome sequence comparisons (52, 84).

Immunogenicity or Protective Characteristics

Avian paramyxoviruses of different serotypes show limited cross-reactivity, and little information is available if APMV isolates of the same serotype might differ (55). In the few published reports, high HI titers were observed in APMV-2 infected chickens, but APMV-4, 6, and 17 infections induced delayed and comparatively low HI titers (34, 81). In a different study chickens were infected with virus isolates belonging to APMV-1–9. The highest antibody titers were observed for APMV-1 and 3; moderate antibody titers were observed for APMV-2, 8, and 6; low antibody titers were observed for APMV-4, 7, and 9, and a very low antibody response was observed for APMV-5. The chickens in this study were challenged with a virulent APMV-1 strain with complete protection in birds previously infected with APMV-1 and 3, partial protection after infection with APMV-2, 7, 8, and 9, and no protection after infection with APMV-4, 5, and 6 (56).

Genetic or Molecular

The determination of full-length sequences for almost all APMV serotype viruses closes an important gap for understanding the biology of the genus *Avulavirus*. The complete genomes vary in length between the serotypes and within serotypes. But all of them fulfill the rule of six for the family Paramyxoviridae (16). The general genomic organization follows the order of proteins NP-M-P-F-HN-L (14, 33, 34, 46, 52, 65). The 3′ leader region was conserved with 55 nt in length. The very first nucleotides of the leader region were highly conserved and were a complement to the trailer sequence located at the 5′ end of the virus genome. Each mRNA encoding for the single protein contains 5′ and 3′ noncoding regions that vary in length between the proteins and between the viruses. In addition, the intergenic regions vary in length and likely function as regulatory regions for the transcription cascade. The presence of RNA editing sites within the region encoding for the P protein has been identified, which might result in mRNAs encoding for the V and W proteins in APMV 1–9 (65). One additional coding sequence for a small hydrophobic protein (SH protein) was identified for APMV-6 (84).

Laboratory Host Systems

The isolation of APMV has most commonly been with 9- to 11-day-old embryonated SPF chicken eggs using either the allantoic cavity (34, 46, 63), intra-amniotic route for APMV-5 (58), or the yolk sac route (29). However, Muscovy duck eggs also have been used for virus isolation (2).

Pathogenicity

Isolates of APMV can be grouped as lentogenic in chickens when using the Newcastle disease virus (NDV) scale for classification. Infection of SPF chickens with isolates from APMV serotypes 2–9 did not show any clinical signs (56). The intracerebral pathogencity index in 1-day-old chickens was that of a lentogenic NDV after inoculation with APMV-2 (74), APMV-3 (41), APMV-8 (54), APMV-10 (52), APMV-12 (75), APMV-13 (85), APMV-14 (76), and APMV 15 (46, 77). Infection of chickens at various ages with either APMV-2, 3, 4, 6, 8, or 17 did not induce any clinical signs (18, 34, 41, 54, 74, 81). Impaired growth of chickens was observed after intramuscular infection with an APMV-3 isolate (5). This indicates likely differences between virus isolates from the same APMV serotype. Infection of 4-week-old turkeys with two isolates of APMV-3 did not induce clinical signs (41). Experimental infection of chickens and pigeons with APMV-5 did not cause disease, but caused fatal enteritis in young budgerigars (29, 55). Virus isolates of APMV-6 serotype caused mild respiratory disease and a drop in egg production in turkeys (3). Interestingly, an APMV-7 was isolated from dead collared doves but whether the virus isolate contributed to the mortality was not proven (27). An APMV-7 isolate induced clinical signs and affected egg production in turkeys (63). After infection of ducks with an APMV-8 isolate from wild birds, no signs of disease were observed (54).

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Pathobiology and Epizootiology

Incidence and Distribution

Based on the reports APMV 2–15 and 17 were isolated from a variety of birds and are distributed worldwide. The natural source for APMV is likely wild birds from which the viruses are transmitted to domestic poultry. Table 3.3 describes where and from which species APMV 2–15 and 17 have been isolated. This is not complete since there are many more reports about isolations of the different APMV.

Natural and Experimental Hosts

The presence of APMVs in their natural hosts has been described above which is certainly just a snapshot of the natural presence of APMV in the wild. The presence of APMV 2–15 and 17 in wild birds suggests that this is their natural reservoir. Infection of chickens and turkeys as experimental hosts does not usually induce clinical signs (56). This might also be dependent on the virus isolate. Infection of mammals such as mice (39) and hamsters (66) did not induce clinical signs except for an APMV-9 isolate, which induced moderate clinical signs in the hamsters.

Transmission, Carriers, Vectors

The route of transmission for APMVs appears similar to that of AMPV-1 where the fecal–oral as well as the respiratory route are the primary routes of transmission. The role of carriers and vectors is unknown.

Incubation Period

Because almost no clinical disease is observed, the determination of an incubation period is difficult. After infection of young budgerigars with APMV-5, all diseased birds died within 14 days postinfection (58). A similar observation was described for another APMV-5 isolate after infection of young budgerigars (55).

Clinical Signs

Morbidity and Mortality

In general, data for experimental infection of naïve birds are rare. Most experimental infections in naïve chickens did not result in any clinical signs (52, 56, 81). The infection of African Cut-throat finches with an APMV-2 isolate resulted in a decrease in activity (26). Mild respiratory signs were observed after infection with APMV-3 in 1-day-old chickens and turkeys, while no clinical signs were observed in older birds (41). APMV-3 has been associated with encephalitis and high mortality in caged birds and with respiratory disease in turkeys (78). A severe drop in egg production has been observed in turkeys in which APMV-3 was isolated (10). It also has been reported that APMV-3 causes acute pancreatitis and central nervous system symptoms in Psittacine and Passerine birds (13). An uncommon but severe clinical disease (depression, dyspnea, diarrhea, torticollis, death) for APMV-5 in budgerigars has been reported from Japan and England (58). APMV-6 might cause a mild respiratory disease and problems in egg production in turkeys (3). The infection of turkeys with virus isolates which have been grouped with APMV-7 caused respiratory signs that were expressed as rhinitis and airsacculitis (63). For viruses that belong to APMV-4, 8, 9, 10, 13, 14, 15, and 17, no clinical disease has been described after infection.

Pathology

Gross Pathology

When turkeys become infected with APMV-2, sinusitis has been observed (45). Enlarged liver and spleen were observed in red-crowned parakeets where APMV-3 was isolated (35). Interestingly, postmortem examination of 2-week-old chickens and turkeys revealed enlargement of the pancreas with focal necrosis at five days after infection with APMV-3 (41). Budgerigars infected with APMV-5 showed hemorrhages in the proventriculus, duodenum, jejunum, and rectum, and sometimes discoloration of the liver and splenomegaly (55). Enlarged pancreas and airsacculitis was observed in turkeys after infection with an APMV-7 isolate (63). In doves in which an APMV-7 was isolated, enlarged and congested livers and spleens were observed (27).

Microscopic Pathology

Catarrhal tracheitis, mild enteritis, and gastrointestinal associated lymphoid tissue (GALT) hyperplasia were observed in chickens infected with APMV-2, 4, and 6 (81). Mild interstitial pneumonia and lymphocytic infiltrates in the pancreas were observed in chickens infected with APMV-4 and 6 (81). In parakeets infected with APMV-3, lymphocyte infiltration in the kidneys, moderate lymphoid cell infiltration in hepatic and pulmonary tissue was documented while other organs showed no microscopic changes (brain, inner ear, pancreas, spleen, and intestine), indicating a specific tissue tropism (35). The infection of budgerigars with APMV-5 resulted in extensive loss of mucosal epithelium, edema of the intestinal wall, and vascular engorgement (55). APMV-7 infection in turkeys caused a mild multifocal or nodular lymphocytic airsacculitis (63).

Pathogenesis of the Infectious Process

The pathogenesis of infection with APMV other than APMV-1 is not well investigated. Usually the infection is restricted to the respiratory tract and sometimes also to

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the digestive tract. With the exception for APMV-5 in budgerigars, where death upon infection was observed, no serious disease was described. It is assumed that there is transient self-limited replication immediately after infection. The cleavage of the F protein (F0 to F1 and F2) is a prerequisite for a subsequent infection of the deeper layer of tissues. Virulent APMV-1 contain a polybasic amino acid sequence between F1 and F2, and this allows the cleavage by endogenous proteases present in most tissues resulting in a systemic infection as well as efficient replication in cell culture. In contrast, some APMV serotypes (APMV-2, 4, 6, and 7) do not have such a polybasic amino acid sequence but they can still replicate efficiently in cell culture without presence of exogenic proteases (83). On the other hand they do not induce a systemic infection. The underlying molecular mechanism is unknown.

Immunity

Infection of domestic birds (chickens and turkeys) with APMV 2–10 and 17 resulted in a seroconversion of the birds (34, 52, 56, 81). The level of antibody response might vary between single isolates of the same APMV serotype and among different serotypes (56). Infection of chickens with APMV 2–9 induced protection from death after an NDV challenge infection only in APMV-3 infected chickens and limited protection in chickens infected with APMV-2, 7, 8, and 9 (56).

Diagnosis

Isolation and Identification of Causative Agent

Identifications of APMV 2–15 and 17 were performed mainly after inoculation of sample material in the allantoic fluid of embryonating SPF chicken eggs. The allantoic fluid was harvested and used in a hemagglutination assay using chicken RBCs. APMV-5 was isolated from the amniotic cavity of embryonating SPF eggs. Propagation of APMV-5 was best in cells obtained from embryonating chicken eggs (58). A passage via the yolk sac might help to isolate APMV-5 (29). The virus isolates can be identified by electron microscopy as paramyxoviruses. Furthermore, with the recent availability of complete sequences of the coding regions from all APMV serotypes, diagnostic tests such as reverse transcriptase polymerase chain reaction (RT-PCR) to perform specific serotype assays might become available.

Serology

Infection of birds in addition to mammals with APMV serotypes results in seroconversion (39, 52, 56). It needs to be mentioned that in the presence of high APMV-1 antibody titers cross-reactivity with APMV-2 serotype-specific antigen was observed (80). A similar phenotype was observed with APMV-17 where a low cross-reactivity was observed with APMV-9 (34). The serologic method of choice is the HI test, either with known APMV serotype-specific antigens or well-characterized antiserum. Seroconversion was observed between 7 and 14 days after infection regardless of species infected.

Differential Diagnosis

For differential diagnosis all viruses causing respiratory signs or macroscopic changes in the intestine in birds could be suspicious. Viruses such as low pathogenicity avian influenza virus, infectious bronchitis virus, infectious laryngotracheitis virus (ILTV), and avian metapneumovirus need to be excluded by either molecular assays or virus propagation and detection of the antigen by appropriate tests. The combination of molecular assays, classical methods such as virus isolation in the embryonating SPF chicken eggs combined with electron microscopy for distinguishing morphology, has the highest probability of success for excluding other viruses such as APMV-1.

Intervention Strategies

Management Procedures

High biosecurity levels on the farm certainly minimize the risk of introduction of APMV and other important diseases in the poultry house. For APMV the possibility that wild birds gain access to poultry houses is probably still the main source for virus introduction. Wild birds on poultry farms have been considered potentially important in the transmission of APMVs in part because they have been found to transfer NDV from farm to farm (30).

Vaccination

Types of Vaccine

Vaccines to prevent APMV-1 infections are well-known and broadly used. In contrast, the development of vaccines other than APMV-1-based vaccines is restricted to specific situations. Several APMV-3 vaccine candidates that could be used to vaccinate parakeets against the disease were described (13). Licensed oil-emulsion vaccines against APMV-3 infection and disease have been available for several years in Europe and the United States (15, 21). They have been used to prevent egg production losses associated with APMV-3 infections in laying turkeys. Autogenous vaccines for different APMV-serotypes have also occasionally been used in poultry. The long-term goal is the use of APMV viruses as potential live vaccine vectors for use in different species, including humans (39).

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Treatment

There are no reports of treatment of animals affected with APMV 2–15 and 17. The main tool to prevent infection, especially those that cause clinical disease of birds, is high biosecurity with a quarantine management program to prevent the introduction of any new infectious agents. In the case of APMV the quarantine should be a minimum of two weeks, which will reduce the chance of virus shedding in birds just infected at the time of purchase.

Avian Metapneumovirus

Silke Rautenschlein

Summary

Agent, Infection, and Disease. Avian metapneumovirus (AMPV) belongs in the genus *Metapneumovirus* of the family Paramyxoviridae. Different antigenic subtypes (A–D) have been described. Avian metapneumovirus may lead to respiratory disease and reproductive disorders in poultry. Wild birds are considered as natural reservoirs. Avian metapneumovirus-induced disease may result in direct economic losses but is often associated with secondary bacterial infections.

Diagnosis. Clinical signs and lesions do not allow a definitive diagnosis of AMPV-infection. Molecular tests are commonly used to identify infected flocks in the acute stage of the disease. Serological tests are also important tools, because the virus is cleared quickly from the respiratory tract.

Intervention. Biosecurity measures and vaccination in endemic regions are usually implemented to control the disease in poultry dense areas.

Introduction

Definition and Synonyms

The clinical diseases that may result from AMPV infections of turkeys or chickens have been termed turkey rhinotracheitis (TRT), avian pneumovirus infection of turkeys (APV), swollen head syndrome (SHS), and avian rhinotracheitis (ART). Although these clinical signs and lesions are not specific for AMPV infections, it is accepted that the conditions referred to as TRT, SHS, or ART can occur as a result of AMPV infection. The more severe form of associated disease probably results from dual or secondary infection with other organisms, and for SHS, the characteristic swollen head appears as a result of coinfection with secondary adventitious bacteria, usually *Escherichia coli* (75).

Economic Significance

Avian metapneumovirus infections of poultry are associated with economic and animal welfare problems. Despite vaccination approaches AMPV is now one of the main respiratory agents in turkeys in turkey dense regions. Decreases in egg production may often be the only clinical sign in layers and breeders. In broilers AMPV infections are often associated with SHS (reviewed in 75). Avian metapneumovirus subtype C (AMPV/C) was first isolated during 1996 in Colorado, and caused serious economic problems for many years mainly in the Midwest (reviewed in 75). Currently AMPV is observed only sporadically in the United States, mainly in breeders associated with a drop in egg production, and therefore no intervention measures are taken. Avian metapneumovirus subtype C of a different genetic linage have emerged recently in Europe and Asia (127), where they may lead to economic losses due to egg drop and/or respiratory symptoms mainly in ducks but also chickens and pheasants (89, 123, 134).

Public Health Significance

Avian metapneumovirus subtype C has a close genetic and antigenic relatedness to human metapneumovirus (HMPV) (130). In one experimental study turkeys were successfully infected with different HMPV strains and showed transient respiratory disease, but a different study did not lead to a productive infection in birds (reviewed in 75). So far there are no reports of human disease from AMPV. One study of human adults showed that occupational exposure to turkeys may increase the odds of infection with AMPV compared to nonexposed controls (83).

History

Avian metapneumovirus was first described in turkeys in South Africa in the late 1970s (21), and was later detected in many countries all over world (reviewed in 75). The infectious agent was initially placed in the genus *Pneumovirus* (reviewed in 75). In the late 1980s a disease

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was described in chickens, which was associated with the same infectious agent (23). It became later known as SHS as it consisted of upper respiratory tract signs followed by a small number of flocks exhibiting swollen heads (4).

During the early 1990s attenuated vaccines were developed. In 1994 it was shown that two subtypes of AMPV existed, termed A and B. In 1996 AMPV was first described in turkeys in the United States (113). The Colorado outbreak was eradicated, but later the virus was also detected in turkey dense Minnesota and neighboring states (16) in turkeys and a variety of wild bird species but not in chickens (15, 128). The virus was shown to be antigenically distinct from subtypes A and B (112) and was referred to as subtype C AMPVs.

Viruses similar to the United States AMPV/C, but of a different genetic lineage, also have been reported to occur in diseased ducks, and chickens in France and China (123, 125, 134) as well as in pheasants in Korea (89). A recent study detected this subtype C lineage in water-associated wild birds in the Netherlands (129).

A retrospective molecular analysis of viruses isolated from turkeys in France in the 1980s indicated the presence of a fourth subtype of AMPV, designated subtype D (14, 126), which was not reported again after 1985.

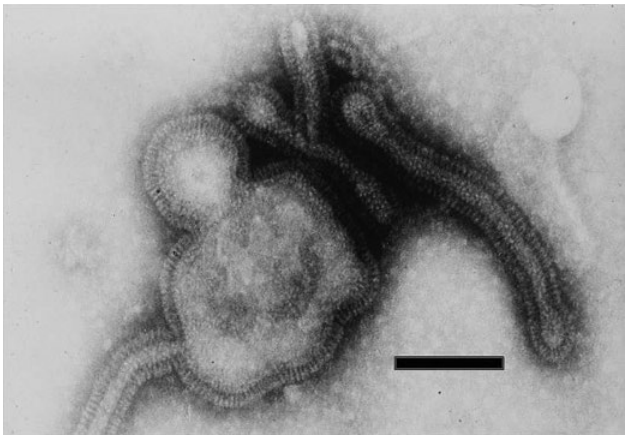


Figure 3.4 AMPV particles. Bar = 100 nm. Reproduced with permission (96).

Etiology

Classification

Avian metapneumoviruses are members of the family Pneumoviridae. Two genera belong to this family consisting of *Orthopneumovirus* and *Metapneumovirus*, in which the species avian (a) and human (h) metapneumoviruses are placed (8). Avian metapneumoviruses have been classified into four subtypes (A, B, C, and D) based on nucleotide and deduced amino acid sequence data (45). While AMPV/A, B, and D group together more closely (19), a close genetic relationship between HMPV subgroup A and AMPV/C was proposed by comparing sequence identity, genomic organization, codon usage bias, and phylogenetic location (19, 68, 130). Two genetic sublineages of AMPV/C have been defined, one from Europe and Asia (125, 134) and in the United States (19, 116). It was suggested that the most recent common ancestor of HMPV and AMPV existed around 200 years ago (50). While recombination events between lineages may contribute to the diversity of HMPV strains (85), there is only limited information on genetic diversity of AMPV (19, 116, 125).

Morphology

Negative contrast electron microscopy of AMPV reveals pleomorphic fringed particles, usually roughly spherical, 80–200 nm in diameter, although occasionally round particles with diameters of 500 nm or more can be seen (Figure 3.4). Fringed filamentous forms of 80–100 nm in diameter and up to 1,000 nm long may also be present, particularly in preparations from organ cultures. Surface projections were reported to be 13–14 nm in length and the helical nucleocapsid to be 14 nm in diameter (reviewed in 75).

Chemical Composition

The virus genome is nonsegmented and composed of single-stranded negative-sense RNA of approximately 14 kilobases. Genome length of AMPV/C viruses may vary mostly due to the different length of their G genes (19). In sucrose gradients the buoyant density of an isolate from turkeys was 1.21 g/mL with an approximate molecular weight of 500×10^6. The organization of the genes encoding structural and nonstructural proteins is presented in Figure 3.5. The F protein, which is a type I membrane protein formed by a homotrimer, is synthesized as a precursor (F0) and was shown to be highly conserved, at least in the region involved in the fusion, folding, and homotrimerization (19, 62). The G protein is a heavily glycosylated type II membrane protein with higher heterogeneity in length and sequence identity even within AMPV subtypes (27). Further details regarding protein structures are found in Brown et al. (19).

Virus Replication

Few detailed studies on AMPV replication have been published and mechanisms are suggested to be similar to the HMPV (reviewed by [56]). Avian metapneumovirus attaches through the G protein to the cell surface receptor, which has not been clearly identified for AMPV (19, 56). The AMPV/B F proteins contain a conserved Arg-Asp-Asp (RDD) motif, suggesting that they may

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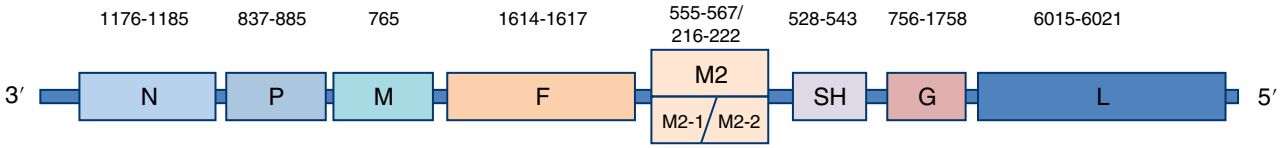


Figure 3.5 Schematic presentation of the genomic organization of AMPV. The genes encode the following proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), second matrix proteins (M2-1 and M2-2), small hydrophobic protein (SH), surface glycoprotein (G), and a large, viral RNA-dependent RNA polymerase (L). The genome is flanked by a leader and trailer at the 3′ and 5′ ends, respectively. The number of nucleotides is indicated on top of the coding regions (23).

mediate membrane fusion via the binding of RDD to integrin (141). Avian metapneumovirus may undergo direct fusion in neutral pH conditions mediated by the refolded F protein with the host cell plasma membrane without the G protein (107). A glycine at position 294 in the AMPV F proteins seems to play a critical role in F protein-mediated fusion under low pH conditions (139). Residue 149 contributed to the hyperfusogenic activity of the F protein of certain AMPV/B strains (138). Avian metapneumovirus replication is trypsin independent *in vitro* but medium supplementation with trypsin may enhance virus replication rates (107, 139). Trypsin enhanced AMPV/C F protein fusogenicity was regulated by the amino acid residues at positions 100 and 101. The transmembrane serine protease TMPRSS12 is suggested to facilitate the cleavage of the AMPV/B F protein (140). The viral genome uncoats in the cytoplasm and serves as a matrix for viral transcription and replication starting at the 3′ leader sequence (51, 55). Because of polymerase dissociation, genes are transcribed and expressed at reduced levels ongoing from the leader to trailer of the genome. The development of a reverse-genetics system for AMPV/A and B (88, 100) has confirmed that the minimum replicative unit is the ribonuclear complex. The SH protein can be deleted in AMPV/A to leave viable but slower growing viruses and an altered syncytial phenotype (93, 100). Replication of G- and M2-2-gene deleted viruses was more severely impaired in turkeys than in cell culture (67, 93). Variation in the length of the G gene among type C AMPVs suggests that a large portion of the extracellular domain (333 aa) is not essential for virus viability but may play a role in enhancing virus attachment specificity in the natural host (137).

A single polymerase (L) mutation in an AMPV/B enhanced the virulence and virus viability at an elevated temperature, indicating that increased viral virulence may be related to better replication in tissues away from the cool respiratory tract (20). Further details on AMPV replication have been described by Easton et al. (51).

Susceptibility to Chemical and Physical Agents

Avian metapneumovirus was reported to be sensitive to lipid solvents, stable at pH 3.0 to 9.0, and inactivated at 56 °C after 30 minutes. An AMPV/C lost viability by 12 weeks at 4 °C, 4 weeks at 20 °C, 2 days at 37 °C, and 6 hours at 50 °C. Several disinfectants were effective in reducing the viability (reviewed in 75).

Avian metapneumovirus remained viable after 7 days of drying at room temperature. An AMPV/C isolate survived for up to 60 days at a temperature between -12 °C and 8 °C in built-up turkey litter, and viral RNA was still detectable in the litter kept at 8 °C after 90 days (as reviewed in 75).

Strain Classification

Early studies using antibody-based test systems and polypeptide profiling demonstrated considerable antigenic differences between strains (e.g., 41). Subtype C United States isolates were shown to have no significant serological relationship with subtype A and B strains from Europe (48).

Phylogenetic comparison of the four subtypes has shown that A, B, and D viruses are more closely related to each other than to subtype C (7, 14, 126), which is in turn more closely related to the HMPV than the other three. Sequence analyses among American subtype C viruses indicated 89–94% nucleotide sequence identity within the N, P, M, F, and M2 protein genes. Further confirmation of subtype differences was obtained following nucleotide sequence analysis of the G glycoprotein (44, 82). The G gene sequences of different subtypes also may vary in size and number of nucleotides (131). Comparison of American AMPV/Cs with European subtype A and B showed between 41% and 77% nucleotide sequence identity (112, 114). The subtype C lineages of AMPV, which were isolated in Europe, Asia, and the Middle East, share an overall nucleotide identity of 92.3–94.3% (89, 123, 127).

Laboratory Host Systems

Inoculation of infective mucus of different subtypes into the yolk sac of turkey or chicken embryos resulted in embryo mortality after 4 or 5 passages, but virus remained at a low titer (5). Inoculation of turkey or chicken tracheal organ cultures (TOCs) with subtype A and B viruses but not subtype C resulted in ciliostasis, but again, viruses only replicated to low titers (reviewed by [75]). The fusion protein of AMPV/C was shown in

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in vitro studies to be an important determinant of host tropism (49). Isolates adapted to embryos or TOCs, replicated also in cultures of primary chicken and turkey embryo cells, mammalian cells such as Vero, BS-C-1, and MA104 cells with a characteristic cytopathic effect of syncytium formation and relatively high virus titers (86, 87). A continuous quail tumor cell line (QT-35) and an immortal chicken embryo liver-derived cell line were also used to propagate the virus (69, 90). In some studies, serial passages in Vero cells led to truncation of the G gene, while in others up to 50 passages did not affect its length (38).

Pathogenicity

Despite the high morbidity and occasionally increased mortality rate associated with AMPV in the field, the pathogenicity of AMPV isolates has been difficult to assess in the laboratory, and varies between strains. Experimentally, infected birds often show recognizable signs of rhinotracheitis, but these are milder than those seen in the field (reviewed in 75). Chickens show, at most, mild respiratory disease in laboratory infections (Figure 3.6) and nasal mucus may often only be discernible after light squeezing behind the nostrils. Transmission and infection studies in 2- or 3-week-old broiler chicks with AMPV-turkey isolates of subtype A, B, and C induced clinical signs of coughing and sneezing for up to 8 days postinoculation (PI) (reviewed by 75). An isolate of AMPV from chickens with SHS as well as wild bird isolates of subtype C induced rhinotracheitis in turkey poults (34, 75).

Field investigations support the presence of multiple infectious agents contributing to AMPV infection and disease (74, 118), and a longitudinal study suggested a direct correlation between field AMPV infection and colibacillosis-associated mortality (63). Therefore, the difference in pathogenicity between laboratory and field infections is related to the conditions under which the birds are kept and the presence or absence of exacerbative organisms (2). In laboratory studies concurrent infection of turkey poults with AMPV and respiratory bacteria, such as *E coli, Bordetella avium, Mycoplasma gallisepticum, Riemerella anatipestifer*, *Ornithobacterium rhinotracheale*, and lentogenic NDV, significantly exacerbated the disease. Turkey poults preinfected with *Chlamydia psittaci* showed a significant increase in the severity of subsequent AMPV infection (reviewed by 75).



Figure 3.6 Avian metapneumovirus (AMPV) infection in a chicken. Experimental infection leads to foaming eyes and nasal discharge. *Source:* S. Rautenschlein.

Pathobiology and Epizootiology

Incidence and Distribution

In most countries where AMPV has appeared as a new disease, it has spread rapidly. It is distributed all over the world and different subtypes have been detected in different countries in the world (as reviewed by [1, 3, 75, 109]). The incidence in many countries correlates with the density of poultry, and in Europe it is one of the main respiratory agents in turkeys. Layers and breeders are also infected but mostly subclinically. While North America had been free of AMPV/A and B, for some years subtype C has led to economic losses in the Midwest, predominantly in Minnesota (113), but currently, the incidence is low.

During recent years, a different sublineage of AMPV/C was detected in other parts of the world, predominately in ducks, but other poultry species may also be affected (123, 134).

Natural and Experimental Hosts

Turkeys and chickens, apparently of any age, are known natural hosts of AMPV. In field as well as experimental infections with AMPV/A or B, susceptibility with clinical signs was demonstrated in turkeys, chickens, and pheasants (64, 65). Serological studies suggest that these subtypes are widespread in game birds and also appear in farmed ostriches and in seagulls. It was possible to reproduce rhinotracheitis-like disease in guinea fowl (*Numida meleagris*) with virus isolated from AMPV-affected turkeys (reviewed by [75]).

Avian metapneumovirus subtype C was detected by RT-PCR and occasionally virus isolation in nasal turbinates of clinically healthy sparrows, ducks, geese, swallows, gulls, and starlings sampled in the north central region of the United States (as reviewed in 75). The presence of AMPV/C was also confirmed in further wild bird species in other regions of the United States suggesting that wild birds can be a reservoir for AMPV, even in regions where diseases in poultry have not been reported (128). Outside the United States, subtype C-like viruses have also been reported to occur in com-

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mercial Muscovy ducks, pheasants, and chickens and have been associated with respiratory signs and egg production problems (89, 125, 134).

Subtypes A and B related to chicken isolates have been detected in ducks and pigeons. Pigeons and sparrows may also only act as mechanical vectors. Experimentally, pigeons, geese, and ducks appeared to be refractory to infection with the virus (reviewed in 75).

In transmission studies AMPV/C from the United States was detected for up to 14 days in mice and 6 days in rats where there was seroconversion but no clinical disease (98). A virus from the Asian AMPV/C lineage led to lung associated pathological lesions in BALB/c mice after experimental infection (133).

Transmission, Carriers, Vectors

Migratory birds are considered a natural reservoir of infection, which may explain the seasonal pattern of introduction into poultry as seen in some studies (35, 114). However, movement of viruses through migratory birds does not explain how closely related strains have been detected from China, Nigeria, the United Kingdom, and Brazil where natural intercontinental spread with wild birds seems unlikely (103). There has been no apparent spread of subtype A and B viruses from South and Central America to the United States, and no evidence of subtype C viruses spreading south from the United States, although, Minnesota, which was the epicenter of AMPV/C infection in the United States, lies in major wild fowl flyways which reaches from Canada to Central and South America.

Farm proximity (78), particularly for turkeys (76), as well as multiple age settings (1) have been discussed as important prerequisites for virus transmission. Even on a single site, spread is unpredictable. Contaminated water and movement of affected or recovered poults, of personnel and equipment, feed trucks, and so on, have all been implicated in outbreaks. It was suggested that chickens could play a role in the maintenance and circulation of AMPV in a region with chicken and turkey production (31). At present, only contact spread has been confirmed. The virus was transmissible from infected to susceptible turkey poults placed in direct contact, but transmission failed if susceptible birds were housed in the same room but in a different pen (42). Still, airborne spread cannot be fully excluded under certain conditions. There is no published evidence that AMPV can be vertically transmitted even though high levels of virus can be detected in the reproductive tract of laying birds (81). Inoculation with mucus, nasal washings, or other materials from the respiratory tract of affected birds established AMPV infection (5, 97).

To date there is no experimental evidence of long-term AMPV-persistence in chickens or turkeys. Although, both vaccine as well as field strains may cocirculate in poultry dense regions (94, 95).

Incubation Period

After experimental infection the incubation period is short. The clinical signs appear between three and five days postinoculation (PI) (9, 46, 92).

Clinical Signs

The clinical disease in turkeys has been described in detail before (44, 75, 99). Clinical signs in young poults include snicking, rales, sneezing, nasal discharge, foamy conjunctivitis, swollen infraorbital sinus, and submandibular edema. Coughing and head shaking are frequently observed, particularly in older poults. In laying birds there may be a drop in egg production of up to 70% with an increased incidence of poor shell quality and peritonitis (81). Coughing associated with lower respiratory tract involvement may lead to prolapses of the uterus in breeding turkeys. When disease is seen, morbidity in birds of all ages is usually described as up to 100%. Flock mortality ranges from 0.4% to 50%, particularly in fully susceptible young poults. In uncomplicated infections recovery usually occurs between 10 and 14 days. Morbidity and mortality is often influenced by coinfecting pathogens.

Infection with AMPV in chickens is less clearly defined and may not always be associated with clinical signs (44, 77). The virus has been associated with SHS, which is characterized by swelling of the periorbital and infraorbital sinuses (9), torticollis, disorientation, and opisthotonos, probably due to virus effects on the ear. Usually less than 4% of the flock is affected, although widespread respiratory signs are usually present. Mortality rarely exceeds 2% and in broiler breeders egg production is frequently affected. In commercial layers AMPV infection may also affect egg quality (44, 77). Layers may be infected before the onset of lay and without significant respiratory symptoms (30). In laboratory studies the intravenous route of infection had a significant effect on the severity of clinical signs and egg production compared to the oculonasal route, in which egg production remained normal (46, 72).

Avian metapneumovirus was detected with infectious bronchitis virus in the testes of cockerels in a flock with reduced fertility (132) but the role of AMPV in this condition needs further investigation. Infection with AMPV/C led to upper respiratory symptoms and reduction of egg production by approximately 40–85% in sick Muscovy ducks. The eggs of affected birds were soft, thin-shelled, or cracked (123).

Pathology

Gross

Species-related differences may occur in the onset and severity of lesion development. Following infection of laying turkeys a watery to mucoid exudate was found in

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the turbinates 1–9 days PI, with excess mucus in the trachea (81). Various reproductive tract abnormalities were also reported, including egg peritonitis, folded shell membranes in the oviduct, misshapen eggs, ovary and oviduct regression, and inspissated albumin and solid yolk. Similar observations were also made in AMPV/C-infected Muscovy ducks (123). During natural field outbreaks, exacerbated by secondary pathogens, a variety of other gross lesions have been described (44, 77, 113).

In AMPV-infected chickens the only significant lesions noted are those associated with SHS in broilers or broiler breeders. The major gross lesions include extensive yellow gelatinous to purulent edema in the subcutaneous tissues of the head, neck, and wattles. Varying degrees of swelling of the infraorbital sinuses also may be seen (77, 97).

Similar gross lesions have been reported to occur in commercially raised pheasant poults (102).

Microscopic

Detailed histological studies have been performed in experimentally inoculated turkey poults and chickens (reviewed by 75). Experimental infection of susceptible 5-week-old turkeys with a European AMPV isolate resulted in complete deciliation of the trachea by 96 hours PI (79). Increased glandular activity, focal loss of cilia, hyperemia, and mild mononuclear infiltration of the submucosa is found in the turbinates at one to two days PI. Between three and six days PI the peak of damage to the epithelial layer and a copious mononuclear inflammatory infiltration in the submucosa is observed. Some transient lesions may also be seen in the trachea or primary bronchi. The lesions in chickens may be milder and of shorter duration than those observed in turkeys (reviewed by 75).

Ultrastructural

After experimental infection of chickens, AMPV-antigen was observed in the cytoplasm and associated with cilia of the turbinate epithelial cells. Virus particles were observed in ciliated as well as non-ciliated epithelial cells. Infection was associated with cytoplasmic blebs, clumping, and loss of cilia in the apical cell membrane. At five days PI, substitution of ciliated and non-ciliated epithelial cells was observed and many desquamated epithelial cells were detected within the lumina. By day seven PI, regenerative changes were visible in the ciliated epithelium (96).

Pathogenesis of the Infectious Process

Avian metapneumovirus targets the epithelial cells of the respiratory tract as well as the reproductive tract (reviewed in 75). Virus replication induces ciliostasis and loss of cilia (71), allowing secondary pathogens to invade the host. Some AMPV strains also may have transient immunosuppressive abilities, supporting further secondary infections and reducing vaccine responses (36, 37). *In vitro* studies suggest that AMPV may interfere with the antiviral immune response (71). In chicken tracheal organ cultures (TOC) AMPV/A induced an increased apoptosis rate as well as upregulation of nitric oxide release (71). Viral genome may not only be detected in tissue of the respiratory tract such as nasal turbinates, Harderian gland, trachea, and lung, but also in spleen and bursa cloacalis. These tissues may facilitate AMPV/B infection in chickens via cleavage of the F protein by the type II transmembrane serine protease TMPRSS12 (140). Macrophages may distribute AMPV from the site of replication in the respiratory tract to other peripheral tissues. The clearance of viral infection coincides with the induction of AMPV-antibodies and the waning of respiratory signs in the case of AMPV-mono-infections (92, 115, 117).

Immunity

A review on immunological aspects in AMPV infection is given by Smialek et al. (121).

Active Immunity

Cell-Mediated Immunity (CMI). The CMI response is suggested to provide the main resistance to AMPV infection (84, 110). Chemically bursectomized poults given a live attenuated AMPV vaccine when they were one day old were unable to seroconvert but were still resistant to virulent AMPV-challenge (80). Chemically T cell-compromised turkeys showed a slower recovery from clinical signs and histological lesions, as well as an extended virus shedding compared to T cell-intact birds (110). T cells may locally infiltrate the Harderian gland and possibly control AMPV replication at the site of virus entry (92, 108).

Vaccination of maternally derived antibody (MDA)-negative turkeys may result in strong stimulation of CD8(+) T lymphocytes in the Harderian gland and tracheal mucosa. Vaccination of MDA+ birds stimulated mainly CD4(+) T cells locally (119).

Humoral Immunity. Humoral immunity cannot be considered as an indicator of protection against AMPV infection, but specific antibodies inhibit AMPV replication and alleviate the course of the disease. Many authors have described the humoral immune response (reviewed by 99). Following infection of turkeys, AMPV antibodies were detected as early as 7 days PI in serum but not in tracheal washes (119). Antibodies were maintained for up to 89 days, when the trial was terminated (81). The peak of virus neutralizing (VN) antibody levels in tracheal washes and tears was detected around 10–14 days PI, which

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coincided with the clearance of detectable AMPV, and then VN titers declined (92, 108). Accumulation of different B cell populations locally in the nasal turbinates, and the release of IgA into nasal secretions were reported (32). Increased levels of AMPV-specific IgA were also detected in bile, lacrimal fluid, and tracheal washes (60). Antibodies protect the oviduct in the mature hen (72). Two regions (211–310 and 336–479) of the F protein are recognized by VN antibodies, which are highly conserved between AMPV/A, B, D but less with AMPV/C (17). High MDA may disturb antigen specificity acquisition of IgA+ B lymphocytes as well as production of IgA in the upper respiratory tract (120).

Passive Immunity

Hens with circulating AMPV antibodies pass these to their progeny via the egg yolk. The presence of high levels of MDA in 1-day-old turkey poults did not prevent clinical disease following challenge with virulent AMPV (101), but it may interfere with vaccine-virus replication and induced immunity (119). High levels of experimentally, passively transferred AMPV-antibodies, which were found in the circulation as well as locally in tracheal fluid, did not protect against homologous challenge with virulent AMPV (111).

Diagnosis

Isolation and Identification of AMPV

Choice and Timing of Samples for Isolation

Although virus has been isolated from trachea, lung, and viscera including ovary and uterus of affected birds (123), ocular and nasal secretions, choanal swabs, or tissue scraped from the sinuses or turbinates were shown to be better sources for virus detection (9). It is extremely important to obtain samples as early as possible after infection because virus may only be present in the sinuses and turbinates for 6–7 days (9, 45, 64). Isolation of virus is rarely successful from birds showing severe signs; presumably the extreme signs are a result of secondary bacterial infections in birds predisposed by earlier AMPV infection. This probably accounts for the difficulties in isolating virus from chickens, especially with SHS, because the characteristic signs appear to be due to secondary *E. coli* infection.

Due to the labile nature of the virus it is essential that samples for attempted virus isolation are sent immediately to the laboratory on ice (45, 75). Where delays are unavoidable samples should be frozen at –50 °C to –70 °C or on dry ice. FTA cards were shown to be suitable for collecting and transporting AMPV-positive samples including smears and homogenate supernatant of respiratory tissue for molecular detection and characterization of viral RNA (10).

Virus Isolation

The various methods used for the primary isolation of AMPV have been published elsewhere (45, 75). What is apparent from the European and more recently, the American experience, is that multiple approaches to diagnosis should be used to maximize the chances of successfully isolating the virus.

Tracheal Organ Cultures. TOC from turkey or chicken embryos can be used for virus isolation. Following inoculation with samples they are observed for ciliostasis, which may take several passages before a consistent effect is observed (45). Mixed primary culture of lung and trachea has also been used for virus isolation (109). Tracheal organ cultures were found to be unsuitable for the isolation of subtype C viruses because the isolates did not cause ciliostasis (48).

Culture in Embryonating Eggs. Six- to eight-day-old embryonating turkey and chicken eggs as well as eleven-day-old duck eggs from AMPV-negative flocks have been used to isolate the virus following inoculation by the yolk sac route. Usually, serial passage is required before the agent causes consistent growth retardation and embryo mortality. This technique was used to isolate the original AMPV strain in South Africa in 1980 and the original subtype C AMPV strains from the United States or more recently from China (22, 104, 123).

Cell Cultures. Once adapted to growth in embryonating eggs and TOC the virus can be cultivated to high titers in a range of avian and mammalian cells. Occasionally, chicken embryo cells, QT-35, and Vero cells have been used successfully for primary isolation (69, 123). Where positive results were obtained multiple blind passages were required before the virus produced a consistent cytopathic effect (CPE). This CPE is characterized by formation of syncytia within seven days. A direct plaque assay was developed for the quantitation and evaluation of the biological properties of AMPV/C in Vero and rhesus monkey kidney cells (LLC-MK2) (142).

Virus Identification

The isolated virus shows a paramyxovirus-like morphology when examined by negative contrast electron microscopy. The physiochemical properties can be investigated to aid identification (45, 66). Strains have previously been distinguished using monoclonal antibodies (mAb) but molecular methods are now almost universally used (45).

Direct Detection of Viral Antigens

Polyclonal and monoclonal antibodies have been used to detect AMPV antigen (71). Antibodies reactive to a conserved region in the AMPV nucleoprotein (N) were

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shown to cross-react even with the HMPV N protein (6). The presence of AMPV antigen was detected in both fixed and unfixed tissues and smears using mostly immunoperoxidase (IP) and immunofluorescence (IF) staining (as reviewed by 75).

Molecular Identification

A variety of RT-PCR techniques have been developed and evaluated and these have been extensively described elsewhere (26, 29, 57, 75). Molecular techniques are fast, sensitive, and may detect an equivalent to approximately 0.5 infectious doses (57). Internal positive or negative controls may be included (53). An important consideration when using PCR methods is whether to use subtype-specific or generic-type RT-PCRs designed to detect several AMPV subtypes (129). Reverse-transcriptase PCR methods using primers targeted to the F, M, SH, and G genes have been developed but are limited in specificity and may not detect all subtypes. Primers used in RT-PCR methods directed to the conserved region of the N gene have been described with the ability to detect representative AMPV isolates of subtypes A, B, C, and D (reviewed in 75). Commercial quantitative RT-PCRs (qRT-PCRs) are available to detect all four subtypes (18). Positive products may be further analyzed to determine the subtype using subtype-specific RT-PCRs or by sequencing and restricting fragment length analysis, which may help to distinguish, for example, AMPV/B field and VCO3 vaccine strain, which contains a unique Tru9I site (94).

Serology

The ELISA is the most commonly employed serological method (reviewed in 75). A variety of commercial and in-house kits have been developed for detecting AMPV antibodies. It was shown that vaccinal antibodies may not be detected if heterologous strains of AMPV are used to prepare the coating antigen for the ELISA plates (52). Some competitive ELISA kits incorporate a specific AMPV mAb that facilitates the testing of sera from different avian species. Studies using ELISA kits incorporating subtype A or B antigens were found to be relatively insensitive for detecting antibodies to the Colorado strain of AMPV (48). More recently ELISAs have been designed which incorporate whole virus antigens prepared from United States-isolates of AMPV. More sensitive and specific ELISAs using M and N protein-expressed antigens in sandwich-capture ELISAs for detecting subtype C antibodies were developed (reviewed in 75). The use of antibodies from egg yolk in laying hens has been described (39).

Antibodies to AMPV can also be detected by standard VN techniques (66). Cross-reactivity occurs with subtype A and B viruses and good correlation with ELISA and indirect immunofluorescence test has been reported (reviewed in 75). Both acute and convalescent sera should be submitted for analysis. The sera should be heat treated at 56°C for 30 minutes to remove nonspecific inhibitors and if delays in testing are unavoidable stored at –20°C.

Differential Diagnosis

Strain Variability

Subtype A and B viruses were originally differentiated on the basis of nucleotide sequence analysis of the G protein gene and mAb analysis, although they belong to the same serotype. Subtype C is different both in sequence and antigenically (reviewed in 75). Sequence analysis is the most definitive method to differentiate subtypes. With the detection of subtype C and D, it remains to be seen whether further subtypes of AMPV will be identified. It is possible that most current RT-PCR methods may fail to detect the presence of "new" AMPV subtypes. A multidiagnostic approach is required to detect further subtypes of AMPV (45).

Other Viruses

Paramyxoviruses, particularly NDV and APMV-3, infectious bronchitis, and influenza viruses may cause respiratory disease and egg production problems in chickens and turkeys that closely resemble AMPV infection. Paramyxoviruses and some avian influenza virions are similar in morphology but can be easily distinguished from AMPV because they possess hemagglutinin and neuraminidase activity. Infectious bronchitis virus can be differentiated from AMPV by morphological and molecular characteristics (reviewed by 75).

Bacteria including Mycoplasmas

A wide range of bacteria including mycoplasma species can cause disease signs very similar to those of an AMPV infection. These organisms often act as secondary opportunistic pathogens following AMPV infection and may cause considerable diagnostic problems. Only by isolating or identifying AMPV in the affected birds can a clear distinction be made (reviewed in 75).

Intervention Strategies

Management Procedures

Poor management practices such as inadequate ventilation and temperature control, high stocking densities, poor litter quality and general hygiene, multi-age stock, and the presence of secondary pathogens can all exacerbate AMPV infection. Debeaking or vaccinating flocks at a critical time may influence the severity of clinical signs (reviewed by 75). Good biosecurity is essential in preventing the introduction and spread of AMPV onto

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poultry farms from wild birds or infected poultry on nearby farms. Disinfection of equipment and feed trucks should be routine as well as changing clothes and boots and good hygiene for delivery and catching crews (75). The only known example of AMPV eradication from an affected area was in the state of Colorado, United States, when AMPV/C first emerged. By the application of strict biosecurity, stamping out, and good management practices, the infection was eradicated from the entire state.

Vaccination

Types of Vaccines

Both live attenuated and inactivated AMPV vaccines are available commercially for use in turkeys and chickens. The successful attenuation of AMPV strains and their effective use as vaccines have been described (75). Live attenuated vaccines have been shown to stimulate both systemic immunity and local immunity in the respiratory tract. In turkeys and particularly chickens, the humoral antibody response is poor following primary live vaccination but birds may still be protected against challenge via CMI in the respiratory tract (reviewed by 75). To produce complete protection in adult birds under field situations oil-adjuvanted inactivated AMPV vaccines are administered to birds previously primed with live vaccines.

Further experimental developments in AMPV vaccination strategies are ongoing (33, 105) including *in ovo* delivery of AMPV vaccines (135) and the development of recombinant, virosome, subunit, and DNA vaccines (54, 73, 91, 124, 136). The reverse genetics system for AMPV allows the development of mutated viruses, which may lead to improved vaccines (40, 122). Immunization with AMPV with incorporated chicken Fc into its virions led to higher levels of antibodies and better protection than the unmodified AMPV (106).

Field Vaccination Protocols and Regimes

Live vaccines are normally given by spray or in the drinking water and a recent study showed that with accurate application, both give similar immunity equivalent to that induced by eye drop (59). In meat-type birds AMPV vaccination is focused on reducing the economic losses due to weight loss and general bad performance of the flocks. Different from turkeys, only a low percentage of broiler flocks are immunized, mainly in the poultry dense regions in Europe. Brown layers are more frequently vaccinated against subtypes A and B in the field than white layers. In the United States biosecurity measures are currently emphasized in the control of AMPV in turkey flocks, and less vaccination (targeted only to AMPV/C) due to the low incidence of detectable AMPV cases. Good cross-protection occurs following vaccination with subtype A and B vaccines (43). Although full protection is offered by vaccines under experimental conditions (61), AMPV-associated diseases have been seen in AMPV-vaccinated turkey and chicken farms (12, 13). Only a vaccination program which included two live and one killed vaccines gave complete protection from AMPV infection to the birds, while a single live vaccine application was not efficacious (30). Live vaccination may have to be repeated at least two to three times, especially in male turkeys. Vaccination reactions may occur due to the presence of secondary pathogens. Breeding stock receives an additional inactivated vaccine at 16–20 weeks with the emphasis on protecting the oviduct. The use of a live infectious bronchitis (IB) and NDV vaccines may interfere with the replication of live AMPV vaccines, resulting in a reduction in the AMPV-antibody response but with no apparent adverse effect on the induction of protective immunity (11, 47).

Extended field persistence of a live AMPV vaccine has been described (24). In addition field strains show evolution mechanisms to circumvent protective immunity in vaccinated birds (25, 27). After the use of live vaccines reversion to virulence has been demonstrated in some studies (28), suggesting unstable attenuation of the vaccine virus or the selection of virulent subpopulations (58).

Treatment

There is no available treatment for AMPV infections. Success in reducing the severity of the disease may be achieved by controlling secondary adventitious bacteria with antibiotics (70).

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4

Infectious Bronchitis

Mark W. Jackwood and Sjaak de Wit

Summary

Agent, Infection, and Disease. Infectious bronchitis is caused by the avian coronavirus, infectious bronchitis virus (IBV), which is found worldwide. Infections, depending on the strain, may cause an acute upper-respiratory tract disease, drops in egg production, decreased egg quality, and nephritis. The virus is transmitted by inhalation or direct contact with contaminated objects and morbidity is usually 100%; whereas, mortality can vary depending on a variety of host factors and the strain of the infecting virus. Chickens are the primary host but the virus has also been found in pheasants and peafowl. There exist many different antigenic types of the virus and due to a high rate of mutation, new antigenic variants constantly emerge. As a general rule, different antigenic types and variants have little to no cross-protection.

Diagnosis. The preferred diagnostic test is molecular detection of the viral spike (S1) gene or virus isolation in embryonating chicken eggs. Multiple antigenic types of the virus are identified by sequence analysis of the S1 gene or by the virus neutralization test using serotype-specific antibodies. The disease can also be diagnosed by demonstrating rising antibody titers against IBV between preclinical and convalescent sera.

Intervention. Attenuated live and killed vaccines are used in an attempt to control the disease. However, multiple different antigenic types and constantly emerging new types that do not cross-protect make it difficult to prevent transmission and disease.

Introduction

Definition. Avian infectious bronchitis (IB) is an economically important, highly contagious, acute, upper-respiratory tract disease of chickens and other fowl, caused by the avian gammacoronavirus infectious bronchitis virus (IBV). The virus is found worldwide and is transmitted by inhalation or direct contact with infected birds or contaminated litter, equipment, or other fomites. Vertical transmission of the virus within the embryo has not been reported, but virus may be present on the shell surface of hatching eggs via shedding from the oviduct or gastrointestinal tract. A poor viral polymerase proofreading mechanism resulting in genetic mutations and genome recombination events can lead to the emergence of new serotypes of the virus, which do not cross protect, complicating control by vaccination.

Avian coronaviruses similar to IBV also have been found in pheasants (*Phasianus colchicus*) and peafowl (Galliformes) and IBV-like viruses have been isolated from turkeys, teal (*Anas crecca*), geese (Anserinae), pigeons (Columbiformes), guinea fowl (*Numida meleagris*), partridge (Alectoris), and ducks (Anseriformes) (28). The turkey coronaviruses (TCoV) are described in Chapter 12. Gammacoronaviruses similar to IBV have also been detected in wild birds (71, 92, 138).

Economic Significance

The disease is characterized by respiratory signs, reduced weight gain, and reduced feed efficiency in meat-type broiler chickens infected with the virus. Infection also predisposes broilers to secondary opportunistic bacterial infections that can result in airsacculitis, pericarditis, and perihepatitis. Morbidity is almost always 100%, but mortality can vary depending on the age and immune status of the birds, the strain of the virus, and if secondary bacterial or viral pathogens are involved. Some strains of IBV are nephropathogenic and can cause high mortality due to kidney failure in susceptible birds.

In layer and breeder chickens, infection may result in reduced egg production of up to 70% and declines in eggshell quality. The virus can replicate in the oviduct and cause permanent damage in young hens resulting in limited egg production over a prolonged period of time and birds that fail to come into production (false layers). Eggs

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from breeds with pigmented shells may become pale, and the albumen can have a watery viscosity. Egg production often recovers but may be permanently depressed in flocks with no immunity to the virus.

Public Health Significance

Infectious bronchitis has no known human health significance.

History

Infectious bronchitis was first observed in the United States in North Dakota in 1930, and the first documented description of the disease was published by Schalk and Hawn in 1931 (160). Early descriptions of the disease were consistent with a mild form of infectious laryngotracheitis (ILT) (23), but in 1936, Beach and Schalm using neutralization studies in chicks showed that the virus that caused IB was different from the virus that causes ILT (11).

An important discovery occurred in 1937 when Beaudette and Hudson (13) found that IBV could be propagated in the allantoic cavity of embryonating eggs. In 1941, Delaplane and Stuart (69) suggested that IBV propagated in embryonating eggs might have immunizing value, which led to the first IB vaccine report by van Roeckel et al. (177). The first IBV vaccine in the United States was developed using the van Roekel M41 strain, which is a Mass serotype virus isolated at the University of Massachusetts, Amherst, in 1941. More on the history of the M41 type vaccines used in the United States and other Mass-type vaccines can be found here (96).

Another important discovery relating to the control of the virus was made by Jungherr in 1956 when he reported that an IBV isolated in Connecticut did not cross protect chickens against challenge with the original Mass isolate (108). This led to the awareness that different serotypes of the virus existed and that they did not cross protect.

In the 1960s, it was discovered that IBV could interfere with growth of Newcastle disease virus (NDV) in embryonating eggs and cell culture, which was significant because IBV and NDV vaccines are often given together (12, 150). Also in the 1960s, Winterfield and Hitchner reported that some strains of IBV can cause a nephritis-nephrotic syndrome, and the nephropathogenic strains Gray and Holte were isolated (181).

A significant advancement in the diagnosis of IBV occurred in the 1990s when several laboratories began identifying the type of IBV using molecular techniques (101, 111, 116, 123). This allowed for the rapid identification of many isolates and the comparison of viruses around the world. More about the early history of IB research can be found in the review by Fabricant (73) .

Etiology

Classification

Infectious bronchitis virus is a gammacoronavirus in the subfamily Coronavirinae and family Coronaviridae (https://talk.ictvonline.org/ictv-reports/ictv_9th_report/positive-sense-rna-viruses-2011/w/posrna_viruses/222/coronaviridae). The Coronaviridae family includes two subfamilies, Coronavirinae and Torovirinae, and within the Coronavirinae subfamily there are four genera, alphacoronavirus, betacoronavirus, deltacoronavirus, and gammacoronavirus. Alphacoronaviruses and betacoronaviruses are mammalian viruses. The deltacoronaviruses include species from wild birds and gammacoronaviruses include avian coronaviruses IBV and TCoV, as well as coronaviruses isolated from pheasant, goose, pigeon, and duck (28, 104). The Beluga whale coronavirus SW1 is also a gammacoronavirus (131). Additional coronaviruses, which are not assigned to a genus, include viruses isolated from an Asian leopard cat and Chinese ferret badger (72, 183).

Morphology

Infectious bronchitis virus is an enveloped virus with a round to pleomorphic shape. The virus particles are approximately 120 nm in diameter with club-shaped surface projections (spikes) about 20 nm in length (Figure 4.1), which gives the virus a crown-like appearance and hence the name corona (Latin for crown).

Infectious bronchitis virus strains differ in their density in sucrose gradients; particles with a full complement of



Figure 4.1 Virion of avian infectious bronchitis virus (IBV) illustrating club-shaped projections (Mark Jackwood, Department of Population Health, College of Veterinary Medicine, University of Georgia).

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spikes have a density of 1.18 g/mL, and lesser-spiked particles may be as low as 1.15 g/mL.

Chemical Composition and Structure

The viral genome is a single-stranded positive-sense strand of RNA that is approximately 27.5–28 Kb in length. It is 5′ capped and has a poly-A tail at the 3′ end. The virions are made up of spike (S), envelope (E), membrane (M), and nucleocapsid (N) structural proteins (148). The S glycoprotein is a trimer made up of two subunits, S1 and S2 (approximately 520 and 625 amino acids, respectively). The spike glycoprotein is a type I membrane protein containing a receptor binding domain, cleavage site, precoil domain, fusion peptide, heptad repeat regions, interhelical domain, transmembrane domain, and cytoplasmic tail (115, 148). It is involved in host cell attachment as well as virus and cell membrane fusion and entry into the cell (27, 180). Virus-neutralizing and hemagglutination-inhibiting (HI) antibodies are directed against the first third of the NH_3 end of the spike (32, 93, 115). The E protein is a small integral membrane protein involved in assembly of the virus (159). The M glycoprotein spans the viral envelope three times with a portion of the NH_3 end exposed on the outer surface of the virus. The N phosphoprotein encapsidates the viral RNA genome to form the helical nucleocapsid within the virion and interacts with the M and E proteins for virus assembly (102).

Virus Replication

For an overview of coronavirus replication see (75). The gene organization is 5′ untranslated region (UTR)-leader-1a/1ab-S-3a-3b-E-M-5a-5b-N-3′ UTR (Figure 4.2). The UTRs interact with viral encoded polymerase proteins and possibly host cell proteins for viral RNA transcription and replication (122). The open reading frames (ORFs) 1a and 1ab encode polyproteins that are post-translationally cleaved into 15 nonstructural proteins (Nsps) by a papain-like protease (PLP) and the main protease (Mpro), both of which are encoded on the polyprotein itself. The Nsps are involved in regulation of host cell functions and make up the viral RNA-dependent RNA polymerase (reviewed in [148]). Most coronaviruses have 16 Nsps but IBV as well as other gammacoronaviruses lack Nsp 1. The 1ab protein is translated through a -1 frame-shift at a "slippery" heptanucleotide sequence that is just upstream of an RNA pseudoknot structure. The 3a, 3b, 5a, and 5b ORFs are nonstructural proteins with largely unknown function. The virus replicates in the cytoplasm of the host cell and produces a 3′ co-terminal nested set of 5 subgenomic messenger RNAs. Each mRNA has a 5′ leader sequence that is joined to the mRNA during transcription. Although some of the subgenomic mRNAs are polycistronic, for the most part each mRNA encodes the protein at the extreme 5′ end. The full-length genome (mRNA1) encodes the viral polymerase polyproteins. Subgenomic mRNAs 2, 3, 4, 5, and 6 encode S, 3a/3b/E, M, 5a/5b, and N, respectively.

The S glycoprotein mediates host cell attachment, virus and cell membrane fusion, and entry into the host cell. Once in the cell, the viral genome acts as mRNA encoding Nsps 2 to 16, which forms to create the viral polymerase in double membrane vesicles at the Golgi (86). Transcription of viral subgenomic RNA and subsequent translation of viral proteins occurs in the cytoplasm. The S, E, and M proteins are inserted into the Golgi membrane, whereas the N protein binds to the newly synthesized viral genome to form the nucleocapsid. Interactions between nucleocapsid and E and M proteins result in budding of the virus particles at the cytoplasmic surfaces of the endoplasmic reticulum. The virus particles are transported to the plasma membrane in vesicles where fusion occurs to release the virus particles from the cell. Virus particles can also be released via cell lysis.

Susceptibility to Chemical and Physical Agents

Centrifugation forces of greater than 100,000 xg can sometimes result in the loss of spikes, or at least the S1 subunit. Incubation at 37 °C also can result in the loss of the S1 subunit, which is noncovalently attached to the S2 subunit by disulphide bonds (166).

Thermostability

Coronaviruses are heat liable, being inactivated after 15 minutes at 56 °C, but samples containing protein should be treated at 60 °C for at least 30 minutes to completely inactive the virus (149). Long-term storage of IBV is recommended at –80 °C. Survival up to 12 days and for as long as 56 days when ambient temperatures are below freezing has been reported. Thermo-instability requires that a cold chain always be maintained for samples sent to the laboratory for diagnosis.



Figure 4.2 Organization of the infectious bronchitis virus (IBV) genome.

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Lyophilization

Infectious allantoic fluid lyophilized, sealed under vacuum, and stored in a refrigerator has remained viable for at least 30 years (reviewed in 96). Attenuated vaccines can be lyophilized in the presence of sucrose or lactose to preserve potency and extend shelf life.

pH Stability

The reduction in titer, following extremes of pH, are variable depending on virus strain. A pH 3 treatment at room temperature for 4 hours resulted in reductions in titer of 1–2 log10 for most isolates, but up to 5 log10 for others. Infectious bronchitis virus in cell culture was more stable in medium at pH 6.0 and 6.5 than at pH 7.0 to 8.0 (reviewed in [96]).

Chemical Agents

Infectious bronchitis virus, being an enveloped virus, is sensitive to ether, 50% chloroform, and 0.1% sodium deoxycholate (4°C for 18 hours). Most common disinfectants used in the poultry house inactivate IBV and no one type is recommended over another. The area to be disinfected should be free of organic material and disinfectants should be used at the manufacturer's recommended concentration. Treatment with 0.05% or 0.1% beta-propiolactone (BPL) or 0.1% formalin eliminated IBV infectivity. Only BPL treatment had no adverse effect on IBV hemagglutination (HA) activity, making it a good choice for creating HA antigen and killed vaccines.

Strain Classification

Many methods are used to differentiate and classify isolates of IBV, and they have been thoroughly compared (62). However, serotype and genetic typing, based on the sequence of the S1 protein, are most commonly used to classify strains. Serotype classification involves treatment of the virus with neutralizing antibodies, whereas genetic type classification involves examining the sequence of the S1 protein. Although not a hard and fast rule, strains of the virus that have greater than 90% amino acid similarity in the S1 gene (genetic type) are likely to be serologically related (serotype). Classification of virus types by immunization and challenge of birds, referred to as protectotype has also been reported (46).

There has been a lack of standardization of IBV strain nomenclature in the past, but most scientists have adopted the system suggested by Cavanagh in 2001 (29), which is similar to that used for avian influenza viruses. Basically, IBV strains are identified by the following scheme: IBV/bird type/country of origin/genetic type or serotype/strain designation/year of isolation. See *Genetic Classification* below for genetic type and lineage designations GI to GIV). Often IBV and bird type (assuming the isolate is from a chicken) are dropped, but if the isolate is not from a chicken or the type of chicken (broiler, layer, breeder) is important it is included. Examples of some viruses are US/GI-9,Ark/ArkDPI/81, US/GI-1, Mass/Mass41/41, Italy/GI-21,Italy-02/497/02, China/GI-19,LX4/QX/99, and IBV/Pheasant/UK/24/B171-3/99.

Serotype Classification

Traditionally, IBV serotypes have been defined by 2-way cross-virus neutralization (VN) testing in embryonating specific pathogen free (SPF) chicken eggs (81). This typing method involves reacting the unknown virus with antisera against known strains; then serotype-specific antibody is prepared against the unknown virus and reacted with known virus strains. The data are used to calculate a relatedness value using the Archetti and Horsfall formula (7).

Strain classification by the HI test has also been used, but most IBV strains do not spontaneously hemagglutinate and must be treated with neuraminidase (158). The HI antibody response following a single exposure of the virus can be highly strain specific, and the specificity and limited cross-reactivity of the early immune response are the basis for serotyping isolates using HI tests. However, multiple exposures to the virus, which is common in vaccinated birds, results in high and variable cross-reactions making it difficult to clearly differentiate strains using the HI test (45, 78).

Genetic Classification

Currently, most laboratories use nucleic acid approaches to characterize IBV isolates by genetic type (97, 112, 121). Typically, the reverse transcriptase-polymerase chain reaction (RT-PCR) is used to amplify the S1 gene or the hypervariable region of the S1 gene, followed by nucleic acid sequencing (112, 120) or less frequently, restriction fragment length polymorphism (RFLP) analysis (116, 123). The sequence of the whole genome is available for many isolates (genotype), and are available from Genbank (www.ncbi.nlm.nih.gov) for strains all over the world.

The deduced amino acid sequence of the S1 protein should be used to genetically type IBV isolates. The hypervariable region sequence of the S1 protein can be used to identify field viruses in a diagnostic laboratory setting, but generally is not sufficient for thorough characterization of genetic type. Analyzing the S1 gene phylogeny of 1286 IBV strains, Valastro et al. (175) defined 6 genetic types (GI to GVI) comprising 32 IBV lineages worldwide. The genetic type GI contains 27 different IBV lineages whereas GII to GVI each contain 1 IBV lineage.

There exists a correlation between percentage of similarity between S1 protein sequences and cross-protection (64). Generally, viruses that fall into the same

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genetic type are related serologically, but exceptions do exist. Location of the amino acid differences (31) and sequence analysis of VN-monoclonal-antibody escape mutants (110, 137) indicate that a minimum number of changes can affect the conformationally-dependent neutralizing epitopes on the S1 protein resulting in little or no cross protection.

Strains of IBV and Viral Evolution

It is well known that a number of different types, subtypes, and variants of IBV exist, which is due to a high degree of genetic diversity that occurs through a high mutation rate and recombination events. Mutations include substitutions, which are the result of a high error rate and limited proofreading capability of the viral RNA-dependent RNA polymerase (RDRP), as well as insertions and deletions, caused by recombination events or by RDRP stuttering or slippage. Although IBV (and other coronaviruses) has a 3′ to 5′ exoribonuclease (exon) domain in Nsp 14 that is involved in proofreading and repair (132), the average rate of synonymous mutation is still high at approximately 1.2×10^{-3} substitutions/site/year (87, 89). Recombination has been reported in many coronaviruses including IBV (88, 167, 186). Since the replicase gene was shown to be a determinant of pathogenicity (8), recombination in the 1a/1ab genes associated with the RDRP can affect pathogenicity. Because the S glycoprotein gene is involved in host cell attachment (cell tropism) and contains viral neutralizing epitopes, recombination in the S glycoprotein gene can result in the emergence of new strains or serotypes of the virus as well as new viruses capable of causing disease in other host species (95). New IBV types, subtypes, and variants, whether the result of mutations, recombination, or both, continue to emerge, making control of IBV extremely challenging (96).

Laboratory Host Systems

Chicken Embryos

Infectious bronchitis virus grows well in 8 to 11 days of incubation in SPF chicken embryonating eggs following the inoculation of the allantoic cavity. The maximum virus titer in allantoic fluid (AF) is reached 1–2 days postinoculation (PI), although this peak can be delayed for non-egg-adapted field strains (62). For isolation of non-egg-adapted field strains, several sequential passages may be required to achieve high titers of virus in the AF. The extent of changes to the infected embryos that are induced by IBV vary greatly and are strain, dose, and age of the embryo dependent. Inoculation of 8-day-old embryos results in more extensive lesions and mortality than the same inoculation at 10–11 days of age. Characteristic lesions such as stunting (dwarfing) and curling of the embryo and its feet (125) occur with increasing passage as does the incidence of embryo mortality, which for an embryo-adapted strain can be observed as early as 2–3 days PI. Upon opening the air cell end of the egg, the embryo is seen curled into a spherical form with feet deformed and compressed over the head and with the thickened amnion adhered to it (Figure 4.3). A common internal lesion of the IBV-infected embryo is the presence of urates in the mesonephros of the embryonic kidney. This lesion is not pathognomonic for IBV infection and can also be observed in embryos infected with avian adenovirus. Confirmation of the presence of IBV antigen or viral RNA in inoculated eggs is therefore preferably performed 2–3 days PI and independent from the occurrence of embryo lesions.

Microscopic lesions in embryos infected with the IBV-M41 strain have been studied (125). Congestion with perivascular cuffing and some necrosis of the liver by the sixth day PI was observed. Lungs were pneumonic, characterized by congestion, cellular infiltration, and serous exudate in the bronchial sacs. In the kidney, interstitial nephritis with edema and distension of the proximal convoluted tubules and the presence of casts was noted. Glomeruli were not altered. The chorioallantoic membrane (CAM) and amniotic membrane were edematous. No inclusion bodies were observed.



Figure 4.3 Comparison of normal 16-day-old embryo (left) and curled, dwarfed, and infected embryo of the same age (right).

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Cell Culture

Primary isolation of IBV field strains directly from pathological material in conventional monolayer cell cultures has proved unsuccessful (42). Adaptation of IBV strains is often necessary for sufficient replication leading to induction of cytopathic effect (CPE) (83). The number of passages that is needed for the adaptation can vary widely, even within the same serotype/genotype. Chick embryo kidney (CEK) cells and chicken kidney (CK) cells show the highest sensitivity for adapted IBV strains (126, 145). Chicken kidney cells form syncytia, which quickly round up and detach from the culture surface, appearing as large spheres with refractile contents. A few strains of IBV (e.g., the Beaudette strain) have been propagated successfully in the African green monkey Vero cell line, which has been used for many fundamental studies of IBV (56).

Organ Cultures

The propagation of IBV in organ cultures (OC) of trachea and other tissues has been reviewed (57). Tracheal organ cultures (TOCs) have proved very useful for the isolation, titration, and serotyping of IBV, because no adaptation of field strains is required for growth and the induction of ciliostasis. The sensitivity of TOCs for the detection of IBV strains is comparable to that of the use of embryonating SPF eggs. Following infection with IBV, ciliostasis, which is easily observed by low-power microscopy, usually occurs within 3–4 days (62). The presence of IBV in field samples must be confirmed by an IBV-specific test because ciliostasis also can be induced by many other agents.

Pathogenicity

Infectious bronchitis is primarily a disease of chickens with all ages being susceptible to infection. The pathogenicity of IBV can vary widely between strains. The clinical outcome of an infection in chickens depends on many variables such as the virus strain and type; sex and age of the chicken; immune status (vaccination, immune suppression, and maternally derived antibodies); coinfections; and environmental circumstances such as climate, dust, ammonia, and cold stress.

Infection is initiated via the respiratory tract regardless of the tissue tropism of the strain (respiratory, kidney, reproductive organs). The virus replicates and can produce lesions in many types of epithelial cells, including those of the respiratory tract (nasal turbinates, Harderian gland, trachea, lungs, and air sacs), kidney, and reproductive organs (oviduct, testes). Many strains also grow in many cells of the alimentary tract (esophagus, proventriculus, duodenum, jejunum, bursa of Fabricius, cecal tonsils, rectum, and cloaca) often with little pathobiological clinical effect (reviewed in 70).

All IBV strains produce lesions of varying severity in the respiratory tract depending on their virulence, chicken age at infection, genetic susceptibility of the chicken line, climate, and maternal or active immunity of the chicken. Therefore, IBV infections often increase the susceptibility to secondary respiratory infections or increase the damage of infections with primary respiratory pathogens. These increases have been shown for agents such as *Escherichia coli* (49, 85, 129, 130, 164), *Mycoplasma gallisepticum* (140, 170), *Mycoplasma synoviae* (73, 74, 90, 113, 119), *Mycoplasma imitans* (77), *Avibacterium paragallinarum* (152), Newcastle disease virus (139), and avian influenza virus (142). The damage caused by these secondary infections can be substantial, especially in broilers, resulting in a higher mortality, growth depression, increase of feed conversion, and higher condemnation rates. Proper vaccination against IBV also can be useful to prevent the chickens developing clinical IB and subsequent *E. coli* airsacculitis (129).

Although several strains of IBV are highly nephropathogenic, causing extensive and reproducible kidney disease in experimental conditions, many strains of IBV may be associated with nephritis to some degree in the field; environmental factors are probably important as to whether kidney complications are significant. The virulence of the strains for the kidney also depends on the age of infection. Young birds (less than two weeks of age) typically show more severe nephritis and higher mortality than older birds (1, 24, 188). Cumming (54) and Glahn et al. (84) enumerated some of the management factors that contribute to IB-related kidney disease. Greater mortality was seen in males, with cold stress, in certain breeds, increased levels of dietary calcium or when animal byproducts were the major component of high-protein diets. Some of these factors known to exacerbate the clinical disease have been used in experimental models to evaluate the clinical outcome of interaction between such factors and different IBV strains. Reddy et al. (154) reported significant differences between the replication kinetics of the nephropathogenic B1648 and respiratory M41 IBV strains when compared *in vitro* in respiratory mucosa explants and blood monocytes (KUL01(+) cells), and *in vivo* in chickens that might explain the different behavior of these strains in chickens.

Virulence for the reproductive tract also may differ among IBV strains. Presence of maternal antibody could prevent damage to the oviduct during an early-age IBV infection (21). In susceptible layers, different IBV strains produced a range of effects varying from shell pigment changes with no production drop to production drops of up to 70% (18, 19).

The virulence of IBV strains for other organs such as the alimentary tract seems to be low (reviewed in 70). However, several groups have reported outbreaks of a

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"proventricular-type" IBV infection in chickens (179, 184) by QX and Q1 strains. Those studies did not show proof of local replication of the involved IBV strains, and the mortalities were exceptionally high for a solely IBV-induced disease. It remains unclear whether the reported signs were only caused by the IB strain or that another agent might be involved.

In recent years, coronaviruses have been detected in an increasing number of bird species and turkey, pheasant, goose, pigeon, and duck coronaviruses are considered avian coronaviruses in the gammacoronavirus with IBV. Mostly, the strains were not isolated but were detected by RT-PCR and sequencing, and little to no clinical signs were observed in these birds. Consequently, the majority of the detected strains could not be inoculated into chickens to determine infectivity and virulence. An exception are the coronaviruses isolated from pheasants with respiratory and kidney disease. These viruses differ in gene sequence from IBV to an extent similar to that exhibited by different serotypes of IBV (34). When three coronavirus isolates from pheasants were inoculated into chickens, no signs of disease were observed (124), which led to pheasant coronavirus (PhCoV) being officially considered as a species distinct from IBV. The issue of the coronavirus species determination is thoroughly discussed here (33).

Pathogenesis and Epizootiology

Incidence and Distribution

Infectious bronchitis is distributed worldwide and many dozens of serotypes and genotypes have been detected in all continents except Antarctica (64, 94). The first IBV variants were detected in the 1950s in the United States (73); however, a retrospective study (103) has shown that IBV variants were already circulating in the US poultry industries in the 1940s. Several serotypes can cocirculate in a given region with some strains being detected on several continents, while others seem to be regional.

Natural and Experimental Hosts

As explained previously, it is now accepted that the chicken is not the only host for IBV, although it is possible that IBV only causes disease in the chicken.

Age of Host Commonly Affected

All ages are susceptible, but the disease is most severe in chicks, often causing some mortality, especially with nephropathogenic strains. As age increases, chickens become more resistant to the nephropathogenic effects, oviduct lesions, and mortality due to infection (2, 25, 38, 53).

Transmission, Carriers, Vectors

Infectious bronchitis virus is highly contagious and spreads rapidly among chickens in a flock (65, 128). The disease has a short incubation period: susceptible birds placed with recently infected chickens usually develop clinical signs within 24–48 hours.

Transmission may be by either inhalation or ingestion of infectious virus particles by direct contact between infected and susceptible birds; by indirect contact through aerosol droplets or feces; and by exposure to virus-contaminated fomites, such as clothing, shoes, tools, and so on. Aerosol generation from the respiratory tract is a significant mode of transmission because of high virus concentrations in the respiratory tract during the acute stage of the infection. The highest concentration of IBV can be detected in the trachea during the first 3–5 days PI. After this period, the virus titer in the respiratory tract drops rapidly and in the second week PI can already be below the level of detection, especially in birds with a certain level of protection (62). Most likely, transmission by aerosol is especially effective over short distances, such as within a flock or premises, because the enveloped virion is inactivated relatively quickly in the environment.

The virus is also excreted in the feces and in the uric acid from kidney. During the chronic stage of an IBV infection, virus can be more readily detectable in the intestinal tract (cecal tonsils or cloaca swabs) and for a longer time than in the respiratory tract (62). Several authors report a long-term recovery (2–7 months) of IBV from infected or vaccinated flocks (3, 4, 38, 141); others report a re-excretion of IBV following T cell suppression by cyclosporine (16, 70) or at onset of lay (105). Possible explanations of long-term isolations or re-excretion of an inoculated virus are continual cross-infection within infected or vaccinated flocks, continual excretion of the virus at levels usually below the detection levels of tests, reactivation after treatment with cyclosporin (16), or reinfection due to contact with the infected feces from the previous infection after a decrease in level of protection. The two main candidate sites mentioned in these reports for persistence are cecal tonsils and kidney. The phenomena of possible long-term excretion and re-excretion may result in flock-to-flock transmission by direct or indirect contact with contaminated litter, fomites, or personnel, but further study is needed to understand if this is a common source of transmission.

Vertical transmission does not seem to be relevant for IBV, although Cook (41) could reisolate the challenge virus after infection in laying SPF hens and cockerels for 2 weeks from semen, for 1–7 weeks from the vitelline membrane of the eggs, and even from a small number of hatched chicks. However, the implication of this last finding for the field remained unclear, because these

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chicks developed no clinical signs, did not seroconvert, and were not protected against challenge.

In view of the recent discovery of IBV in species other than the chicken, it should be considered that other species of birds might not only be able to transport IBV mechanically but also may actively multiply IBV or be a source of IBV outbreaks.

Incubation Period

The incubation period of IB is dose dependent and can be as short as 18 hours for intratracheal inoculation and up to 36 hours for ocular application.

Clinical Signs

The nonspecific respiratory signs of IB in susceptible chicks are gasping, coughing, sneezing, tracheal rales, and nasal discharge. Watery eyes may be observed, and an occasional chick may have swollen sinuses. The chicks appear depressed and may be seen huddled under a heat source. Feed consumption and weight gain may be significantly reduced. In chickens, older than six weeks of age, the signs are usually less clear, and the disease may even go unnoticed unless the flock is examined carefully by handling the birds or listening to them at night when the birds are normally quiet. The severity of the respiratory signs is influenced by the quality of the climate, housing, kind of bird, strain involved, IB vaccination program, and presence of coinfections including secondary infections.

Broiler chickens infected with a nephropathogenic virus may appear to recover from the respiratory phase and then show signs of depression, ruffled feathers, wet droppings, increased water intake, and mortality (54). Young age, cold stress, breed of chicken, increased levels of dietary calcium, and high-protein diets containing animal byproducts as the protein source are predisposing factors for the development of clinical signs during an infection with a nephropathogenic strain (54, 114).

In laying hens, the respiratory signs can be absent or very mild even in cases of clear production drops and the production of eggs with pale, unpigmented shells. The severity of the production decline may vary from slight up to 70% (18, 19) and depends on factors such as the causative virus strain and level of immunity against that strain, the timing of infection within the period of lay, and by coinfections. Following IBV infection at the onset of production a more severe drop in total production of normally shelled eggs, an increase in the number of abnormally shelled eggs, and more lasting adverse effects on egg weight and internal egg quality were observed, in comparison with infection after peak production. With mild drops in production, a normal level of production can be restored in 1 or 2 weeks. With severe drops of production, 6–8 weeks may elapse before production returns to the preinfection level, but in some cases, this is never attained.

In addition to production declines, IBV infections can cause a range of effects on the egg quality varying from loss of shell pigment, shell quality (misshapen, thin, soft-shelled, and rough-shelled eggs, Figure 4.4), thin to watery albumen (Figure 4.5) in a fresh egg, and decreased hatchability. Flocks with false layers fail to reach the normal rate of lay, whereas the flock looks healthy, behaves normally, and produces good quality eggs. The peak of production can be as low as 35% of expected production values (21).

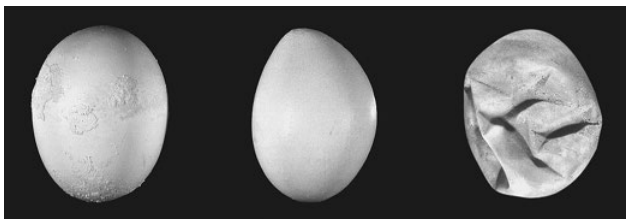


Figure 4.4 Thin-shelled, rough, and misshapen eggs laid by hens during an outbreak of IB. (Van Roekel)

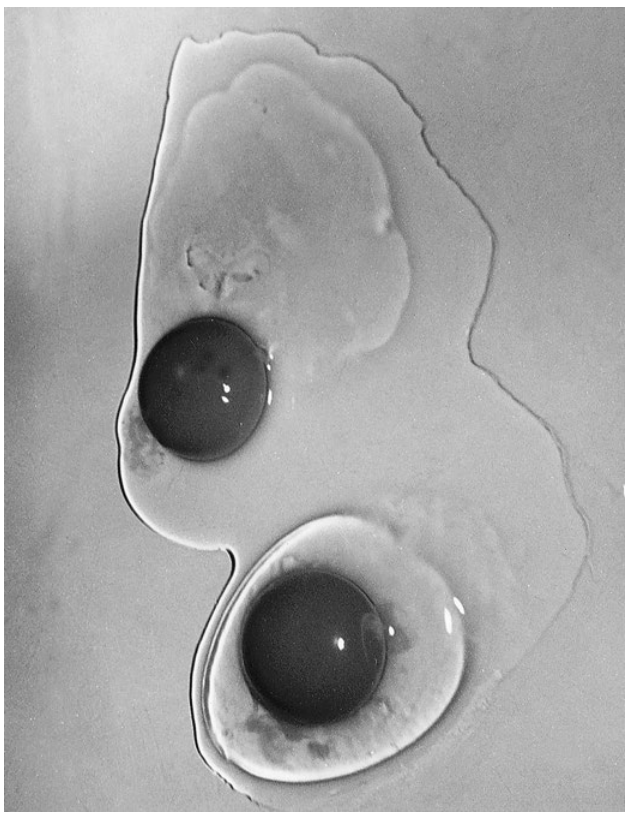


Figure 4.5 Contents of two eggs. Normal egg (bottom). Egg from chicken exposed to IBV at 1 day of age (top). Note watery albumen with yolk separated from thick albumen (51).

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Pathology

Gross

Infected chickens have serous, catarrhal, or caseous exudate in the trachea, nasal passages, and sinuses. Air sacs may be foamy during the acute infection and then may become cloudy and contain a yellow caseous exudate. Areas of pneumonia may be observed around the large bronchi. Nephropathogenic infections may produce swollen and pale kidneys with the tubules and ureters often distended with urates (55, 187) (Figure 4.6).

Fluid yolk material may be found in the coelomic (abdominal) cavity of chickens that are in production, but this is also seen with other diseases that cause a marked drop in egg production. Cystic left oviducts may be a consequence of IBV infection of an unprotected bird at a young age (14, 21, 37, 52, 68, 106) and are a cause of false layers resulting in reduced peak in egg production when the flock reaches maturity. Effects of IBV infection on the reproductive tract of chickens in production have been detailed by Sevoian and Levine (163). They observed reduced length and weight of the oviduct in infected birds as well as regression of the ovaries.



Figure 4.6 Kidney lesions associated with infectious bronchitis (IB) caused by T strain of virus. Note swollen kidneys with tubules and ureters distended with urates (55).

Microscopic

The tracheal mucosa of chickens with IB is edematous. There is loss of cilia, rounding and sloughing of epithelial cells, and minor infiltration of heterophils and lymphocytes within 18 hours of infection. Regeneration of the epithelium starts within 48 hours but cilia recovery begins 7 to 8 days later. Hyperplasia is followed by massive infiltration of the lamina propria by lymphoid cells and the formation of a large number of germinal centers often forming after 7 days. If air sac involvement occurs, there is edema, epithelial cell desquamation, and some fibrinous exudate within 24 hours. Increased heterophils can be observed later with lymphoid nodules, fibroblast proliferation, and regeneration by cuboidal epithelial cells (155) (Figure 4.7A, B, C, D, E, F).

The histological changes post IB vaccination and challenge in the Harderian gland includes a sharp increase in the number of plasma cells, hyperemia, and extensive lymphoid follicle formation (59, 172).

The kidney lesions of IB are principally those of an interstitial nephritis (Figure 4.7G and H) (35, 36, 155). The virus causes granular degeneration, vacuolation and desquamation of the tubular epithelium, and massive infiltration of heterophils in the interstitium in acute stages of the disease. The lesions in tubules are most prominent in the medulla. Focal areas of necrosis may be seen along with indications of attempted regeneration of the tubular epithelium. During recovery, the inflammatory cell population changes to lymphocytes and plasma cells. In some cases, degenerative changes may persist and result in severe atrophy of one or all of the divisions of the nephrons. In urolithiasis, the ureters associated with atrophied kidneys are distended with urates and often contain large calculi composed mainly of urates.

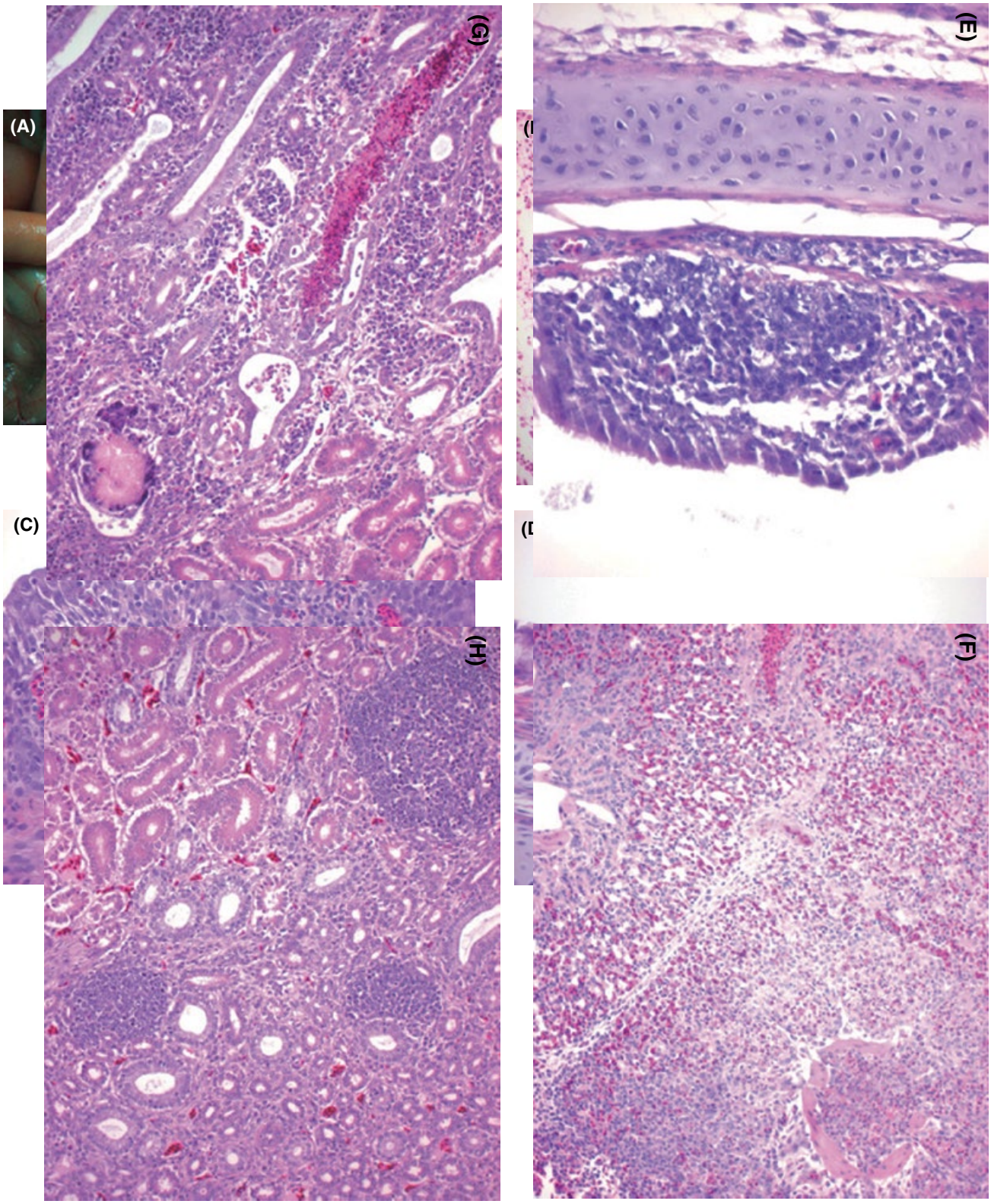
Experimental IBV infection of the oviduct of mature hens resulted in decreased height and loss of cilia from epithelial cells; dilation of the tubular glands; infiltration by lymphocytes, other mononuclear cells, plasma cells, and heterophils; and edema and fibroplasia of the mucosa of all regions of the oviduct (155, 163).

The histological lesions in the enteric tract appear to be mild for IBV infections that are free of coinfections, reviewed in (70). No lesions were detected in any part, from proventriculus to ileum, in chickens that had been infected with the enterotropic G strain; only the rectum showed desquamation of the cells from the tips of villi, congestion, and focal infiltration with lymphocytes, macrophages, and some heterophils (6). More recent reports of proventriculitis associated with IBV strains of the QX and Q1 genotype need confirmation of the role of IBV in these lesions (66).

Immunity

Aspects of immunity to IBV have been reviewed previously (30, 70). Although differences in both

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breed- and strain-related genetic resistance to IBV infection have been described in chickens (10, 22, 47, 48, 144, 165), relevant data about the genetic resistance of commercial lines of chickens are not available.

Innate Immunity

The innate immune system is involved in directing the adaptive responses to IBV and can also act as expresser of the adaptive immunity (109). It has been shown that IBV induced a diversity of local innate effectors and Th1-based adaptive immunity during the early phase of IBV infection, and that these immune effectors are responsible for the rapid clearance of virus from the trachea (178). Mannose-binding lectin (MBL), an innate pathogen pattern-recognition molecule, is involved in the regulation of the adaptive immune response to IBV (109) and is able to bind specifically to the spike S1 protein of IBV and subsequently block the attachment of S1 to IBV-susceptible cells in chicken tracheal tissues (185). Recent work suggests that macrophages might play a role in spreading of the virus within the bird (5, 154).

Active Immunity

Cell-Mediated Immunity. Work on cell-mediated immune responses to IBV has been reviewed (70), and specifically for the cytotoxic T lymphocytes (CTL) (40). Work with the nephropathogenic Gray strain showed that the MHC-class 1 restricted CTL response correlated much better with the initial elimination of virus from lungs and kidneys during the acute phase of infection than the humoral IgM and IgG response (161). Adoptive transfer of different kinds of immune T cells to chicks prior to infection with the Gray strain demonstrated that IBV-primed CTLs with αβ T cell receptors (TCR2) could protect chicks from acute infection in the respiratory tract (162).

Humoral Immunity. Vaccination or infection with IBV results in an antibody response that can be detected using different kinds of group-specific and serotype-specific techniques. Usually, the first antibodies can be detected in serum and lacrimal fluid between one and two weeks PI. However, the level of antibody response depends on many variables including age at inoculation, presence and level of maternally derived antibodies, level of immunity at the time of vaccination/infection, application route, genetics, and immunosuppression, reviewed in (62). In serum, IgA, IgG (IgY), and IgM can be detected; in lacrimal fluid and tracheal washings, IgA and IgG are the commonly detected antibody classes. An important part of the IgA in the lacrimal fluid originates from the Harderian glands (9, 61). The IgG concentration in the same fluid is largely the result of passive transport of IgG from the serum (61, 171). IgM is only present for a few weeks after infection or vaccination; therefore, its detection is indicative of a recent exposure (62).

Serotype-specific virus neutralizing antibodies are induced by the amino-terminal S1 subunit of the S glycoprotein (31). In young birds, IBV ELISA and VN antibody levels in tears were not accurate indicators of IBV immunity as determined by challenge with Mass/Mass41/41. High tear IBV antibody titers were observed in some chickens determined to be susceptible to IBV challenge and low tear antibody titers were detected in some protected chickens (80). No correlation was found between the serum ELISA antibody titers and the degree of kidney protection against the nephropathogenic Belgium/B1648/96 strain (147). In another study, no antibody was detectable post vaccination in post hatch cyclophosphamide bursectomized chickens and still the birds resisted challenge (39). In laying birds, a clear correlation was found between the level of HI antibodies against the challenge virus and the level of protection against egg drop (18–20, 76) as long as these were the result of a live priming and subsequent boost using an inactivated vaccine.

Immunosuppression

Despite many studies that have detected neutralizing IBV serum and local antibodies post-vaccination, the relevance of antibodies and cellular immunity in the mechanism of protection against infection and disease is still largely unknown. Infections with virulent infectious bursal disease virus (IBDV) strains in birds at one to eight days of age prior to the IBV vaccination have shown to decrease the efficacy of an IBV vaccination, including a decrease in antibody response to the vaccination and longer excretion of the challenge strain postinoculation (146, 157, 182). A similar effect was reported after *in ovo* bursectomy in line C White leghorns. These birds experienced a more severe and longer lasting infection than the intact birds and also developed less respiratory protection against a secondary challenge (43). A US/Ark/Ark-DPI/81 challenge at 15 days of age in immunodeficient SPF birds by a combined chicken anemia virus and IBDV inoculation at 7 days of age resulted in more severe and persistent clinical signs and lesions, a delayed and reduced antibody response, and increased and persisting viral shedding (173).

Maternally Derived Immunity

High levels of maternally derived antibodies significantly reduced the extent of clinical signs or damage to trachea, kidneys, and oviduct due to IBV infection in chicks during the first days of life (21, 54, 58, 68, 134, 135). Several groups have reported a negative effect of high levels of maternal antibodies against the vaccine strain when it is applied on day of hatch, whereas others did not detect a

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lower efficacy of the day-old vaccination in the presence of maternal antibodies (58, 60).

Diagnosis

Diagnosis of IB is based on the clinical history, lesions, seroconversion (rising IBV antibody titers), IBV antigen detection by a number of antibody-based antigen capture assays, virus isolation, and detection of IBV RNA (81). Thorough diagnosis of IBV includes identification of the serotype or genetic type of the virus so that appropriate vaccines can be used. The many approaches used to detect the virus or antibodies induced by it have been described and critically compared (62) .

Isolation and Identification of the Causative Agent

Although primarily a respiratory pathogen, IBV also can infect epithelial cells in the kidney, oviduct, and gastrointestinal tract. Knowledge of the pathogenesis of IBV, reviewed in (153), has been instructive for effective sample collection to detect the virus.

Virus Isolation

Tracheal swabs or fresh tracheal tissue is the preferred sample, especially within the first week of infection, and samples should constantly be kept cold (on wet ice) until tested. Titers of IBV reach a maximum in the trachea by day 3–5 PI, after which they decline rapidly. Because the virus initially grows in the upper respiratory tract and then spreads to non-respiratory tissues, kidney and cecal tonsils collected at postmortem examination can be of value in cases in which more than one week has elapsed since the start of infection. However, it should be recognized that vaccine viruses could also be found persisting in cecal tonsils. Although the virus can replicate in the gastrointestinal tract, cloacal swabs or fecal material are difficult samples from which to isolate the virus. Additionally, samples from the lung, kidney, and oviduct should be considered, depending on the clinical history of the flock (3).

When collecting samples from a large flock, both healthy birds and those with clinical signs should be sampled. Typically, clinical signs begin 3–5 days following infection when the virus is no longer at peak titer. In mild cases of the disease, clinical signs due to the virus may go unnoticed until secondary pathogens become involved, at which time IBV is no longer present. Alternatively, the placement of susceptible sentinel chickens has been described (78) and can be beneficial when direct sampling is unsuccessful.

Samples for virus isolation commonly are inoculated into the allantoic cavity of 9- to 10-day-old embryonating chicken eggs or TOCs, preferably from an SPF source. Fluids should be harvested after 48–72 hours from either culture system and passed at least 3–4 times before being called negative based on failure to cause lesions or death in embryos, or ciliostasis in TOCs (81). However, these observations are not in themselves sufficient to confirm the presence of IBV; the presence of the virus must be confirmed by VN, HI, immunofluorescence, immunohistochemistry, detection of the viral nucleic acid, or electron microscopy (15).

Coronaviruses similar to IBV in wild or domestic birds other than chickens may or may not replicate in embryonating chicken eggs (34, 104).

Confirmation and Typing of IBV by Antibody-Based Methods

Detection of IBV directly or indirectly in postmortem material (scrapings of tracheal mucosa or other tissues) or virus grown in embryonating eggs using serotype specific or monoclonal antibodies has been done but the results are not always easy to interpret, especially from direct field specimens because of nonspecific reactions (reviewed in [62]). All IBV serotypes appear to have common epitopes (group-specific antigens) likely due to the moderately high amino acid sequence identity within the N and M proteins and conserved regions of the S2 protein. Following a first infection with IBV, most of the antibody response is serotype-specific. A second infection, especially with a different serotype, results in a more broadly reactive serum. Because chickens in the field will almost certainly have been vaccinated (sometimes multiple times) with attenuated live vaccines (broilers and pullets) and killed vaccines (layers and breeders) against IB, field sera are not very useful for serotyping unknown viruses. Only sera induced experimentally (the procedure can be found in [81]) using SPF chickens should be used for determining the serotype of a virus.

The serotype of IBV has traditionally been determined by the VN or HI test (see Serotype Classification, above). However, virus-neutralization testing, whether conducted in embryonating eggs or in TOC, is time consuming and labor intensive, and the HI test suffers from nonspecific cross-reactivity. Thus, molecular-based tests have for the most part replaced VN and HI testing.

Confirmation and Typing of IBV by Nucleic Acid-Based Methods

The real-time RT-PCR test, also known as quantitative RT-PCR, is becoming more widely used to detect IBV directly from clinical samples (26, 98). Advantages of this test are that many samples can be examined in a short

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period of time, it is cost-effective, and it gives an indication of the level of viral nucleic acid in the sample. Recently IBV type specific primers and probes for some strains of the virus have been developed for real-time RT-PCR IBV type specific testing (156).

Conventional RT-PCR also can be used to detect the presence of IBV nucleic acid in a clinical sample; however, passage in embryonating eggs is sometimes necessary to obtain a positive result. Identification of the type of IBV in the sample is determined by sequence analysis of amplicons from the S1 gene (see Genetic Classification, above). Advantages of genetic typing includes a rapid turnaround time and the ability to detect a wide variety of IBV types. The hypervariable region of the S1 gene can be used to genetically type IBV in a diagnostic laboratory setting but the entire S1 gene should be used for complete characterization. Spike sequence data can be used to identify any IBV type as well as previously unknown field isolates and variants and phylogenetic analysis of unknown field isolates and variants with reference strains can be used to establish relatedness (100, 112, 120, 136, 143, 176).

Serology

Demonstration of rising antibody titers against IBV between preclinical and convalescent sera can be used to diagnose IBV infection. Because the ELISA, immunofluorescence, and agar gel precipitan (AGP) tests all bind antibody to group-specific antigens, they cannot be used to differentiate serotypes. Currently the ELISA test is the most widely used serologic test for antibodies against IBV because it is inexpensive and can be used to test a large number of samples in a short time. Commercial tests are available and typically detect antibodies (IgG) after one week postinfection (67, 127, 133).

Routine serology also can be done with the AGP and HI tests, reviewed in (62). Although the AGP test can detect antibodies within the first week of infection, the strongest precipitating antibodies (IgM) are short-lived, and IgG is poorly reactive. Thus, the AGP test is not recommended for detection of antibodies beyond two weeks PI (67). Although cross-reactive antibodies can be detected in the HI test, sensitivity may suffer, because this is largely a serotype-specific reaction. Nonetheless, the low cost, simple equipment, and speed of the HI test makes it a useful procedure as long as the test limitations are considered.

Differential Diagnosis

The clinical presentation of IB may resemble mild forms of other acute respiratory diseases such as Newcastle disease (ND), ILT, low-pathogenicity avian influenza, avian metapneumovirus, and infectious coryza. Newcastle disease caused by velogenic viscerotropic or neurotropic strains of paramyxovirus type 1 produces much higher mortality than IBV. Lentogenic ND virus infections with pneumotropic strains and low pathogenicity strains of avian influenza like H9N2 produce mild to moderate respiratory disease with low mortality and, thus, may resemble IB. Infectious laryngotracheitis tends to spread more slowly in a flock, but respiratory signs may be more severe than with IB, and infectious coryza typically causes facial swelling that occurs only rarely in IB. Egg production declines and shell quality problems in flocks infected with ND virus, avian influenza virus, ILT virus, avian metapneumovirus, and *Avibacterium paragallinarum* (infectious coryza) as well as avian mycoplasmas and EDS adenovirus are similar to those seen with IBV, except that in the case of EDS adenovirus, internal egg quality is not affected.

Intervention Strategies

Management Procedures

Ideal management includes strict isolation, high biosecurity, and repopulation with a single age of chicks, following the cleaning and disinfection of the poultry house and equipment in contact with poultry or poultry litter and composting or removal of the feces from the premises. Because IBV is highly infectious, immunization is needed in many areas in an attempt to prevent production losses due to IB.

Vaccination

Chickens just recovered from infection or recently vaccinated are protected from challenge with the same virus (homologous protection), but the extent of protection against challenge with other IBV strains (heterologous protection) varies. Challenge of vaccinated birds with homologous virus results in much lower shedding of challenge virus, and for a shorter period, than in unvaccinated birds (50, 65, 118, 147).

Types of Vaccine

Both live and inactivated virus vaccines are used for IBV immunization. Live vaccines are used in meat type (broiler) chickens and for the initial vaccination and priming of breeders and layer pullets. Infectious bronchitis virus strains used for live vaccines are attenuated by serial passage in embryonating chicken eggs (17), sometimes in combination with heat treatment (99). Evidence that some vaccines increased in virulence after back-passage in chickens (91) demonstrates the potential for enhancement of virulence of such vaccines by contin-

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ued circulation of the vaccine virus, a rolling infection, in a flock that is often caused by poor vaccination procedure, resulting in vaccination of only a fraction of birds in the house. The use of fractional doses of IB-attenuated vaccines has been associated with enhancing cyclic infections in a flock and an increase in vaccine-associated virulence.

The Mass/Mass41/41 strain, Mass/H120/55, and other vaccines of the Massachusetts serotype are commonly used in most countries. The goal of a vaccination program is to cover the antigenic spectrum of isolates in a particular country or region. When vaccinations with a single serotype are not providing sufficient protection against the prevailing field strains, vaccines of other serotypes can be added to the program. The broadening of the protection can be achieved by adding vaccines to the program that are homologous to the most important prevailing field strains (80, 81, 100, 104, 108) and/or by using combinations of vaccines that are able to induce a broad cross-protection against many strains (45, 63, 79).

Several studies have shown that live IBV, Newcastle, ILT, and avian metapneumovirus vaccines might interfere with each other's replication, humoral response, and induced level of protection when administered together or in relatively short intervals of each other (44, 79, 151, 168, 169, 174). The level and direction of interference is likely dependent on strains, dose, method and interval of application, maternally derived antibodies, and active immunity.

Inactivated oil-emulsion vaccines are administered to breeders and layers prior to the onset of egg production. Pullets may be vaccinated between 10 and 18 weeks of age depending on the immunization program. The efficacy of inactivated vaccines depends heavily on proper priming with live vaccine(s). Inactivated vaccines must be administered to birds individually, by intramuscular or subcutaneous injection. Inactivated vaccines induce high levels of serum antibody and increase protection to internal tissues, kidney, and reproductive tract (19, 20, 64, 117, 120). In contrast to live vaccines, inactivated vaccines are not nearly as effective at preventing infection of the respiratory tract following challenge with the homologous virulent virus (49).

New "variant" strains may be used to prepare inactivated autogenous vaccines for controlling IB in laying birds without the risks of using a live variant that could spread to and potentially cause disease in nearby flocks. Inactivated variant vaccines may offer better protection against challenge with the virulent live variant IBV than inactivated vaccines containing standard serotypes such as Mass and Conn (117).

Application Methods

Under experimental conditions, live vaccines are usually administered individually by eye drop or intranasal application. In the field, live vaccines are usually applied by the mass application methods including coarse spray, aerosol, or drinking water (107). The mass application of IBV vaccines in the field is known for its many variations in: (1) application technique (eye drop, coarse spray, drinking water, aerosol); (2) quantity, quality, and temperature of the water used to dilute the vaccine; (3) dosage; and (4) the combination of different vaccines (e.g., IBV with NDV vaccines). Many of these factors can have a negative effect on the efficacy of the vaccine under field conditions.

Despite the potential negative effect of the maternally derived antibodies on the efficacy of the vaccination in the first days of life (see Immunity), vaccination by spray application of maternally immune 1-day-old commercial chicks is efficacious and routinely performed, especially in the broiler industry. Besides the convenience, vaccination in a hatchery can be much better controlled than that in the poultry house.

Application by the drinking water system requires management measures to be taken to ensure that all birds can drink a sufficient amount of freshly prepared vaccine within a few hours, and should include the complete emptying of the water system before filling it with the vaccine. The water that is used with the vaccine should be of high quality, cold, and free of chemicals that can harm the vaccine such as sanitizers (many municipal sources of water contain chlorine, which can inactivate the vaccine). The incorporation of powdered skim milk at a 1:400 concentration or another suitable product has been shown to stabilize the virus titer during vaccine administration and resulted in better IBV and NDV immune responses in a field trial with 76 flocks (82).

The complexity of mass application methods can easily be underestimated resulting in decreased efficacy and undesired circulation of the vaccine through the flock. This might lead to an increased susceptibility to secondary bacterial infections (49, 85, 129, 130, 164) and reversion of the vaccine virus to virulence (91). As yet, no IB vaccines have been applied *in ovo*; all commercially available vaccines reduce hatchability to unacceptable levels.

There are probably dozens of serotypes/genotypes of IBV currently awaiting discovery, and IBV by nature is constantly changing through mutations and recombinations, which will pose challenges to the poultry industries and to vaccine developers. Given the current technology, it will only be economically feasible to develop new vaccines against a small number of new types of IBV. Therefore, control of IB will continue to involve "juggling" a small selection of vaccines, in combination with good management practices.

Treatment

No specific treatment exists for IB. Provision of additional heat to eliminate cold stress, good air quality,

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elimination of overcrowding, and attempts to maintain feed consumption to prevent weight loss are flock management factors that may help reduce losses from IB. Treatment with appropriate antibiotics may be used to aid in reducing the losses from airsacculitis resulting from infection by secondary bacterial pathogens. In case of clinical nephritis, a decrease in protein levels in the food and a supply of electrolyte replacers in the drinking water might be helpful to compensate for the acute loss of sodium and potassium and thereby reduce mortality from nephritis. The recommended concentration for treatment is 72 mEq of sodium and/or potassium, with at least one-third in the citrate or bicarbonate salt form (54).

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5

Infectious Laryngotracheitis

Maricarmen García and Stephen Spatz

Summary

Agent, Infection, and Disease. Infectious laryngotracheitis (ILT) is an economically important respiratory disease of poultry that affects the industry worldwide. This highly contagious disease is caused by *Gallid herpesvirus type 1* (GaHV-1), and is easily transmitted by infected birds and fomites. Lax biosecurity, transportation of infected birds, and spread of contaminated litter, facilitates the spread of the virus.

Diagnosis. Clinical signs of respiratory disease are not pathognomonic, even when tracheal plugs with mucosal hemorrhage are present. The use of real-time PCR and histopathology are most commonly used to confirm infection.

Intervention. Biosecurity procedures are necessary for prevention, but vaccination is commonly used for control of the disease in endemic regions. Both attenuated vaccines and recombinant viral vectored vaccines can be used alone or in combination. A balance of disease protection with side effects of vaccines must be considered. Control in a region requires both government and industry cooperation.

Introduction

Infectious laryngotracheitis (ILT) is an upper respiratory tract infection of chickens caused by *Gallid herpesvirus type 1* (GaHV-1). This virus can cause severe production losses due to mortality and/or decreased egg production. Severe epizootic forms of the infection are characterized by signs of respiratory distress including gasping and expectoration of bloody mucus, with high morbidity and moderate mortality. The infection is controlled by vaccination and implementation of biosecurity.

Economic Significance

The overall economic significance of ILT has not been precisely determined. However, the poultry industry in the United States can expect to experience multimillion dollar losses each year as a consequence of GaHV-1-induced mortality, decreased egg production, cost of vaccination, and performance penalty due to vaccination reactions particularly in broilers. Similar losses are likely to occur in North America, South America, Europe, and Asia, continents with dense poultry production where ILT is endemic (90).

Public Health Significance

There is no evidence to suggest that GaHV-1 is transmissible to human beings or other mammals.

Etiology

Classification

Gallid herpesvirus type 1 (GaHV-1) is a member of the genus *Iltovirus*, subfamily *Alphaherpesvirinae* of the *Herpesviridae* family within the order Herpesvirales (30). *Psittacid herpesvirus type 1(PsHV-1)*, the causative agent of Pacheco's disease, a lethal respiratory infection of psittacine birds, is a related virus in the genus *Iltovirus*.

Morphology

The GaHV-1 virion has a nucleocapsid of icosahedral symmetry surrounded by a protein tegument layer, encapsulated by an outer envelope with incorporated virus encoded glycoproteins (Figure 5.1A, B).

Chemical Composition

The GaHV-1 genome, contained within the nucleocapsid, is a linear double-stranded DNA molecule composed

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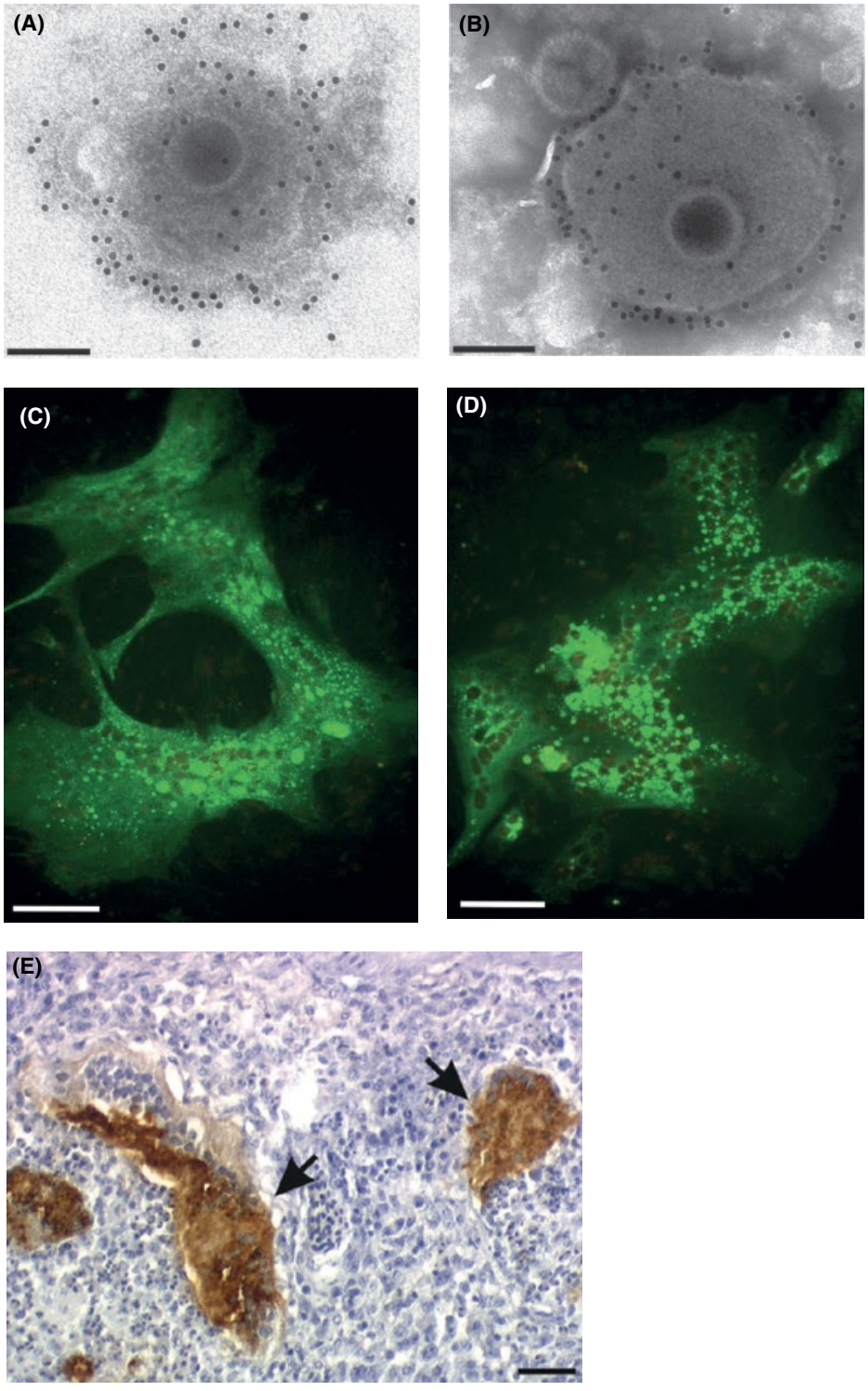


Figure 5.1 GaHV-1 reactivity to monoclonal antibodies (mAb) against viral glycoproteins gC and gJ. (A, B) Immunoelectron microscopy of purified GaHV-1. Virus particles were stained with phosphotungstic acid, and the virion's surface envelope glycoproteins detected by anti-gC mAb (A) and anti-gJ (B) followed by gold-tagged secondary antibodies. (C, D) Indirect immunofluorescence assay in infected chicken kidney cells fixed 24 hours after infection with methanol and acetone (1 : 1). Monoclonal antibody binding was detected with fluorescein-conjugated secondary antibodies, and chromatin was counterstained with propidium iodide. (E) Immunohistochemical staining of a lung section from a GaHV-1 infected chicken was performed by the ABC method. A gJ-specific mAb detected viral antigen in the cytoplasm of several syncytia (arrows). Bars represent 150 nm (A, B), 100 μm (C, D), and 50 μm (E). Used with permission Fuchs et al. (42).

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of a unique long (U_L) and unique short (U_S) region flanked by inverted repeats. Two isomeric forms with differently oriented U_S regions are possible (81). Figure 5.2 shows a schematic diagram of the complete GaHV-1 genome for virulent isolate 63140 (118). On average, the total length of the GaHV-1 genome is 151,607 nucleotides (nt) with unique long and unique short regions of 111,275 and 13,094 nt, respectively, and 13,619 nt comprising the inverted repeats. The complete genome sequences of 34 strains of GaHV-1 have been determined and are available in NCBI GenBank nucleotide database.

Seventy-nine open reading frames (ORFs) are predicted for the GaHV-1 genome: 64 located within the U_L,

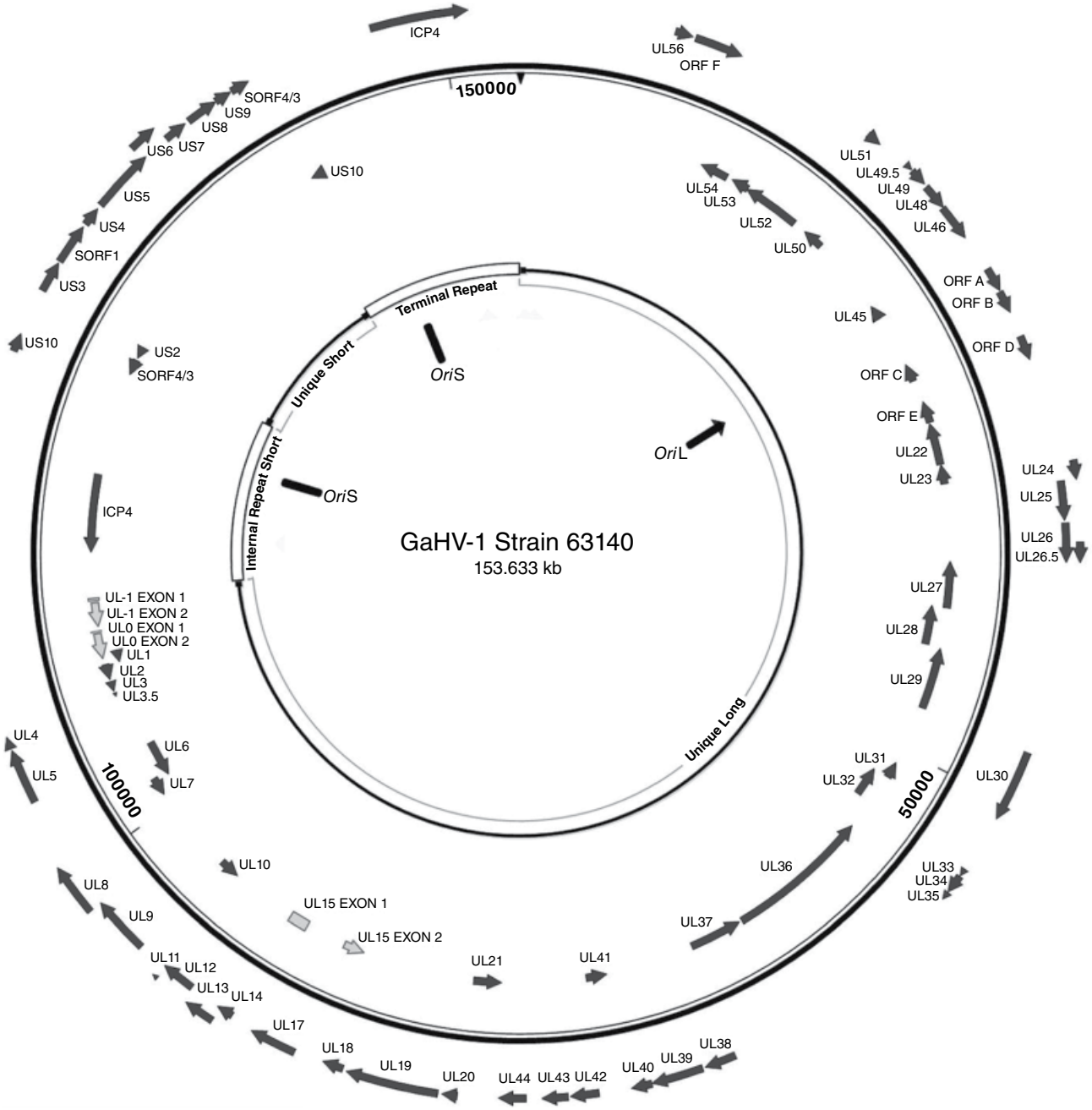


Figure 5.2 Genomic organization of the GaHV-1 strain 63140. The terminal and internal repeat short regions, the unique long region, and the unique short region are shown in the inner circle. The positions of the origins of DNA replication (OriL and OriS) are indicated by a black arrow and black bars, respectively, within the inner circle. Black arrows bracketing the outer circle represent the locations and directions of transcription of the respective open reading frames. The exons of spliced gene products are shown as open gray boxes and gray arrows (Stephen Spatz).

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eight within the U_S, six within the inverted repeats, and one spanning the U_S/TR_S junction. With the exception of eight ORFs (Figure 5.2, ORFs A, B, C, D, E, F, U_L-0 and U_L-1) that are unique to the members of the *Iltovirus* genus, the majority of the remaining ORFs are homologues to genes of other alphaherpesviruses. Other unique characteristics of the GaHV-1 genome is the translocation of the U_L47 gene to the U_S region, which is usually localized within the U_L genome region of other alphaherpesviruses. The GaHV-1 genome also exhibits a large internal inversion of a conserved gene cluster within the U_L region, this inversion comprises the U_L22 to U_L44 genes (42). Besides protein encoding genes, the genome of GaHV-1 also encodes 10 microRNAs (approximately 22 nt) (104). The roles of these microRNAs in the silencing of viral or host transcription/translation are unknown.

Twelve glycoprotein-coding genes have been identified in the GaHV-1 genome (120). Based on chicken antibody responses and mAbs, glycoproteins J and C have been identified as the most abundant surface proteins of GaHV-1 (1, 127, 131, 132). For further information on the identified GaHV-1 gene products and their potential function the reader is referred to a review on GaHV-1 molecular biology (42).

Virus Replication

GaHV-1 initiates infection by attachment to the cell membrane followed by fusion of the viral envelope with the cell plasma membrane. The nucleocapsid is released into the cytoplasm and transported to the nuclear membrane. Viral DNA is released from the nucleocapsid and transported into the nucleus through nuclear pores. Transcription and replication of viral DNA occur within the nucleus (54). The transcription of herpes simplex virus (HSV-1) genes occurs in an ordered pattern of gene expression, immediate–early (IE), early (E), early–late (E/L), and late (L). Transcription of GaHV-1 genes classified the ICP4 as the only immediate–early gene, 30 genes are classified as early, 28 as late, while the transcription kinetics of around 15 genes appeared quite "leaky" because these genes have features of both early and late genes. These findings suggest that the kinetics of GaHV-1 transcription is subjected to a more complex pattern of regulation than those classically described for herpes simplex virus (HSV-1) (88). Following DNA replication concatemeric virus DNA is cleaved and packaged into nucleocapsids that acquire an envelope by migration through the inner lamellae of the nuclear membrane (Figure 5.3A, B) (42, 54). Enveloped particles then migrate through the endoplasmic reticulum and accumulate within vacuoles in the cytoplasm where mature capsids are formed by the incorporation of the tegument material (Figure 5.3C, D) and a secondary envelopment step (Figure 5.3D). Mature virions are then released from cells by exocytosis (Figure 5.3E, F) (42, 54).

Strain Classification

Antigenicity

Early findings using *in vitro* virus-neutralization and *in vivo* cross-protection studies indicated that GaHV-1 vaccine strains and field isolates were antigenically homogeneous (111, 116). More recently the same live attenuated GaHV-1 vaccines continued to offer effective protection against newly evolving field isolates, suggesting that these isolates also remain antigenically related to vaccine strains (75, 80, 108).

Molecular Classification

Due to the conserved antigenicity of GaHV-1, discrimination among viruses has been done mostly at the genome level. In the past twenty years, amplification of a single or multiple viral genes by polymerase chain reaction (PCR) followed by restriction fragment length polymorphisms (RFLP) and/or sequencing analysis have been the most widely used methodology to genotype GaHV-1 strains. A summary of methods utilized to genotype GaHV-1 strains and determine whether viral genotypes from geographically distinct regions correspond to vaccine, field isolates, or both are shown in Table 5.1. In most countries the outbreak related-strains were either viruses closely related to the chicken embryo origin (CEO) vaccines "Vaccinal LT" (CEO-like virus), "vaccinal LT" cocirculating with field viruses, or field virus by itself (31, 90). The identification of "target" genes useful for GaHV-1 strain genotyping has been facilitated with the ever-increasing availability of GaHV-1 full genome sequences. Multi-loci PCR-RFLP, sequencing assays (2, 9, 17), as well as single nucleotide polymorphism analysis by real-time PCR assays (83) have recently been developed.

Full genome sequences of GaHV-1 vaccines and field strains have greatly contributed to our current understanding of the molecular epidemiology of ILT and has established the origins of GaHV-1 strains responsible for disease outbreaks. The phylogenetic relationship among the sequenced GaHV-1 genomes is presented in Figure 5.4. GaHV-1 genomes are distributed into four clades. Clade I contains the CEO vaccine strains from Europe and the United States including the US Hudson strain (Trachivax®, LT Blen®), the Cover strain (Laryngo-Vac®), the Salisbury 146 strain (Poulvac ILT®), the European CEO vaccine Serva and its derivative Nobilis Laringovac, as well as the Russian "O" vaccine strain. In addition, Clade I comprises virulent GaHV-1 strains from Italy (4787 80, 193435 07, and 757 11), China (LJS09 and K317), Australia (ACC78), and the United States (14.939 and 63140). Based on their close relationship to CEO

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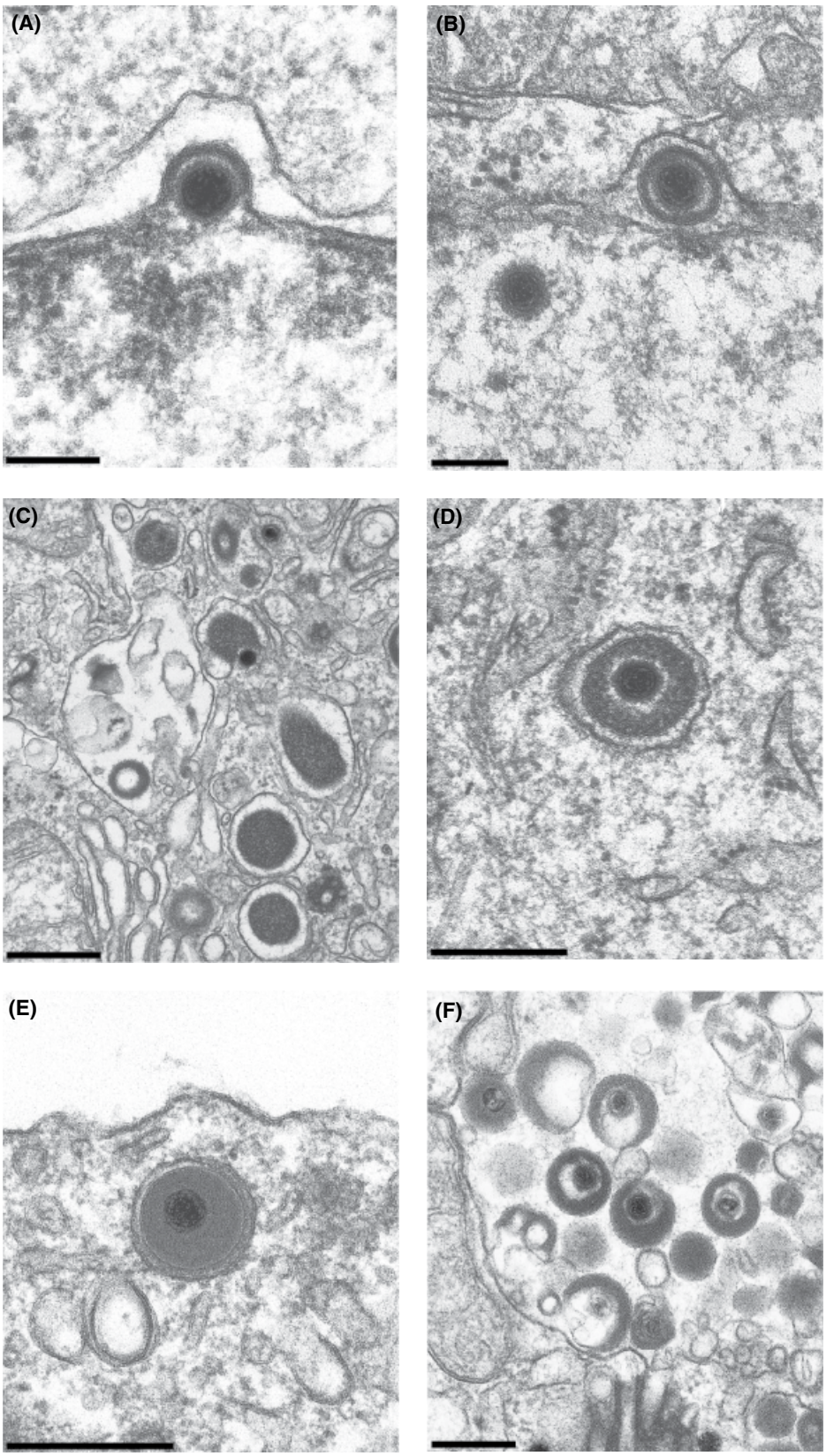


Figure 5.3 Electron microscopy of GaHV-1 morphogenesis. Chicken hepatoma (LMH) cells were fixed and embedded 18 hours after infection, and ultrathin sections were stained with uranyl acetate and lead salts. The micrographs show nuclear egress by primary envelopment of capsids at the nuclear membrane (A, B), and formation of mature and capsid-less particles by assembly of tegument and secondary envelopment in the trans-Golgi region of the cytoplasm (C, D). Virions are released from cells by exocytosis (E, F). Bars represent 150 nm (A and B), 500 nm (C), and 300 nm (D–F). Modified and used with permission of Fuchs et al. (42).

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Table 5.1 Methods and target genes utilized for genotyping GaHV-1.

Region/Country	Year isolated	Method	Genes	Genotype of outbreak viruses	Reference
North America					
Canada	2004	PCR-RFLP	ICP4, gE, UL47/gG	CEO vaccine-like and field virus	(93)
		Sequencing	UL47-gG junction		
United States	1998–2005	PCR-RFLP	ORF B-TK, ICP4, gM/ UL9, UL0/UL-1	CEO vaccine-like and field virus	(94)
South America					
Brazil	2002	PCR-RFLP	TK, gE, gG, and ICP4	CEO vaccine-like and field virus	(12, 13)
		Sequencing	Two fragments ICP4 gene		(12)
Brazil	2004–2011	Sequencing	Two fragments ICP4, TK fragment	CEO vaccine and field virus	(14)
Europe					
United Kingdom	1991–1997	PCR-RFLP	ICP4	CEO vaccine-like	(53)
	2004	PCR-RFLP	ICP4 fragment	CEO vaccine-like	(26)
Varied countries	1994–2007	PCR-RFLP	TK fragment	CEO vaccine-like	(92)
		Sequencing			
Italy	2007–2008	PCR-RFLP	gE, gG, TK, ICP4, ICP18.5, ORF B/TK	CEO vaccine-like	(91)
		Sequencing	gE, gG, TK, ICP4, ICP18.5, ORF B/TK		
Australia					
	1999, 2002–2004	PCR-RFLP	gE, gG, TK, ICP4, ICP18.5, ORF B/TK	Field virus	(74)
	2004 and later	Sequencing	TK fragment	CEO vaccine-like	(36)
	2007–2009	PCR-RFLP	TK, ICP4, ICP18.5	CEO vaccine-like and field virus	(9)
	2009–2015	PCR-RFLP	TK, ICP4, ICP18.5	CEO vaccine-like and field virus	(2)
East Asia					
Taiwan	1985, 1990–1991	PCR-RFLP	TK, gG, gC, ICP4	CEO vaccine-like and field virus	(15)
South Korea	1982–1998	PCR-RFLP	TK, gG	CEO vaccine-like and field virus	(59)
	1986–2012	PCR-RFLP	TK, ICP4, ICP18.5, ORF B/TK, UL47/gG	CEO vaccine-like and field virus	(72)
	2005–2014	Sequencing	UL54, UL52, gB, ICP18.5, gJ, ICP4	CEO vaccine-like	(17)
Israel	2005–2006	PCR-RFLP	TK	CEO vaccine-like and field virus	(28)
Egypt	2007–2010	Sequencing	Two fragments ICP4 gene	CEO and TCO vaccine-like	(115)
India	2010–2011	Sequencing	Fragment gG gene	CEO vaccine-like	(52)

vaccines, these later strains are largely suspected to have emerged from CEO vaccines. Clade II includes the tissue culture origin (TCO) US vaccine LT IVAX, the virulent USDA reference strain, and the US virulent isolate 81658. Clade III encompasses strains from the United States, Australia, and China. This clade includes the US virulent strain J2 isolated from a backyard flock in 2008 and the 1874C5 strain isolated from commercial broilers in 2004 (48). Also, within this clade is the virulent Australian virus CL9 which resulted from natural recombination events between Australian vaccines, A20 and SA2, and the European Serva CEO vaccine (79). More recently genome analysis revealed the emergence of yet another recombinant virus in Australia, identified as class 10 (Figure 5.4). The class 10 virus possibly resulted from recombination events between the SA2 vaccine and natural recombinant virus class 8 (ACC78) included in Clade I (Figure 5.4). These "mosaic" viruses, class 9 and class 10, included in Clade III, have become the dominant strain in Victoria and New South Wales, Australia,

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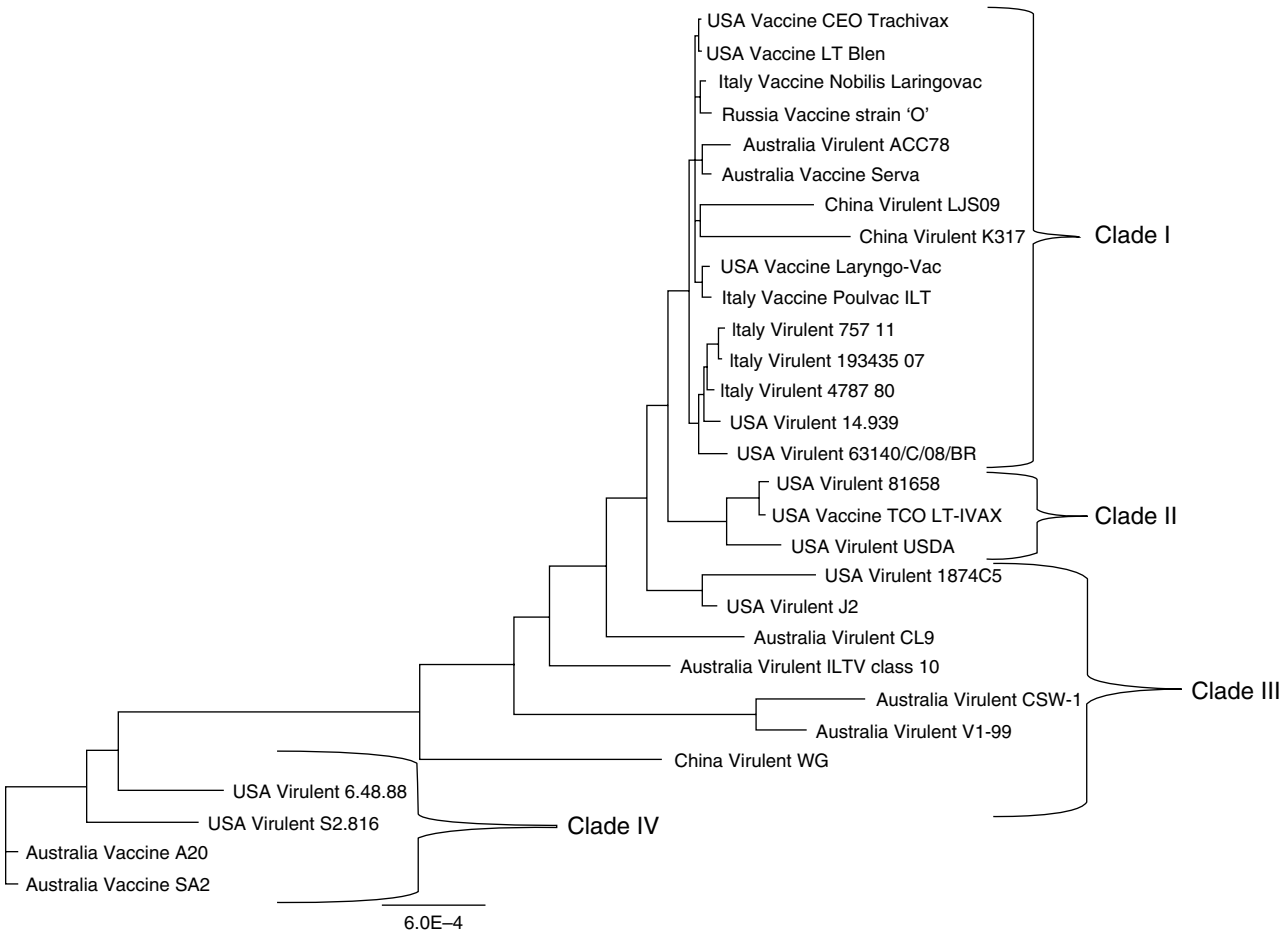


Figure 5.4 Phylogenetic analysis of Gallid herpesvirus 1 genomes. The tree was generated from a multiple alignment of whole genome sequences using the web- based MAFFT program (https://mafft.cbrc.jp/alignment/software/) and phylogeny was derived by the neighbor-joining applied to sequence distance calculated by the Jukes–Cantor method with 100 bootstraps.

respectively (2). Clade III also contains the genomes of independently evolving GaHV-1 viruses from the United States and China. Clade IV contains virulent strains from the United States that originated from backyard flocks and the Australian vaccine strains A20 and SA2. The strain 6.48.88 was isolated from chickens in 1988 and the S2.816 strain was isolate from Peafowl in 2002 (47).

Several comparative genome studies have been conducted with the objective to identify mutations related to virus attenuation/virulence. One study revealed a nonsense mutation within the ORF C gene of the Clade II isolates, which includes the TCO vaccine, indicating that the truncation of this viral protein may be associated with virus attenuation (47). However, a shared conclusion from GaHV-1 comparative genome studies was that rather than any specific single nucleotide polymorphism (SNP) a constellation of genetic differences most likely are responsible for GaHV-1 attenuation and/or virulence (47, 77, 99). More comprehensive mutagenesis and/or gene deleted studies are necessary to define the genotypic determinants of virulence in GaHV-1.

Twenty individual genes have been successfully deleted from the GaHV-1 genome resulting in strains with a wide range of *in vitro* growth defects (42). Among the 20 gene-deleted strains, eight have been evaluated to assess their degree of attenuation *in vivo*. Table 5.2 presents the GaHV-1 gene deleted strains and their relative degree of attenuation.

Laboratory Host Systems

GaHV-1 was first propagated in the embryonating chicken egg, but the virus can also be propagated in a variety of avian primary cell cultures including chicken embryo liver (CELi), chicken embryo lung (CELu), chicken embryo kidney (CEK), and chicken kidney (CK) cells. The susceptibility of CELi, CK, CEK, CELu cells, and embryonated chicken eggs to support GaHV-1 replication was compared and revealed that CELi and CK cells were more susceptible to GaHV-1 infection than CEK, CELu, or embryos, since both CELi and CK cells allowed primary isolation of the virus (66). In

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Table 5.2 Degree of *in vivo* attenuation of GaHV-1 gene deleted strains.

Deleted gene	Protein function	*In vivo* attenuation[a]	Reference
UL0	Nuclear protein	++	(128)
UL23	Thymidine kinase	+	(60)
UL44	Glycoprotein C	< +	(98)
UL47	Tegument protein	< +	(62)
UL50	dUTPAse	< +	(44)
US4	Glycoprotein G[b]	++	(34)
US5	Glycoprotein J	+	(43)
ORF C	Unknown	++	(46)

[a] Estimated degree of attenuation were categorized as low (< +), moderate (+), or pronounced (++) since standardized experiments would be required for precise evaluation.
[b] Chemokine binding protein (35).

embryonating chicken eggs, the virus induces opaque plaques on the embryo chorioallantoic membrane (CAM) that result from necrosis and proliferation of the affected cells (122). Viral cytopathology in tissue culture is characterized by swelling of cells, cytoplasmic fusion, formation of multinucleated giant epithelial cells (syncytia) with large cytoplasmic vesicles (107). GaHV-1 can also be propagated in continuous avian cell lines. A liver tumor cell line named LMH (leghorn male hepatoma) permits the propagation of GaHV-1. The LMH cell line is infrequently used for primary isolation (113), however, it has been instrumental in the reconstitution of recombinant GaHV-1 strains (42). Based on the presence of viral cytopathic effect the GaHV-1 tissue culture infectious dose 50 ($TCID_{50}$) can be determined in CK, CEK, and CELi cells. While the embryo infectious dose 50 (EID_{50}) is determined based on the presence of plaques in the embryo CAM. GaHV-1 plaque forming units can be quantified in CK, CEK, and LMH cells (122).

Pathogenicity

The intratracheal pathogenicity index (ITPI), which measures the gross pathology and microscopic changes in trachea after intratracheal inoculation with GaHV-1, was first utilized to demonstrated that US field isolates were significantly more virulent than live attenuated vaccines (55). However, the ITPI of contemporary field isolates does not necessarily correlate with the severity of the disease they induced. Therefore, other parameters have been utilized to evaluate pathogenicity which include, mortality, signs of respiratory disease, viral genome load in trachea and the conjunctiva, and reduction in weight gain (73, 76, 95, 125). Under experimental conditions it is relevant to choose routes of inoculation that would better reflect the pathogenicity parameters that are being measured. A recent study revealed that the replication of field and CEO vaccine viruses in the trachea and the conjunctiva was greatly influenced by the route of inoculation (ocular, oral, or intranasal). Findings from this study suggests that interactions of the virus with the head associated lymphoid tissue (HALT) will dictate patterns of infectious laryngotracheitis virus (ILTV) replication, which consequently will determine the outcome of infection (8).

Further details on the virus interaction with the trachea and conjunctival mucosa revealed that GaHV-1 can breach the basement membrane of the mucosa, block apoptosis of infected cells, and induce apoptosis of non-infected cells (105). Therefore, interactions of the virus with mucosal epithelial cells promotes virus replication and mucosal invasion which consequently would facilitate the establishment of latency.

Virus transmissibility is also considered an important virulence trait of GaHV-1 vaccine strains and field isolates. Natural recombinant viruses circulating in Australia have shown to have increased infectivity and enhanced transmissibility (78).

Pathobiology and Epidemiology

Incidence and Distribution

Infectious laryngotracheitis is a serious disease of poultry that is particularly common in regions with large poultry concentrations and intensive poultry production. Trends of growing denser poultry populations in shorter cycles, rearing different types of poultry of various ages in the same area, in combination with lax biosecurity are some of the factors that have contributed to the increase of ILT incidence worldwide.

Natural and Experimental Hosts

Chickens are considered the primary host of GaHV-1, but natural infections have been reported in pheasants, pheasant–chicken crosses, peafowls (25), and turkeys (100). Starlings, sparrows, crows, doves, ducks, pigeons, and guinea fowl appear to be refractory to infection (57).

Transmission, Carriers, Vectors

Although bird to bird transmission is necessary to maintain the virus circulating among susceptible populations, indirect transmission of the virus constitutes a significant mode for spreading the infection. Exposure of naïve birds to contaminated equipment, personnel, contaminated litter, manure, and infected carcasses are recognized as

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the main modes to spread the disease. The spread of non-composted litter from vaccinated and/or infected flocks as fertilizer for pastures is considered a practice that can trigger epizootics of the disease (38). Equally, live hauling of infected broilers has been associated with the spread of the disease as infected chickens most-likely release virus on fomites during transportation (31). High viral genome load in feces and dust from experimentally infected chickens (110) as well as the detection of viral genomes in darkling beetles (96), suggest that these can serve as mechanical vectors to spread the disease among flocks in poultry production regions. The role that these vectors play in disseminating the virus warrants further studies.

The sources of GaHV-1 varies, the most direct sources of the virus are respiratory discharges from affected birds or direct contact of birds with contaminated fomites (6). Birds exposed to live virus or vaccine will mount an immune response and stop actively shedding virus and remain latently infected. Upon stress birds can asymptomatically shed virus. Recovered birds are recognized as potential sources of infection for their lifetime (7). Backyard flocks are also considered potential carriers of virus due to their unknown vaccination status and clinical history. A risk assessment study found that broiler flocks affected with the disease were 36 times more likely to be located within a mile of an infected backyard flock (69). Moreover, breeders and layers vaccinated with CEO vaccines serve as reservoirs of virus that can cause outbreaks of the disease due to lax biosecurity in these production sites (38). Incomplete CEO mass vaccination contributes to the establishment of prolonged infections in broiler flocks (48) which can lead to virulent reversion and enhanced transmissibility.

Incubation Period

The incubation period of the disease can be wide-ranging, since it is contingent on the viral dose, route of inoculation, degree of viral virulence, and bird age. Under experimental conditions, the onset of clinical signs can range from 3 to 7 days postinoculation (dpi) (95, 125). Virus replication in the trachea can be detected as early as 2 to 7 dpi (95). Based on field observations clinical signs of the disease commonly appear between 6 to 14 days post exposure.

Clinical Signs

GaHV-1 virus causes an acute respiratory disease in chickens; both severe and mild forms of the disease have been described. Severe forms of the disease are best characterized by increased nasal discharge, moderate to severe conjunctivitis, moist rales, followed by marked dyspnea and expectoration of blood-stained mucus (70). Virus replication causes severe epithelial damage and hemorrhages of the larynx and the trachea mucosa. Mucoid casts/plugs in the trachea obstruct airways and predispose chickens to asphyxiation (106). Mild forms of the disease are characterized by unthriftiness, mild tracheitis and conjunctivitis with swelling of infraorbital sinuses, persistent nasal discharge, and mild respiratory rales (82, 114, 121). In natural outbreaks of the disease most birds will recover in 10 to 14 days after the onset of clinical signs (70).

Morbidity and Mortality

Severe epizootics of the disease cause high morbidity (90–100%) and mortalities of 20% or higher, but usually mortalities are in the range of 5–20%. On the other hand, mild enzootic forms of the disease result in morbidity as low as 5% and very low mortality (0.1–2%) (6).

Pathology

In severe forms of the disease, birds develop nasal discharge and conjunctivitis with frothy ocular secretion and swelling of the infraorbital sinuses (Figure 5.5A). Early during the infection mucoid inflammation of the trachea mucosa can be observed, while degeneration, necrosis, and hemorrhage of the trachea mucosa can occur at later stages. Diphtheritic changes are common in the trachea mucosa and may be seen as mucoid casts that extend its entire length (106). In some cases, severe hemorrhage into the tracheal lumen may result in blood casts (Figure 5.5C) and blood may be found in mucus and necrotic tissue. Inflammation may extend down to the bronchi and into the lungs and air sacs. Mild forms of the disease cause edema and congestion of the epithelium of the conjunctiva and infraorbital sinuses (Figure 5.5B) with gross lesions in the trachea that consist of moderate mucoid tracheitis with varying degrees of hemorrhage localized to the larynx and upper trachea (Figure 5.5D).

Microscopic and Ultrastructural Changes

Early microscopic changes include the loss of goblet cells and infiltration of the trachea mucosa with extensive hyperplasia of respiratory and conjunctival epithelial cells at later times. Multinucleated cells (syncytia) are produced predominantly in ciliated cells of the epithelium (Figure 5.5E, F). Small syncytia, often of circular shape, can be also found beneath the normal ciliated epithelium (102). Nuclear inclusion bodies in syncytia cells are characterized by strong eosinophilic staining surrounded by clear haloes (2 to 5 days) (Figure 5.5G, H).

Between 0 to 72 hours postinfection there is a mild infiltration of lymphocytes and polymorphonuclear cells, presumably heterophils, into the lamina propria and areas where syncytia are present. Between 3–5 dpi

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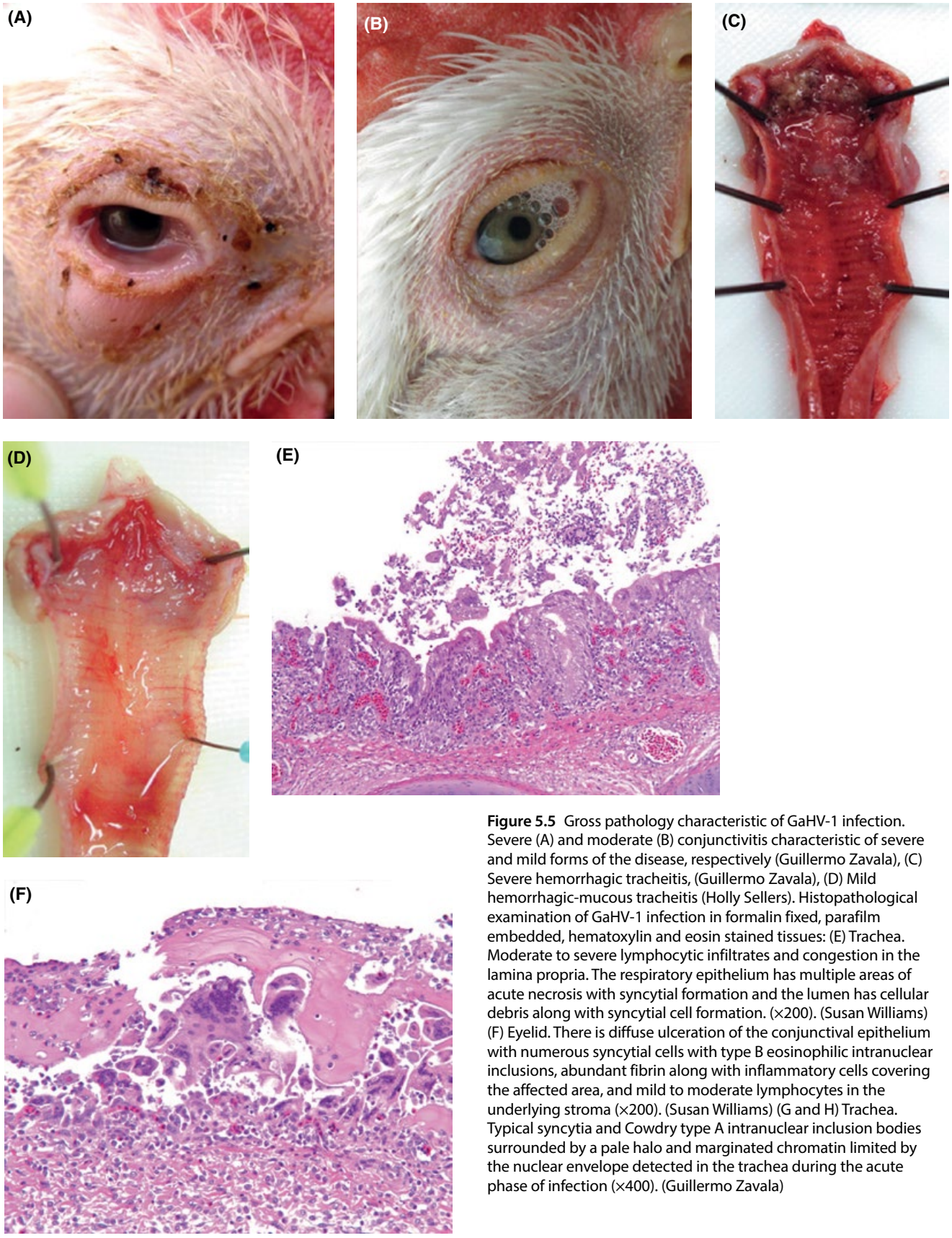


Figure 5.5 Gross pathology characteristic of GaHV-1 infection. Severe (A) and moderate (B) conjunctivitis characteristic of severe and mild forms of the disease, respectively (Guillermo Zavala), (C) Severe hemorrhagic tracheitis, (Guillermo Zavala), (D) Mild hemorrhagic-mucous tracheitis (Holly Sellers). Histopathological examination of GaHV-1 infection in formalin fixed, parafilm embedded, hematoxylin and eosin stained tissues: (E) Trachea. Moderate to severe lymphocytic infiltrates and congestion in the lamina propria. The respiratory epithelium has multiple areas of acute necrosis with syncytial formation and the lumen has cellular debris along with syncytial cell formation. (×200). (Susan Williams) (F) Eyelid. There is diffuse ulceration of the conjunctival epithelium with numerous syncytial cells with type B eosinophilic intranuclear inclusions, abundant fibrin along with inflammatory cells covering the affected area, and mild to moderate lymphocytes in the underlying stroma (×200). (Susan Williams) (G and H) Trachea. Typical syncytia and Cowdry type A intranuclear inclusion bodies surrounded by a pale halo and marginated chromatin limited by the nuclear envelope detected in the trachea during the acute phase of infection (×400). (Guillermo Zavala)

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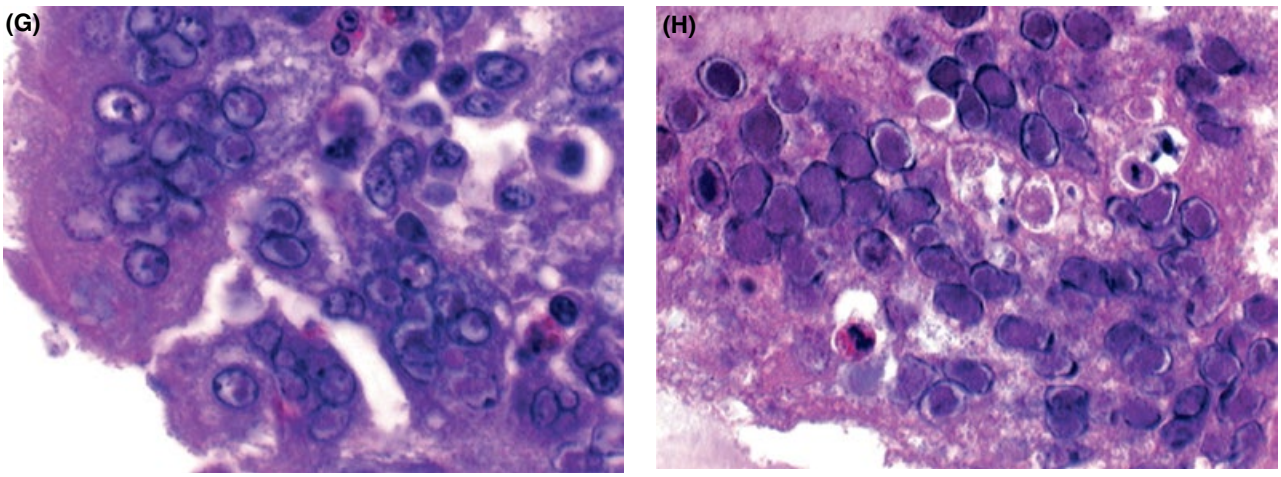


Figure 5.5 (Continued)

there is increased severe edema of the lamina propria and the underlying tissues, with influx of numerous macrophages, lymphocytes, histiocytes, and plasma cells throughout the lamina propria. After 5 dpi blood vessels protrude into the tracheal lumen and hemorrhage occurs. Regeneration of the epithelial lining starts as early as 6 dpi and is well-defined by day 8 (102).

Pathogenesis of the Infectious Process

The progression and outcome of GaHV-1 infection are contingent on multiple factors. Differences in genetic resistance or susceptibility to GaHV-1 infection were first documented in inbred lines of chickens with slight differences in their major histocompatibility complexes (MHC) (85). The inability of inbred one-day-old chickens to efficiently clear GaHV-1 infections was associated with the low number of peripheral CD8+ cells present in younger birds (101). Similar to inbred chickens, vaccination of one-day-–old outbred chickens produced severe reactions caused by the spread of the vaccine virus to the lungs (5). Birds of almost any age can become infected and develop disease. In the field, outbreaks of the disease have been reported as early as three weeks of age (38).

During the acute phase of the disease, between 2–6 dpi, the virus is commonly isolated from the tracheal epithelium (5, 63, 95, 103), around 7–8 dpi there is an abrupt disappearance of virus from the trachea (5). However, low levels of viral genomes can be detected in tracheal swabs 20 to 60 days after vaccination in the trachea using PCR (18, 19). Outside the trachea, GaHV-1 can replicate in the mucosal membranes of the esophagus, pharynx, larynx, conjunctiva, nasal cavity, air sacs, lungs, and bronchi (5, 63, 95, 103, 112). GaHV-1 has also been isolated from brain, spleen, kidney (103), thymus (95), pancreas, and ceca after experimental inoculation (129). Furthermore, viral DNA can be detected in heart, liver, bursa, proventriculus, duodenum, small intestine, cecal tonsils, large intestine, cloaca, and feather pulp (28, 137). Because of widespread tissue distribution, it has been postulated that GaHV-1 establishes extensive viremia (29, 129, 137). However, there is no evidence that peripheral blood lymphocytes or activated T cells support GaHV-1 replication. While macrophages can express viral antigens, they are somewhat refractory to the production of high viral titers (11, 84).

Birds that recover from a GaHV-1 infection do become long-term carriers of the virus. During latency, the virus is dormant in the trigeminal ganglia (TRG) of the ocular sensory nerve until reactivated. Stress inflicted by moving birds and the onset of laying can stimulate the re-excretion of virus in recovered chickens (65). Reactivation has been difficult to reproduce experimentally because unlike other alphaherpesviruses, treatment with immunosuppressive drugs (e.g., cyclophosphamide, dexamethasone) does not reactivate GaHV-1 (67). Virus reactivation can be demonstrated when organ culture explants of TRGs from infected chickens are cocultured with permissive cells (4). The carrier state has been confirmed by successfully isolating virus from trachea organ culture collected from recovered birds (4). Intermittent viral shedding to the trachea has also been demonstrated in live recovered or vaccinated chickens (67).

Immunity

GaHV-1 glycoproteins appear to be the most immunogenic viral antigens capable of eliciting both humoral and cell-mediated responses (42, 130). Early reports showed that passive transfer of antibodies to the offspring (61) or passive transfer of hyperimmune serum to chickens does not protect against disease (40). Furthermore, vaccinated bursectomized chickens are still resistant to infection (40, 41). After GaHV-1 vaccination or challenge the

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levels of either neutralizing or IgG antibodies increased in serum while intermittent increases of IgA, IgM, and IgG antibodies were detected in the trachea mucosa (64, 133). However, the appearance of these antibodies did not correlate with the timing of virus clearance from the trachea. In contrast, thymectomized chickens showed a much lower degree of protection against challenge than non-thymectomized chickens (64). All these early reports affirm the importance of cell-mediated immunity in ILT. The host inflammatory response to GaHV-1 infection plays a crucial role in the outcome of the disease, by controlling viral replication, contributing to the pathology of the disease, and in modulating the adaptive immune response of the host (20). GaHV-1 encodes a viral chemokine-binding protein (glycoprotein G) that regulates the recruitment of inflammatory cells to the trachea. Infection with a glycoprotein G deleted strain showed an increased influx of CD4+ and CD8+ cells into the trachea, while the recruitment of B lymphocytes decreased. The decline of B lymphocytes' influx to the trachea correlated with decreased levels of circulating antibodies (35).

Recent transcript profiling of tracheas from chickens immunized via the ocular/nasal route with the live attenuated CEO vaccine indicated that genes involved in the MHC Class I and MHC Class II antigen presenting and processing pathways were upregulated (86). Activation of the MHC Class I antigen pathways is critical for the stimulation of antigen specific CD8+ T cells, while activation of MHC Class II pathways is critical for the proliferation of specific T helper cell subsets. Also, significant upregulation of IFN-γ mRNA was detected in the trachea of CEO vaccinated chickens as early as six hours post-challenge. This result suggests that the rapid expression of IFN-γ in CEO vaccinated birds may induce early pathways of antiviral responses (124).

Diagnosis

Diagnosis of GaHV-1 requires laboratory confirmation because a diagnosis solely based on clinical presentation can be misleading. Other respiratory pathogens of poultry can cause similar clinical signs and lesions. GaHV-1 diagnosis should be based on more than one test including either histopathological examination of tissues, virus isolation, detection of GaHV-1 antigens, or detection of GaHV-1 genomes (122). The most common assays utilized for the rapid diagnosis of GaHV-1 infection are histopathology examination of tissues paired with real-time PCR.

Histopathology

Lesions produced by GaHV-1 infection are characterized by pathognomonic intranuclear inclusion bodies in respiratory and conjunctival epithelial cells. Intranuclear inclusion bodies can be detected in tissues stained with Giemsa or hematoxylin and eosin. Histopathological examination is considered a rapid diagnosis (results within 24 hours) (56).

Isolation and Identification of Causative Agent

Methods utilized for the isolation of GaHV-1 are listed in the Laboratory Host Systems section. Trachea, conjunctival swabs as well as larynx and lung tissues can be collected for virus isolation. Confirmation of GaHV-1 isolation is achieved by fluorescent antibody (FA) (Figure 5.1C, D) or immunohistochemistry (IHC) staining (Figure 5.1E), and PCR (122). To increase the chances of virus isolation, samples should be collected from birds that are not coughing blood-stained mucus. Although virus isolation is the benchmark criteria in the diagnosis of GaHV-1, it can take up to 1–2 weeks to reach a diagnosis. Therefore, to implement the appropriate measures and avoid the spread of the disease to neighboring flocks the laboratory needs to provide an accurate diagnosis in 24–48 hours. A rapid diagnosis requires collecting samples early in the course of infection since diagnosis based on lesions, viral antigen, and virus nucleic acid detection are inconsistent after 6–10 dpi (10, 56).

Several diagnostic assays have been established for GaHV-1 including the conventional histopathological examination of formalin-fixed, paraffin-embedded tissues. These results are often confirmed with one of the following assays: antigen detection by fluorescent antibodies (FA) in frozen sections (27), antigen detection by IHC in formalin-fixed, paraffin-embedded sections of trachea, larynx, and conjunctiva (Figure 5.1E), or viral nucleic acid detection and quantification from tissues by PCR.

Overall PCR procedures are more sensitive than antigen detection by FA and IHC (10, 27). Due to its inherent speed, sensitivity, and ability to obtain a relative quantification directly from clinical samples, real-time PCR assays have slowly replaced conventional PCR in many poultry diagnostic laboratories. Table 5.3 summarizes the commonly used real-time PCR assays for detection and quantification of GaHV-1 genomes, the targeted genes, and the assay sensitivity as compared to virus isolation and to other PCR methods.

Serology

Among the methods available to measure GaHV-1 antibodies, agar gel immunodiffusion (AGID) was found to be the least sensitive, while enzyme-linked immunosorbent assay (ELISA) and virus neutralization (VN) were found to be equally sensitive. ELISA has the advantage of

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Table 5.3 Nucleic acid detection assays for GaHV-1.

Frequently used real-time PCR methods			
Chemistry	**Gene target**	**Compared to other diagnostic methods**	**Reference**
5′ Taq nuclease	gC	Higher sensitivity than FA[b] or virus isolation	(10)
5′ Taq nuclease MGB probe	TK	Higher sensitivity than virus isolation	(23)
5′ Taq nuclease	ICP4	Higher sensitivity than conventional PCR	(97)
Prime- probe energy transfer	TK	Equally sensitive to 5′ Taq nuclease PCR	(89)
5′ Taq MGB probe	TK	Equally sensitive to 5′ Taq nuclease PCR	(89)
Syber Green	UL15	Higher sensitivity that conventional PCR	(87)
Syber Green	TK	Higher sensitivity than virus isolation	(129)

speed and suitability for testing large sample numbers. However, identification of GaHV-1 infected flocks by ELISA is usually not of diagnostic value because using conventional ELISAs cannot differentiate antibody responses of infected carriers from vaccinated birds. With the advent of recombinant viral vector vaccines carrying GaHV-1 glycoproteins, combination of glycoprotein-specific ELISAs can be used to differentiate infected from vaccinated (DIVA) populations of birds (51, 117).

Differential Diagnosis

GaHV-1 must be distinguished from other respiratory pathogens of poultry that may cause similar clinical signs and lesions. These include the diphtheritic form of avian poxvirus (119) and infections caused by *Mycoplasma gallisepticum* (24), Newcastle disease virus, avian influenza virus, infectious bronchitis virus, and fowl adenovirus.

Intervention Strategies

Control of ILT generally involves routine vaccination of broiler-breeders, layers, and vaccination of broilers if necessary, while implementing biosecurity. Currently there are two categories of vaccines utilized in the control of ILT; the live attenuated vaccines used since the early 1960s and the recombinant vaccines first introduced in the United States in 2004. Since the introduction of recombinant vaccines, the variety of vaccination programs in commercial layers and broilers has significantly expanded particularly in the United States.

Vaccination

Since their introduction live attenuated vaccines have proven to induce effective protection against the disease. However, latent carriers, incomplete mass vaccination, and lapses in biosecurity has allowed vaccine strains to circulate in the field, regain virulence, and become the source of outbreaks (see Molecular Classification). As a response of "vaccinal LT" outbreaks, recombinant viral vector vaccines expressing GaHV-1 antigens were developed. Unlike live attenuated vaccines, these vectored vaccines can't revert to virulence, lack the ability to transmit from bird to bird, cannot reactivate from latency, and are less likely to recombine to generate virulent progeny.

Live Attenuated GaHV-1 Vaccines

Most GaHV-1 live attenuated vaccines originated from virulent field isolates that were attenuated by sequential passage in embryonating chicken eggs and/or tissue culture. Vaccines produced in embryonating eggs are categorized as chicken embryo origin (CEO) vaccines. There is a large variety of GaHV-1 CEO vaccines produced worldwide (90). Currently the only TCO vaccine produced is the LT-IVAX (Merck, Animal Health). For a review on the origins of US, Australian, and European GaHV-1 live attenuated vaccines the reader is referred to two review articles (21, 45).

Chicken embryo origin vaccines can spread horizontally (19, 109) and can revert to virulence after limited back passages in naïve chickens (55). These experimental findings are in agreement with molecular epidemiology data suggesting the CEO related vaccines are the source of some outbreaks of the disease (see molecular epidemiology Table 5.1). Because of its modest ability to spread horizontally, the TCO vaccine has a lower potential for virulence reversion (55, 109).

Live attenuated CEO vaccines can be given via eye drop, or through mass vaccination via the drinking water or coarse aerosol spray, whereas the TCO vaccine can only be given via eye drop. In the United States most breeders and commercial layers are vaccinated with either CEO or TCO vaccines.

Although both CEO and TCO vaccines induce effective protection against disease, the protection efficacy of

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the CEO vaccines is better than that induced by TCO vaccination (123). In the face of a US outbreak, broilers are vaccinated with CEO vaccines via mass vaccination, while in many other countries either CEO or TCO vaccines are applied individually via eye drop route. Vaccination of broilers with CEO can cause significant post-vaccination reactions associated with performance penalties (135). To increase flock coverage and decrease CEO vaccination reactions, mass vaccination via the drinking water is preferred over coarse aerosol spray vaccination in broilers (48).

Viral Vectored GaHV-1 Vaccines

GaHV-1 viral vector vaccines were introduced more than 10 years ago into the United States and are available in North America, South America, Europe, the Middle East, and Asia. Only two types of vector vaccines are currently available; the fowlpox virus (FPV) and the turkey herpesvirus (HVT). The FPV vector expresses the GaHV-1 glycoprotein B and UL32 protein. This vaccine was first licensed in the United States for wing-web vaccination of breeders and subcutaneous vaccination of one-day-old commercial layers (32). Turkey herpesvirus vaccines, expressing GaHV-1 glycoproteins I and D, and a more recent one expressing glycoprotein B are also available (39).

In the United States HVT vector vaccines were originally only licensed for subcutaneous and transcutaneous application. Currently layers and breeders are vaccinated subcutaneously at one day of age, while broilers are vaccinated *in ovo*. Experimental evidence had shown that when applied *in ovo* HVT vector vaccines improve broiler performance, reduce clinical signs, and prevent mortality. However, they are not as effective in curtailing shedding of the challenge virus as the CEO vaccines (68, 126). The onset of protective immunity elicited by HVT vector vaccines is between 4 to 6 weeks after *in ovo* vaccination (39, 126). Other technical aspects that diminish the protection efficacy of GaHV-1 vector vaccines, are faulty *in ovo* delivery of the vaccine, use of fractionated vaccine doses, and interference of GaHV-1 protection by coadministration of other recombinant vaccines (3, 45).

Future GaHV-1 Vaccines

In an effort to produce more stable vaccine strains with diminished possibilities of regaining virulence, strains of GaHV-1 have been attenuated by deleting genes associated with virulence and delivered via eye drop, drinking water, and *in ovo* (22, 33, 46). Alternatives to the HVT and FPV vector vaccines, the LaSota strain of Newcastle disease virus (NDV) (71, 134, 136) and Marek disease virus (MDV) (50) expressing GaHV-1 genes have been assessed for safety and protective efficacy. Specifics on the protection efficacy and safety of these potential GaHV-1 vaccines has been reviewed elsewhere (45).

Vaccination Programs

Because of the high risk of infection and unacceptable economic consequences, most long-lived birds are vaccinated against GaHV-1. With the introduction of vector vaccines, and the use of CEO vaccines banned in some countries, the diversity of vaccination programs has markedly expanded. Based on a survey conducted among poultry veterinarians from the United States, GaHV-1 vaccination of breeders is mainly performed via eye drop with the TCO vaccine. In instances where breeder flocks are located in endemic zones or in regions with a high density of broiler and commercial layers vaccination occurs twice with the CEO vaccine via the drinking water, at 4–5 and 1012 weeks of age. Commercial layer flocks are initially vaccinated subcutaneously at one day of age with an HVT or FPV vector vaccine followed by eye drop vaccination with CEO or TCO, or CEO applied in the drinking water between 8–12 weeks of age. Alternatively, FPV vector vaccine is administered via the wing web between 5–12 weeks of age. Currently vaccination programs for broilers frequently consists of recombinant vaccines paired with CEO vaccines. Producers of larger birds (56 to 70 days of age) use either CEO by itself, or in combination with vector vaccines. While small bird producers (40 to 45 days of age) solely rely on vector vaccines to avoid the potential of harsh respiratory reactions induced by CEO vaccination. In instances when outbreaks of the disease cannot be contained with vector vaccines, some companies are forced to use CEO vaccines and absorb the performance penalty. Vaccination with CEO is regularly done in the drinking water between 7–12 days of age. However, in some situations companies will first vaccinate with CEO and use vector vaccines to wean off of CEO usage once the outbreak has been controlled. Companies with flocks in highly endemic areas may vaccinate with vector vaccines in anticipation of outbreaks. Contingent on the severity of broiler outbreaks, companies and government authorities may delineate a "vaccination zone" around the area where cases are reported. State veterinarians can also negotiate an agreement with companies for a "blanket vaccination" program (38).

Management Procedures

"Infectious laryngotracheitis is a community disease and if your neighbor has it, you may get it". This emphasizes the need for communication and transparency among producers. Since geographic regions are densely populated with a variety of poultry, open channels of communication among poultry producers regarding vaccine use, vaccination methods, bird movement, personnel movement, and litter disposal are pivotal to manage ILT. During outbreaks of the disease collaboration between government,

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industry, and universities had proven to be advantageous in establishing swift countermeasures (38).

Spread of "vaccinal LT" from commercial multi-age layer production facilities can be reduced with appropriate biosecurity measures such as cleaning and disinfecting equipment and vehicles, in-house litter composting, appropriate dead bird disposal, rodent and insect control measures, and discouraging visitors from visiting other production sites (58). Compared to commercial layers, breeder flocks have an enhanced level of biosecurity. Special precautions should be taken to obtain a complete vaccination history when mixing breeding stock particularly when introducing "spiking males" to the flock.

Management practices to prevent ILT in broiler complexes is a more challenging process because uninterrupted biosecurity is needed not only during outbreaks, but during vaccination and clean-outs. In order to decrease the carryover of CEO vaccine through successive grow-outs, adequate cleanup and extended downtime are necessary. Between CEO vaccinated grow-outs, birds should not be introduced in the house for a period of 14 to 21 days. For downtime periods of 21 days it is recommended to heat the house to 100 °F for 72 to 100 hours. For downtime periods shorter than 21 days, in addition to heating and disinfection, wash down of the house and waterlines are recommended (37). Broilers in the United States are grown in built-up litter and management of litter during ILT outbreaks or CEO vaccination is crucial to control the spread of the disease. After heating the house, it is recommended to remove caked litter, windrow the litter and compost it in-house for three days before new birds are placed (49). In addition, to remove litter from the house some companies request growers to produce one flock without CEO vaccination before spreading litter onto pastures. In some instances, longer downtime and enhanced biosecurity audits are required to clear the virus (16).

Treatment

No drug has been shown to be effective in reducing the severity of lesions or relieving clinical signs.

Eradication

When GaHV-1 vector vaccines were first introduced the idea was to slowly eliminate CEO vaccination particularly in broiler and commercial layers. It was expected that this reduction would stop the continuous "vaccinal ILT" outbreaks. But since viral vector vaccines are not as effective as the CEO vaccine in containing outbreaks, CEO vaccination is still being utilized to maximize protection. However, faulty applications and lax biosecurity may still perpetuate vaccinal revertants. In the past decade, research efforts had delineated the weakness and strengths of both vector vaccines and live attenuated GaHV-1 vaccines. Alternative vector vaccines and new vaccination strategies against GaHV-1 are likely to become available in the next decade. In the United States ILT seems to be a more serious problem in broilers than in layers. The broiler industry had concluded that the ideal vaccine should be cost-effective, safe, and significantly reduce viral shedding. It should also induce protection comparable to that of the CEO vaccines and have the following characteristics: inability to revert to virulence, unable to establish latency, compatibility with other vaccines, and easily administered by mass vaccination.

Whether or not the perfect vaccine against ILT could be developed, the control of ILT cannot be sustained only by vaccination. Effective disease control will always require strict implementation of biosecurity alongside vaccination. New experimental evidence has indicated that feces, dust, feathers, and beetles from contaminated environments carry high loads of viral genomes. Whether these are truly alternative modes of virus spread needs to be investigated. If proven true, more rigorous biosecurity practices may need to be implemented. Although it appears that the prevalence of GaHV-1 is increasing worldwide the knowledge acquired in the past decade may be deemed invaluable for future eradication stratagems.

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6

Influenza

David E. Swayne, David L. Suarez, and Leslie D. Sims

Summary

Agent, Infection, and Disease. Avian influenza (AI) is caused by type A influenza virus classified into 16 hemagglutinin (H1–H16) and nine neuraminidase (N1–N9) subtypes. Most infections are subclinical in poultry, but some low pathogenicity (LP) AI strains (H1–12) have produced respiratory disease, diarrhea, and/or drops in egg production. High pathogenicity (HP) strains (H5 and H7 strains) produce severe systemic disease in gallinaceous poultry but variable disease and mortality in waterfowl. Low pathogenicity avian influenza (LPAI) viruses are found worldwide in wild aquatic birds. The emergence and intercontinental spread of Goose/Guangdong lineage H5Nx HPAI viruses and H9N2 LPAI viruses has exposed vulnerabilities in disease prevention and control systems especially within large, complex bio-insecure production and market chains.

Diagnosis. Reverse transcription-polymerase chain reaction (RT-PCR) is commonly used to diagnose avian influenza infections, with type A detection targeted to the matrix or nucleoprotein genes, and subsequent subtype detection of at least the H5 and H7 subtypes. Sequencing directly from clinical samples is being more commonly used. Isolation of virus, primarily in embryonating chicken eggs, is still recommended to allow full characterization of an isolate. Pathotype is determined by sequencing and/or *in vivo* tests (intravenous pathogenicity test). Serologic detection of exposure to AI virus utilizes enzyme-linked immunosorbent assay (ELISA) (type A), agar gel immunodiffusion (type A), and/or hemagglutination-inhibition (HI) tests (H subtype specific).

Intervention. Biosecurity is the primary preventive measure but weaknesses in biosecurity systems results in infection on some farms. Virus elimination is the preferred strategy for HPAI and H5/H7 LPAI control when outbreaks occur in previously AI-free countries or areas. Vaccination is also being used as a preventive and emergency control measure for both LPAI and HPAI.

Introduction

Disease in poultry caused by Type A influenza viruses in the family *Orthomyxoviridae* has been recognized since the late nineteenth century causing mainly sporadic, but serious disease outbreaks. Aquatic birds are the natural hosts of Type A influenza viruses. A dynamic cycle of infection occurs within aquatic avian species, many of which are migratory, and between these birds, poultry, and other animals. Spillover of viruses from wild aquatic birds to poultry and other species occurs frequently. Many of these spillovers are transient unless these influenza viruses evolve to become adapted to specific hosts. Until 2003 most spillover events from wild birds involved low pathogenicity viruses some of which subsequently converted to HPAI viruses in poultry. Since 1959, most HPAI viral outbreaks have been handled by stamping-out programs with the majority being eradicated.

In 1997, a H5N1 HPAI virus related to a virus first detected in Guangdong province in China (A/goose/Guangdong/1/1996 [Gs/GD] lineage viruses) in 1996 caused severe fatal disease in both poultry and humans in the Hong Kong Special Administrative Region (SAR). Subsequent spread of these Gs/GD lineage H5Nx viruses (initially H5N1, but later other N subtypes) from China into other parts of Asia, the Middle East and onwards to Europe, Africa, and North America occurred from 2003 onwards in a series of (mainly) wild bird-mediated intercontinental waves (85) as well as through cross border trade in poultry. During the same period H9N2 viruses capable of causing moderate to severe disease in chickens, but still meeting the definition of LPAI virus, also emerged and spread widely in Asia, the Middle East, and North and West Africa. The Gs/GD lineage H5Nx HPAI

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and H9N2 LPAI viruses remain endemic in poultry in a number of Asian, Middle Eastern, and African countries.

Today, orthomyxoviruses are recognized as the cause of significant numbers of natural infections and disease, usually of the upper and lower respiratory tract, in humans, horses, pigs, dogs, and various avian species. Sporadic infections and disease have occurred in mink, cats, and a variety of marine and terrestrial mammals (326). Infection of domestic poultry by AI viruses typically produces syndromes ranging from asymptomatic infection to respiratory disease and drops in egg production to severe, systemic disease with near 100% mortality (301). The clinical disease produced depends on the virus strain, and the species, age, and immune status of the infected poultry, and concurrent infections. Disease is usually absent with AI virus infection in most free-flying waterfowl species unless the strain involved is a Gs/GD lineage H5Nx HPAI virus (251).

For more detail, see these books (46, 48, 210, 244, 245, 305, 307), special issues of journals (165), proceedings of International Symposia on Avian Influenza (16, 73, 74, 265, 267, 268, 280, 287, 292) and websites of international animal health and public health agencies (www.fao.org, www.offlu.net, www.oie.int, www.who.int).

Definitions and Synonyms

"Avian influenza" is a broad term used to describe any infection or disease in birds caused by Type A influenza viruses. The term "avian influenza viruses" is used to describe influenza A viruses found customarily in birds. Wild waterfowl and other aquatic birds are the primordial reservoir of all influenza A viral genes (301). The appellation "bird flu" is used as a simplified alternative to AI by the media to describe infections in poultry, humans, and other mammals with Type A influenza viruses derived from birds.

The highly lethal systemic disease caused by AI viruses is referred to as "highly pathogenic avian influenza" or "high pathogenicity avian influenza" (HPAI) (301). Prior to 1981, HPAI was known by various names including fowl plague (most common), fowl pest, peste aviaire, Geflügelpest, typhus exudatious gallinarium, Brunswick bird plague, Brunswick disease, fowl disease, and fowl or bird grippe (257). Milder forms of AI have been termed low pathogenic, non-highly pathogenic, and low pathogenicity AI (LPAI) (301). Their impact on poultry production and trade has generally been much lower than with HPAI. The official designation since 2002 is "low pathogenicity" for AI viruses that are not HPAI viruses (301).

HPAI and H5/H7 LPAI are of interest to the World Organization for Animal Health (Office International des Epizooties [OIE]). For the latter, because they can mutate unpredictably from LP to HP viruses naturally in poultry. For more information see Pathotype subsection under Strain Classification.

Economic Significance

Economic losses from AI have varied depending on the strain of virus, species of bird infected, number of farms involved, control methods used, and the speed of implementation of control or eradication strategies. Many of the economic losses associated with outbreaks of HPAI are due to disruption of the poultry supply chain, especially if wide area culling around infected premises is used. In most high income countries, HPAI and LPAI have not become endemic diseases in the commercial poultry industries. Most outbreaks and economic losses have occurred from epidemics of HPAI or LPAI in commercially raised poultry, predominately chickens (*Gallus gallus domesticus*) and turkeys (*Meleagridis gallopavo*) and have been managed by stamping out. In some lower and middle income countries, LPAI is endemic in commercially raised poultry especially viruses of the H9N2 subtype that have spread across Asia, the Middle East, and North and West Africa. In countries where live birds are sold through live poultry markets (LPM), a range of avian influenza virus subtypes including Gs/GD lineage H5Nx HPAI viruses, H9N2, H7N9 (China only), and H6N1 LPAI viruses have been detected. As of January 2018, Gs/GD lineage H5Nx HPAI has become endemic in poultry in China, Southeast Asia (Cambodia, Indonesia, Myanmar, Viet Nam), South Asia (Gangetic plain), the Middle East (Egypt, Iran), and West Africa (Nigeria) (301). Spillover of virus to other countries (through local cross-border trade or long distance wild bird carriage) also occurs, but virus may not persist especially if poultry density is low or preventive and control measures can be implemented quickly following detection of incursions. The manner in which Gs/GD-lineage H5 HPAI virus is maintained differs between countries. Domestic ducks play an important role in China, Cambodia, and southern Viet Nam because of their capacity to be infected subclinically whereas in other parts of Asia the virus is likely maintained because of the size of the industry (farms with varied biosecurity systems located too close together), uncontrolled movement of millions of live poultry through complex market chains (including poorly managed LPM), and weak surveillance systems that do not detect or report the majority of "HPAI-compatible" events.

The most accurate reports on losses have come from HPAI eradication programs (Table 6.1). Direct losses in HPAI outbreaks include costs associated with high morbidity and mortality in affected flocks, depopulation and disposal costs, cleaning and disinfection, quarantine and

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Table 6.1 Examples of economic losses from high pathogenicity (HP) and low pathogenicity avian influenza (LPAI) epidemics as reported in US dollars (reviewed in [299, 304]).

Year	Outbreak	Birds dead or culled	Cost item	Original cost ($US unless otherwise indicated)	Cost in 2017 ($US)	Cost/farm in 2017 ($US)
HPAI						
1924–1925	USA – Fowl plague	Unknown	Direct losses	1 M	14.2 M	—
1983–1984	USA – H5N2 HPAI	17 M (449 farms)	USDA eradication	63 M	153.6 M	342,000
			Non-indemnified industry losses	15 M	36.6 M	8150
			Increased customer costs	349 M	851 M	1.9 M
1985	Australia – H7N7 HPAI	238,518 (1 farm)	Eradication cost	1.4 M	3.2 M	3.2 M
1999–2000	Italy – H7N1 HPAI	13 M (413 farms)	Compensation	100 M	141 M	341,000
			Indirect costs	500 M	705 M	1.71 M
1997	Hong Kong – H5N1 HPAI	1.5 M	Eradication	13 M	19.7 M	—
Feb – May 2003	Netherlands – H7N7 HPAI	30 M (1636 farms)	Government, control of the disease	€270 M	377 M;	230,000
			Industry trade disruption	€500 M	698 M	426,000
Late 2003–2017	Asia – H5Nx HPAI	>300 M	Losses to the poultry industries	>10B	>10B	—
2014–2015	USA – H5N2/H5N8 HPAI	50.4 M (211 premises)	USDA eradication	650 M	669.5	3.2 M
			Indemnity	200 M	206 M	0.93 M
			Direct costs economy-wide	3.3B	3.5B	16.1 M
2016	USA – H7N8 HPAI & LPAI	397,000 (12 premises)	USDA eradication	16.3 M	16.6 M	1.4 M
			Indemnity	4.5 M	4.6 M	383,000
2017	USA – H7N9 HPAI	127,000	Indemnity	1.18 M	1.18 M	590,000
LPAI						
1978	Minnesota USA – various LPAI	141 farms	Losses to the poultry industries	5 M	17.3 M	123,000
1995	Minnesota USA – H9N2 LPAI	178 farms	Losses to the poultry industries	6 M	8.9 M	50,000
1978–1995	Minnesota USA – various LPAI	1058 farms	Losses to the poultry industries	22 M		21,000
1995	Utah USA – H7N3 LPAI	2 M (60 farms)	Losses to the poultry industries	2 M	3 M	50,000
2002	Virginia USA – H7N2 LPAI	4.7 M (197 farms)	USDA eradication	81 M	101 M	513,000
			Losses to the poultry industries	130 M	163 M	827,000
			State government	1 M	1.25 M	6,300

surveillance costs, cost of vaccination (if used), and indemnities paid for birds that are culled. However, indirect costs such as uncompensated losses to the poultry industry including temporary or permanent loss in poultry exports, income lost by farmers and communities during the production downtime, increased consumer costs from reduced supply of poultry products, and losses from decreases in consumer purchases can easily

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dwarf direct losses 5–10 fold. The economic costs for eradication of HPAI have varied greatly, but eradication costs are high and appear to be proportional to the number of birds that died or were culled (Table 6.1). For example, the federal eradication cost, (including indemnities and adjusted for inflation) for the 1983–1984 US H5N2 HPAI epidemic were $342,000 per farm while for the 2014–2015 US H5Nx HPAI epidemic the costs were $4 M per farm. The major change between these outbreaks was an increase in the scale of production. Over this 30 year period the population of egg layer farms increased from 100,000–500,000 birds to 1–5 million birds.

Low pathogenicity AI outbreaks have also caused significant economic losses for producers of chickens, turkeys, ducks, and ostriches, especially when accompanied by secondary bacterial or viral pathogens, but accurate documentation of such costs are generally not available. Losses in general have been less than with HPAI outbreaks because infected flocks have typically been eliminated through controlled marketing programs, the mortality rates have been lower, fewer federal eradication costs were incurred, and national and international trade usually is less disrupted. In some cases bans are placed on imports by some countries when H5/H7 LPAI occurs (Table 6.1) and stamping-out has been used to eradicate H5/H7 LPAI in part because of the prior requirement to report outbreaks to OIE. Losses from LPAI epidemics include mortality losses, losses from reduced egg production that may not recover to preinfection levels, increased condemnations at slaughter, medication against secondary bacterial infections, cleaning and disinfection, delayed placements of new birds, and, for LP H5/H7 outbreaks, restrictions in trade of poultry and poultry products. Poorly documented but also costly have been the endemic H9N2 LPAI poultry infections in much of Asia, the Middle East, and Northern and West Africa; and H5N2 LPAI poultry infections in Mexico, Central America, and the Caribbean with vaccination programs adding to the cost of production (303). Since non-H5/H7 LPAI is usually not dealt with using stamping-out programs, the costs of elimination of LPAI are usually unknown. However, when a stamping-out program was undertaken in the Virginia 2002 H7N2 LPAI outbreak involving mostly meat chickens and turkeys, the eradication program had similar costs as previous HPAI outbreaks (Table 6.1). Since 2005 in high income countries, stamping-out programs have emerged to be a common control method for H5/H7 LPAI, although methods used to achieve this vary it may include vaccination followed by controlled marketing (230).

The large investment (greater than $US1 billion) by donors and governments into control and prevention of avian influenza following the emergence of Gs/GD lineage H5N1 HPAI in Asia was largely due to zoonotic infections in humans and the potential that H5N1 could become a pandemic influenza strain whose costs could exceed $US3 trillion (59).

Public Health Significance

In general, influenza viruses exhibit host species adaptation with transmission occurring most frequently and with ease between individuals of the same species; occasionally interspecies transmission to closely related species occurs (231). Although rare, AI viruses or their genes have been transferred to humans (Table 6.2): (1) transfer of complete AI viruses (*in toto*) with individual sporadic infections, and (2) appearance of individual AI viral gene segments in pandemic human influenza viruses (i.e., reassortment of gene segments). Such transfers have produced rare severe clinical disease with hospitalization, although serological evidence suggests cross-species transmission resulting in subclinical infection or mild disease occurs more frequently (59). The H5Nx Gs/GD lineage HPAI viruses, and H7N9 LPAI and HPAI viruses have caused more clinically significant human infections than all the other AI viruses combined (Table 6.2). Although considerable research has been conducted, we currently can't predict which AI viruses are likely to infect and cause severe disease in humans or other mammalian species. For more details see Chapter One, Disease Prevention, Diagnosis, Control, and Public Health, in the subchapter, Public Health Significance of Poultry Diseases.

History

The term "influenza" originally referred to epidemics of acute, rapidly spreading, catarrhal fevers of humans caused by viruses in the family *Orthomyxoviridae* (135). The main historical features of AI include: (1) initial HPAI cases reported in Northern Italy (H7 HPAI, 1878) and clinical differentiation of HPAI from fowl cholera in 1880, (2) etiology demonstrated as a filterable agent in 1901, (3) spread of HPAI from 1901 New Brunswick Poultry Show throughout Europe with endemicity until the mid-1930s, (4) broad geographic dissemination of HPAI in the early twentieth century (Middle East, Asia, North Africa, and North and South America), (5) recognition of LPAI in chickens (Dinter strain, 1949), (6) HPAI virus classified as an influenza virus in 1955 with all being H7N1 and H7N7 subtypes, (7) identification of H5 HPAI in chickens in 1959 and common terns (*Sterna hirundo*) in 1961 (Table 6.3), (8) H9N2 viruses were first identified in poultry in the 1960s, (9) identification of LPAI viruses from asymptomatic wild aquatic birds (i.e., migratory ducks in California and from a pelagic seabird in Australia, 1972) as being a wildlife reservoir, (10) understanding of HPAI viruses arose from mutation of LPAI viruses at the proteolytic cleavage site of the

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Table 6.2 Listing of confirmed human clinical cases of infection with avian influenza (AI) viruses (data for 1959–2007 cases reviewed in [299]) (59).

Year	Virus	Location	Symptoms	Exposure	Cases	Deaths
1959	H7N7 HPAI	United States	Hepatitis	Unknown	1	0
1977	H7N7 HPAI	Australia	Conjunctivitis	Laboratory accidental exposure	1	0
1978–1979	H7N7 LPAI	United States	Conjunctivitis	Seals with respiratory disease	3	0
1996	H7N7 LPAI	United Kingdom	Conjunctivitis	Tending domestic ducks that mixed with wild ducks on a pond	1	0
1997	H5N1 HPAI	Hong Kong	Influenza-like-illness (ILI), respiratory symptoms, multi-organ failure	Exposure to live poultry market	18	6
1998–2013	H9N2 LPAI	China, Hong Kong, Bangladesh	ILI, respiratory symptoms	1 = contact with live poultry in LPM, 7 = not reported	15	0
2002–2003	H7N2 LPAI	United States	1 = asymptomatic, 1 = respiratory symptoms	1 = outbreak crew, 1 = unknown	2	0
2003	H7N7 HPAI	Netherlands	Conjunctivitis > > influenza-like illness > other symptoms	Depopulation crews, poultry workers, and farmers from poultry H7N7 HPAI outbreak	89	1
2004	H7N3 HPAI	Canada	Conjunctivitis, coryza, and headache	Depopulation crews for poultry H7N3 HPAI outbreak	2	0
2004	H10N7 LPAI	Egypt	Fever and cough	Unclear but father was poultry merchant	2	0
2006	H7N3 LPAI	United Kingdom	Conjunctivitis	Poultry	1	0
2007	H7N2 LPAI	United Kingdom	ILI and/or conjunctivitis	Smallholding poultry	4	0
2003–2017	H5Nx HPAI	Asia, Africa, Canada[a]	ILI and respiratory symptoms > > gastrointestinal	Exposure to live or dead infected poultry in LPM or villages	860	454
2013–2018	H7N9 LPAI/ HPAI	China[b]	ILI, lower respiratory disease, multi-organ failure	Exposure to poultry in LPM	1625	622
2010	H10N7 LPAI	Australia	Mild conjunctivitis and ILI	Exposure to infected chickens in abattoir	5	0
2013	H6N1 LPAI	Taiwan	ILI	Unknown	1	0
2013	H10N8 LPAI	China	Febrile respiratory illness (accompanied by underlying medical problems)	2 = visiting poultry market, 1 = unknown	3	2
				Total	2633	1085

[a] Reflects the cases as of February 03, 2018 (335).
[b] Reflects the cases as of March 28, 2018. Includes three cases of H7N9 HPAI virus (http://www.fao.org/ag/againfo/programmes/en/empres/h7n9/situation_update.html).

hemagglutinin in 1983, and (11) increased reports of epidemics of HPAI from the 1990s onwards, especially the unprecedented H5Nx HPAI (Gs/GD lineage) which has affected 83 countries and remains endemic in a number of lower income countries (reviewed in [299]). Table 6.3 lists the summaries and references of HPAI outbreaks between 1959 and 2017. Of the 42 epidemics, 40 have involved primarily domesticated poultry, principally chickens and turkeys, one involved exclusively wild birds (i.e.,, common terns), and one involved both domestic poultry, including ducks and geese, and wild birds.

Since AI is an international concern, global interest in AI resulted in the convening of 10 international symposia since 1981, specifically addressing AI issues (16, 72, 74, 265, 267, 268, 280, 287, 292). Because influenza is an international problem, solutions will require international, national, and state/provincial efforts and cooperation (75). Additional information on the history of AI can be found elsewhere (299, 305).

Etiology

Classification

Avian influenza viruses are classified in the family Orthomyxoviridae, genus *Influenzavirus A* (163).

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Table 6.3 Forty-two documented pandemics, epidemics, or limited outbreaks of highly pathogenic avian influenza (HPAI) since discovery of avian influenza viruses (AIVs) as cause of fowl plague in 1955, modified from (12, 283, 301). (Specific citations for 1–35 are listed in [301]).

No.	Dates	Prototype AI virus	Subtype	Hemagglutinin proteolytic cleavage site deduced amino acid sequence	Number affected with high mortality or were depopulated[a]
1	1959	A/chicken/Scotland/1959	H5N1	PQRKKR/GLF	Scotland, Aberdeen: one premise, chickens (*Gallus gallus domesticus*), unknown number of birds affected
2	1961	A/tern/South Africa/1961	H5N3	PQRETRRQKR/GLF	South Africa, Western and Eastern Cape provinces (coastline from Port Elizabeth to Lambert's Bay); 1300 common terns (*Sterna hirundo*) (reviewed [301])
3	1963	A/turkey/England/1963	H7N3	PETPKRRRR/GLF	England, Norfolk County: 2 farms; 29,000 breeder turkeys (*Meleagridis gallopavo*) in outdoor and indoor pens
4	1966	A/turkey/Ontario/7732/1966	H5N9	PQRRRKKR/GLF	Canada, Ontario province: 2 indoor farms; 8,100 breeder turkeys
5	1976	A/chicken/Victoria/1976	H7N7	PEIPKKREKR/GLF	Australia, Victoria province: 2 farms; 25,000 indoor laying chickens, 17,000 indoor broilers, and 16,000 indoor and outdoor ducks (*Anas platyrhynchos*)
6	1979	A/chicken/Leipzig(Germany)/1979	H7N7	PEIPKKKKR/GLF, PEIPKRKKR/GLF, PEIPKKRKKR/GLF, PEIPKKKKKKR/GLF	Germany (formerly East Germany), Saxony: 2 farms: 600,000 chickens, 80 geese
7	1979	A/turkey/England/199/1979	H7N7	PEIPKKRKR/GLF, PEIPKRRRR/GLF, PEIPKKREKR/GLF	England, Norfolk county: 3 commercial farms, 9,262 turkeys
8	1983–1984	A/chicken/Pennsylvania/1/1983 (LPAI) A/chicken/Pennsylvania/1370/1983 (HPAI)	H5N2	PQKKKR/GLF (LP) PQKKKR/GLF (HP) – lost a glycosylation site on amino acid 13	USA; Pennsylvania, Maryland, and Virginia: 452 flocks, 17 million birds; mostly chickens or turkeys, a few chukar partridges (*Alectoris chukar*) and guinea fowl (*Numida meleagris*)
9	1983	A/turkey/Ireland/1378/1983	H5N8	PQRKRKKR/GLF	Ireland, Monaghan county: 4 farms; 8,120 turkeys, 28,020 chickens and 270,000 ducks died or were depopulated
10	1985	A/chicken/Victoria/1/1985	H7N7	PEIPKKREKR/GLF	Australia, Victoria province: 1 farm; 24,000 broiler breeders, 27,000 laying chickens, and 61,000 broilers. Used untreated surface water
11	1991	A/turkey/England/50-92/1991	H5N1	PQRKRKTR/GLF	England, Norfolk county: 1 farm; 8,000 turkeys
12	1992	A/chicken/Victoria/1/1992	H7N3	PEIPKKKKR/GLF	Australia, Victoria province: 2 farms, 1 backyard flock and 1 hatchery; 17,000 broiler breeders, 5,700 ducks, 105,000 day-old chicks, 540,000 hatching eggs
13	1995	A/chicken/Queensland/667/1995	H7N3	PEIPRKRKR/GLF	Australia, Queensland province: 1 farm; 22,000 laying chickens
14	1994–1995	A/chicken/Mexico/31381-7/1994 (LP) A/chicken/Puebla/8623-607/1994 (HP) A/chicken/Queretaro/14588-19/1995 (HP)	H5N2	PQRETR/GLF (LP) PQRKRKTR/GLF (HP) PQRKRKRKTR/GLF (HP)	Mexico, Puebla and Queretaro: Chickens—concurrent circulation of LP (1993–present) and HP AIV (late 1994 to mid-1995) strains. 360 commercial chicken flocks "depopulated" (1995) via vaccination and controlled marketing. Unknown number HP infected birds.
15	1994–1995, 2004	A/chicken/Pakistan/447/1995 A/chicken/Pakistan/1369-CR2/1995 A/chicken/Pakistan/16/1995	H7N3	PETPKRKRKR/GLF PETPKRRKR/GLF PETPKRRNR/GLF	Pakistan: Two incursions: (1) 3.2 million broilers and broiler breeder chickens (northern part of country—1994–1995), and (2) 2.52 million layers (Karachi – 2004). "Stamping-out" policy was not used for control, but use of vaccine and controlled marketing. The numbers affected are crude estimates for two separate outbreak periods of clinical disease, but same virus lineage.

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No.	Dates	Prototype AI virus	Subtype	Hemagglutinin proteolytic cleavage site deduced amino acid sequence	Number affected with high mortality or were depopulated[a]
16	1996–present	A/goose/Guangdong/1/1996 (Gs/GD)	H5Nx	PQRERRRKKR/GLF (majority)	76 countries in Asia, Europe, Africa, and North America: Unknown number of commercial and non-commercial flocks (village poultry); over 400 million birds dead or culled from 2003 to early 2012, mostly chickens, but also ducks, geese, Japanese quail, and some wild birds.[b] Variations in hemagglutinin cleavage sites: (1) Clade 1, PQREGRRKKR/GLF; (2) Clade 2.1, PQRESRRKK/GLF; (3) Clade 2.2, PQGERRRKKR/GLF; (4) Clades 2.3.1, 2.3.2, 2.3.3 and 2-like PQRERRRKR/GLF; (4) Clade 2.3.4, PLRERRRKR/GLF; and (5) Clade 7, PQIEGRRRKR/GLF. See http://www.offlu.net/fileadmin/home/en/resource-centre/pdf/Influenza_A_Cleavage_Sites.pdf
17	1997	A/chicken/New South Wales/1651/1997	H7N4	PEIPRKRKR/GLF	Australia, New South Wales province: 3 farms; 160,000 indoor broiler breeders and 261 outdoor emu (*Dromaius novaehollandiae*)
18	1997	A/chicken/Italy/330/1997	H5N2	PQRRRKKR/GLF	Italy, Veneto and Friuli-Venezia-Giulia regions: 8 flocks (hobby/backyard only); 2,116 chickens, 1,501 turkeys, 731 guinea fowl, 2,322 ducks, 204 quail (species unknown), 45 pigeons (*Columba livia*), 45 geese (species unknown), 1 pheasant (species unknown)
19	1999–2000	A/turkey/Italy/977/1999 (LP) A/turkey/Italy/4580/1999 (HP)	H7N1	PEIPKGR/GLF (LP) PEIPKGSRVRR/GLF (HP, majority), PEIPKGSRMRR/GLF (HP, minor), PEIPKRSRVRR/GLF (HP, minor)	Italy, Veneto and Lombardia regions: 413 farms, 8.1 million laying chickens; 2.7 million meat and breeder turkeys; 2.4 million broiler breeders and broilers; 247,000 guinea fowl; 260,000 quail, ducks, and pheasants; 1,737 backyard poultry and 387 ostriches
20	2002	A/chicken/Chile/176822/2002 (LP) A/chicken/Chile/4322/2002 (HP) A/chicken/Chile/4325/2002 (HP)	H7N3	PEKPKTR/GLF (LP) PEKPKTCSPLSRCRETR/GLF (HP) PEKPKTCSPLSRCRKTR/GLF (HP)	Chile, Valparaíso region: Two farms of one company, multiple houses; 617,800 broiler breeders, 18,500 turkey breeders (2 houses)
21	2003	A/chicken/Netherlands/621557/2003	H7N7	PEIPKRRRR/GLF	(1) Netherlands, Gelderse Vallei and Limburg region: 255 infected flocks, and 1,381 commercial and 16,521 backyard/smallholder flocks depopulated. 30 million affected—mostly chickens. (2) Belgium (Limburg and Antwerp provinces), 8 farms, 2.3 million chickens. (3) Germany (Nordrhein Westfalen state), 1 farm, 419,000 chickens
22	2004	A/Chicken/Canada/AVFV1/2004 (LP) A/Chicken/Canada/AVFV2/2004 (HP)	H7N3	PENPKTR/GLF (LP) PENPKQAYRKRMTR/GLF (HP)	Canada, British Columbia province: 42 commercial and 11 backyard flocks infected (1.2 million poultry) – approximately 16 million commercial poultry depopulated, most were chickens; later HPAI isolates had variations in HA cleavage sites of PENPKQAYQKRMTR/GLF, PENPKQAYKKRMTR/GLF, PENPKQAYHKRMTR/GLF, PENPKQAHQKRMTR/GLF, PENPRQAYRKRMTR/GLF, PENPKQACQKRMTR/GLF
23	2004	A/chicken/Texas/298313/2004	H5N2	PQRKKR/GLF	USA, Texas state: 1 non-commercial farm (6,608 chickens), 2 LPM affected; 3 dangerous LPM contacts culled
24	2004	A/ostrich/South Africa/N227/2004	H5N2	PQREKRRKKR/GLF	South Africa, East Cape province: 2004—8 farms, culled. 23,625 ostriches, 3,550 other poultry (chickens, turkeys, geese, ducks, and pigeons), 1,594 ostrich eggs and 1,707 other farmed bird eggs
25	2006	A/ostrich/South Africa/AI1091/2006	H5N2	PQRRKKR/GLF	South Africa, Western Cape province: 24 farms, 7,334 ostriches culled
26	2005	A/chicken/North Korea/1/2005	H7N7	PEIPKGRHRRPKR/GLF	North Korea: 3 farms, 218,882 layer chickens culled; number dead not reported

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No.	Dates	Prototype AI virus	Subtype	Hemagglutinin proteolytic cleavage site deduced amino acid sequence	Number affected with high mortality or were depopulated[a]
27	2007	A/chicken/Saskatchewan/HR-00011/2007	H7N3	PENPKTTKPRPRR/GLF	Canada, Saskatchewan province: 1 farm, 10 barns, 49,500 broiler breeder hens and roosters; 540 dead, 48,500 culled; LP appeared first, with rapid mutation to HP
28	2008	A/chicken/England/1158-114061/2008	H7N7	PEIPKRKKR/GLF	England, Oxfordshire county: 1 farm – 25,000 free-range layer chickens; 10,000 dead, 15,000 culled; HP derived from LP virus on premise
29	2009	A/chicken/Spain/6279-2/2009	H7N7	PELPKGTKPRPRR/GLF	Spain, Guadalajara province: 1 farm, 5 barns; 308,640 layer chickens; 30,000 dead, 278,640 culled
30	2011–2013	A/ostrich/SA/AI2114/2011 A/ostrich/SA/AI2887/2011	H5N2	PQRRKKR/GLF PQRRRKR/GLF	South Africa, Western Cape province: 45,343 ostriches; 108 deaths, 1,178 culled and 39,812 slaughtered on 50 premises; loss of export markets
31	2012–2013	A/chicken/Taiwan/A1997/2012	H5N2	PQRRKR/GLF	Chinese Taipei; Chang-Hua, Pingtung, Yunlin, and Penghu counties: 6 premises (native chickens = 4; broiler breeder = 1, layer chicken = 1); 47,151 chickens, 5,697 deaths, 41,454 culled
32	2012– present	A/chicken/Jalisco/12283(CPA1)/2012	H7N3	PENPKDRKSRHRRTR/GLF	Mexico; Jalisco, Aguascalientes, Guanajuato, Tlaxcala, and Puebla States: 110 premises, 18,906,702 affected poultry, 1,727,500 deaths, 16,793,020 culled. Two waves of disease: (1) June 13, 2012– September 29, 2012, and (2) January 3, 2013 – ongoing. An accurate estimate of number of affected farms and birds involved are not available.
33	2012	A/chicken/New South Wales/12-3121-1/2012	H7N7	PEIPRKRKR/GLF	Australia, New South Wales province: 1 premise, free-range layers, 50,000 affected, 5,000 deaths, 45,000 culled
34	2013	A/chicken/Italy/13VIR4527-11/2013	H7N7	PETPKRRERR/GLF	Italy, Emilia-Romagna region: 6 premises, layers, 952,658 affected, 5,676 deaths, 946,982 culled
35	2013	A/chicken/New South Wales/13-02811-1/2013	H7N2	PEIPRKRKR/GLF	Australia, New South Wales province: 2 premises, free-range and caged layers, 490,000 affected, 18,620 deaths, 471,380 culled
36	2015	A/chicken/England/26352/2015 (H7N7)	H7N7	PEIPRHRKGR/GLF	United Kingdom, Lancashire county: 1 premise, colony and free-range laying chickens, 179,865 affected, 34,604 deaths, 145,261 culled (33, 183, 185)
37	2015	A/chicken/Germany/AR1385/2015 (H7N7)	H7N7	PEIPKRKRR/GLF	Germany, Lower Saxony state: 1 premise, laying chickens, 10,104 affected, 50 deaths, 10,054 culled (184, 185)
38	2015–2016	A/chicken/France/150169a/2015 A/duck/France/150233/2015 A/duck/France/150236/2015	H5N1 H5N2 H5N9	HQRRKR/GLF	France, 8 southwest/southcentral departments: 81 premises, 155,415 poultry affected, 7,470 deaths, 147,945 culled; primarily affected fattening ducks, and some guinea fowl, geese, and layer and meat chickens in small farms and backyard operations. First appeared as H5N1 virus but of different Eurasian lineage than Gs/GD H5N1 HPAI virus, and quickly reassorted with Eurasian LPAI viruses to produce H5N2 and H5N9 HPAI viruses (185)
39	2016	A/turkey/Indiana/16-001403-1/2016	H7N8	PENPKKRKTR/GLF	United States, Indiana state, Dubois county: 1 premise, 43,500 meat turkeys. HP derived from LP virus on premise. 1 dangerous contact layer farm (156,158 layers) and 9 LP affected turkey farms (195,937 birds) in control zone depopulated (121, 185, 186, 304)

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No.	Dates	Prototype AI virus	Subtype	Hemagglutinin proteolytic cleavage site deduced amino acid sequence	Number affected with high mortality or were depopulated[a]
40	2016	A/chicken/Italy/16VIR-1873/2016	H7N7	PELPKGRKRR/GLF	Italy, Emilia-Romagna region: 1 premise, 17,500 organic/free-range layers (185 187)
41	2017	A/Environment/Guangdong/C16283222/2016	H7N9	PEVPKRKRTAR/GLF	China, initially Guangdong province: 7 detections from chickens in live poultry markets (as of March 7, 2017). HP derived from LP virus circulating in live poultry market system since early 2013 (188)
42	2017	A/chicken/Tennessee/17-007147-1/2017	H7N9	PENPKTDRKSRHRRIR/GLF	United States, Tennessee, Lincoln county: 2 premises, 128,000 chicken broiler breeders. HP derived from LP virus circulating in wild waterfowl and infected poultry on 12 premises (6 backyard and 6 commercial, 125,000 birds) in Tennessee, Alabama, Kentucky, and Georgia (189, 319)

[a] Most outbreaks controlled by "stamping-out" or depopulation policies for infected and/or exposed populations of poultry. Chickens, turkeys, and poultry in the order Galliformes had clinical signs and mortality patterns consistent with HPAI, while ducks, geese, and other aquatic poultry lacked or had low mortality rates or infrequent presence of clinical signs. For detailed references covering outbreaks #1–35, see (301).

[b] The initial H5N1 HPAI outbreaks were reported in China (1996) with three incursions in Hong Kong (1997, 2001, and 2002). This was followed by regional extension with outbreaks in 2003–2005 within Southeast Asia (South Korea, Viet Nam, Japan, Indonesia, Thailand, Cambodia, Laos, China, and Malaysia). In mid to late 2005, outbreaks occurred in both wild birds and poultry in central Asia with extension to Eastern Europe and the Middle East by fall of 2005. In 2006, outbreaks were reported in Africa. Initially, chickens were the main species affected with disease and death, but in many of the outbreaks, domestic ducks have emerged to be a major species in maintenance and epidemiology of the viruses. Various wild birds have succumbed to infection. The H5 and N1 gene lineages have been maintained among the HPAI viruses from outbreaks in various Asian, African, and European countries (1996–2007) with the six internal gene segments having undergone reassortment with other AI viruses in Asia. In 2008, the first reassortant appeared with a different NA gene (H5N5) which in subsequent years appeared as H5N6, H5N8, H5N2 (both Eurasian and North American NA lineages) and H5N3. The hemagglutinin gene has evolved into first (i.e., 0–9), second (e.g., 1.1, 2.1, 2.2, 2.3, 7.2), third (e.g., 2.1.3, 2.3.2, 2.3.4), and fourth (e.g., 2.3.2.1, 2.3.4.4) order genetic clades. In 2014, H5N8 (2.3.4.4A) spread into Europe and North America by wild bird movements; and reassortants of this virus occurred in North America. In 2016, H5N8 (2.3.4.4B) spread from China to Russia, India, the Middle East, Europe, and northern and central Africa; and H5N6 (2.3.4.4C) spread from China to Japan and South Korea via wild aquatic bird migrations.

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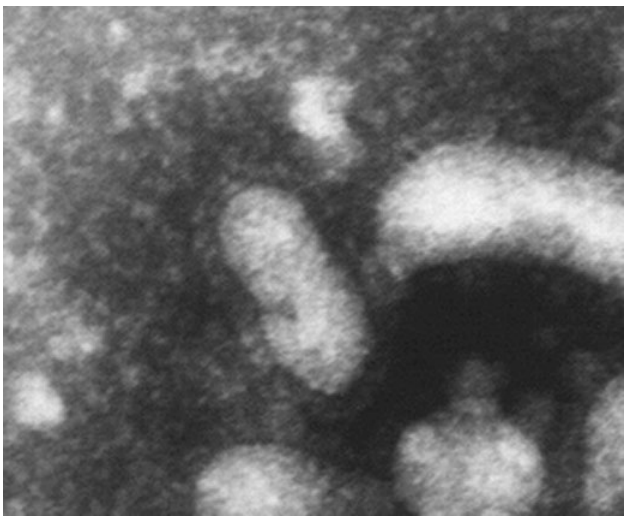


Figure 6.1 Spherical to pleomorphic influenza A virus particles with surface projections of hemagglutinin and neuraminidase. Negative stain with 2% phosphotungstic acid. (David E. Swayne)

Morphology

Virions are typically spherical to pleomorphic (100 nm) but can be filamentous with lengths up to several hundred nm (Figure 6.1) (163, 223). The surface is covered by two types of glycoprotein projections (10–14 nm in length and 4–6 nm in diameter): (1) rod-shaped trimers of hemagglutinin (HA), and (2) mushroom-shaped tetramers of neuraminidase (NA), and a tetrameric Matrix 2 (M2) protein. Virus buoyant density is 1.19 g/cm^3 in aqueous sucrose and single virion molecular weight (Mr) is 250×10^6 (163).

The nucleocapsid is helical (163). The viral genome is composed of eight segments of single-stranded, negative-sense RNA that code for a minimum of 10 or up to 17 proteins depending on the strain (324). Their size and function are listed in Table 6.4. Eight proteins are constituents of the virus (HA, NA, nucleoprotein [NP], matrix 1 [M1], matrix 2 [M2], polymerase basic protein 1

Table 6.4 Gene and protein information on *Influenzavirus A* (58, 143, 192).

Genome		Proteins coded				
Segment	Length (nucleotides[a])	Name	Length (amino acid)	Approx. no. molecules/ virion	Type	Function
1	2341	PB1	759	30–60	Polymerase complex	Transcriptase
2	2341	PB2	757	30–60	Polymerase complex	Endonuclease
3	2233	PA	716	30–60	Polymerase complex	(1) Viral RNA replication. (2) Proteolytic activity
4	1778	Hemagglutinin (HA)	566	500	Integrated type I membrane glycoprotein	(1) Virus attachment to sialylligosaccharide cell receptors including hemagglutinating activity. (2) Envelope fusion. (3) Antibody-mediated viral neutralization
5	1565	Nucleoprotein (NP)	498	1000	Major structural protein—associated with viral RNA segments	(1) Cytoplasmic to nuclear (NP) protein—transport of viral RNP. (2) Necessary for full length vRNA synthesis. (3) Antigen target for cytotoxic T lymphocytes
6	1413	Neuraminidase (NA)	454	100	Integrated type II membrane glycoprotein	(1) Cell receptor-destroying enzyme (sialic acid residues) that causes virus elution. (2) Antibody-mediated virus neutralization restricts virus spread
7	1027	Matrix 1 (M1)	252	3000	Non-glycosylated structural protein beneath viral envelope	Most abundant protein—role in virus budding
		Matrix 2 (M2)	97	20–60	Integrated type III glycosylated membrane protein	Ion channel
8	890	Non-structural 1 (NS1)	230	—	RNA binding protein	(1) Inhibit processing of cellular mRNA. (2) Enhancement of cytoplasmic translation of viral mRNA. (3) Possible inhibition of interferon pathways
		Non-structural 2 (NS2)	121	130–200	Nuclear export protein	Nuclear export of viral RNP

[a] Number of nucleotides based on human influenza strain A/PR/8/34 (H1N1).

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[PB1], polymerase basic protein 2 [PB2], polymerase acidic protein [PA], and a minor amount of nonstructural protein 2 [NS2]), and a nonstructural protein 1 (NS1) is located in the host cell cytoplasm. Expression of PB1-F2 is variable, depending on the virus strain.

Virus Replication

The stages of virus replication have been reported by various investigators in great detail (223). In brief, AI virus HA protein attaches to sialic acid found on host glycoproteins, initiating receptor-mediated endocytosis. The endosomes naturally acidify which triggers a conformational change in the HA2 protein that causes fusion of the viral envelope with the endosome membrane. The HA protein is synthesized as a polypeptide that must be proteolytically cleaved into HA1 and HA2 subunits to allow the virus to be infectious. The viral nucleocapsids are transported to the nucleus where viral transcriptase complex synthesizes mRNA. Six monocistronic mRNAs are produced in the nucleus and transported to the cytoplasm for translation into HA, NA, NP, PB1, PB2, and PA proteins. The mRNA of NS and M gene segments undergo splicing with each producing two mRNAs, which are translated into NS1, NS2, M1, and M2 proteins. The HA and NA proteins are glycosylated in the rough endoplasmic reticulum, trimmed in the Golgi, and transported to the surface where they are embedded in the plasma membrane. The eight viral gene segments along with internal viral proteins (NP, PB1, PB2, PA, and M2) assemble and migrate to areas of the plasma membrane containing the integrated HA, NA, and M2 proteins. The M1 protein promotes close association with the plasma membrane and budding of the virions.

Susceptibility to Chemical and Physical Agents

Avian influenza viruses are relatively unstable in the environment (299). Physical factors such as heat, extremes of pH, hypertonic conditions, and dryness can inactivate AI viruses. Because AI viruses have lipid envelopes, they are inactivated by organic solvents and detergents, such as sodium desoxycholate and sodium dodecyl sulfate. In the presence of organic matter, AI virus can be destroyed by chemical inactivants such as aldehydes (formaldehyde or glutaraldehyde), beta-propiolactone, and binary ethylenimine. After removal of organic matter, chemical disinfectants such as phenolics, ammonium ions (including quaternary ammonium disinfectants), oxidizing agents (such as sodium hypochlorite and Virkon S®), dilute acids, and hydroxylamine can destroy AI viruses.

Laboratory Situation

Avian influenza viruses are relatively stable in protein-containing solutions, but long-term storage should be at –70 °C or following lyophilization (299). Egg grown virus can be maintained for several weeks at 4 °C without loss of infectivity, but hemagglutinating and NA activities can be maintained longer even when the virus is no longer infectious. Inactivation by degrading the viral RNA can retain hemagglutinating and NA activities and antigenicity using various concentrations of formalin, binary ethylenimine, and beta-propiolactone, making these methods valuable for vaccine production. Most commonly used detergents and disinfectants (such as phenolics, quaternary ammonium surfactant compounds, and sodium hypochlorite) inactivate AI viruses, but RNA may be preserved and detected by molecular diagnostic tests long after viable virus cannot be isolated (262).

Field Situation

Influenza viruses when shed are protected by organic material such as nasal secretions or feces, which increase resistance to physical and chemical inactivation (75). Cool and moist conditions favor increased survival of AI viruses in the environment. For example, AI viruses have remained viable in liquid manure for 105 days in the winter and in feces for 30–35 days at 4 °C, for 7 days at 20 °C, and for 4 days at 25–32 °C in the shade (19, 89, 243, 329). In water at 28 °C, HPAI viruses had a decline in infectivity of 1 log in 4–5 days, respectively, and no virus detection after 26–30 days, but at 17 °C, the HPAI viruses persisted from 94–158 days (34). The H5N1 Gs/GD lineage HPAI viruses had shorter environmental survival times compared to most LPAI viruses obtained from wild waterfowl (34), but some H5N1 Gs/GD lineage HPAI viruses have variation in survival times that can be longer (191).

Inactivation and elimination of AI viruses shed into the environment is essential in the control of field infection and can be accomplished through integrated approaches including heating of buildings to 90–100 °F (32–38 °C) for two days to one week, thorough removal and proper disposal of manure and litter, cleaning and disinfecting of buildings and equipment, and allowing a vacancy period before restocking (252). Virus in manure and litter must be inactivated or disposed of by burial, composting, or incineration. Composting, which can reach temperatures of greater than 140 °F, was effective at killing HPAI viruses within poultry carcasses in less than 10 days (217). Effective disinfectants against AI viruses on clean surfaces include sodium hypochlorite (5000 ppm or 0.5% concentration), 2% sodium hydroxide (lye), phenolic compounds, acidified ionophore compounds, chlorine dioxide disinfectants, strong oxidizing agents, and 4% sodium carbonate/0.1% sodium silicate (55). However, organic material must be removed before most disinfectants can work properly. Live poultry

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markets can be decontaminated by removal of poultry and thorough cleaning with detergents and disinfection using any of the agents listed above that are suitable for use on food-producing surfaces (83). Appropriate contact times are required (usually 10 to 30 minutes) for detergents and disinfectants to be effective.

Pasteurization and cooking are effective means of inactivating AI viruses (182). United States Department of Agriculture (USDA) standard cooking times for poultry meat, which achieves an internal temperature of 165 °F (73.9 °C), and egg product pasteurization (54.4–63.3 °C) are adequate to kill AI viruses (302).

Strain Classification

Genera and Subtype Classification

Influenza viruses (A, B C, and D) were classified to genus (e.g., "type") based on serologic reactions of the internal proteins, principally NP and M1 proteins, via immunoprecipitation or agar gel immunodiffusion (AGID) tests, or by sequence analysis of internal gene segments (223, 299). All AI viruses are *Influenzavirus A*. *Influenzavirus B* and *Influenzavirus C* occur in humans and rarely in other mammalian species (339). *Influenzavirus D* was recently described in cattle and pigs (109).

Influenza virus A is further subtyped based on serologic reactions of the HA and NA surface glycoproteins or sequence analysis of HA and NA gene segments (223, 339). Sixteen subtypes of HA (H1–16) and nine subtypes of NA (N1–9) are recognized for AI viruses. Serologic subtyping of HA is done by the HI test and subtyping of NA by neuraminidase inhibition (NI) test (90, 296) or gene sequencing. Most combinations of the 16 HA and 9 NA AI virus subtypes have been reported in either domestic or wild birds, but distribution varies by year, geographic location, and host species. Based on HA gene sequences, H5Nx Gs/GD lineage HPAI viruses have been further classified into first order clades (e.g., 0–9) and some second (e.g., 2.2), third (e.g., 2.2.1), fourth (e.g., 2.3.2.1), and fifth (e.g., 2.3.2.1c) order clades to reflect molecular variation and evolution in genes encoding the HA protein (240). The H9N2 viruses also have also established multiple unique lineages in poultry, but a standardized nomenclature system has not been established.

Convalescent sera from chickens and ferrets and monoclonal antibodies have been used for determining antigenic relatedness of influenza viruses within the individual subtype. Such studies typically have used HI, and/or virus neutralization tests with resulting data being used in cartographic analysis to define intrasubtypic antigenic diversity (1, 300).

Strain Nomenclature

Standard international nomenclature for the designation of influenza virus strains has been established (339). The naming of the influenza virus strains includes the genus or type (A, B, C, or D), host of origin (except for human where the "host of origin" is omitted), geographic site, strain number (if any), and year of isolation followed by the antigenic subtype designating HA (H) and NA (N) in parentheses for type A influenza viruses. For example, a type A influenza virus isolated from turkeys in Minnesota during 2015 and classified as H5N2 is designated "A/turkey/Minnesota/12582/2015 (H5N2)."

Antigenic Variation of Strains—Drift and Shift

As has been demonstrated with seasonal human influenza viruses (H1N1 and H3N2), influenza A viruses have a high frequency of antigenic variation in the surface glycoproteins (HA and NA) because of two phenomena, drift and shift (339).

Antigenic drift in influenza viruses arises from point mutations in the HA and/or NA genes that results in antigenic changes in the coding proteins (339). In poultry populations, immune pressure from vaccination or infection play a role in selecting antigenic variants (49). In areas where LPAI viruses are endemic, such as H9N2 LPAI viruses in the Middle East, Africa, and Asia, H5N2 LPAI viruses in the Americas, and H5Nx HPAI viruses in Asia and Africa, infection by field viruses is widespread and antigenic variants have arisen. Vaccination programs that do not produce strong population immunity has been associated temporally with appearance of drift variants in the field, although causation has not been established (297).

Antigenic shift arises from reassortment between influenza gene segments coding the surface proteins, most importantly the HA, and occurs when two influenza viruses infect the same cell. Most significantly, novel HA and/or NA antigen combinations emerge in the infected population because of the lack of preexisting immunity. Reassortment of internal gene segments occurs frequently, may affect the phenotype of the virus, and occurs commonly when more than one influenza subtype is circulating in the same country (299). Wild ducks are commonly infected with multiple influenza A viruses that provide opportunities for reassortment to occur (222).

Immunogenicity or Protective Characteristics

After natural infection or vaccination, antibodies are produced to a number of viral proteins, but only antibodies produced to the surface proteins HA, NA, or M2 are protective. The HA protein is the major antigen that elicits protective antibodies against death and clinical signs and they are HA subtype specific (i.e., they only neutralize influenza virus of the homologous HA subtype both in *in vivo* and *in vitro* assays) (299). Anti-NA antibodies can also provide protection against homologous NA subtypes in birds (308), but equivalent protection requires higher levels of anti-NA than anti-HA antibodies.

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Antibodies to the M2e protein can provide partial protection in mice, although the mechanism of protection is not understood but likely includes Fc receptor involvement (322). Although several different M2e vaccination approaches have been evaluated in chickens, no vaccine protects for HPAI challenge and only partial protection has been observed in combination with other vaccines or with a LPAI challenge (148, 242).

Antibodies against the internal proteins do not confer protection from death or clinical signs following challenge by HPAI viruses (333). However, immunization with NP or other influenza proteins in a system that stimulates cell-mediated immunity can reduce virus replication during the late stages of the infectious process (139), and there is some experimental evidence of protection from mortality (126, 219). However, such broad immunity has not been of sufficient length and level to provide cross HA subtype protective vaccines in the field.

Genetic or Molecular Characteristics

With the widespread availability of sequencing technology, over 100,000 influenza viruses have been either partially or fully sequenced. This has resulted in detailed phylogenetic comparisons of strains for purposes of molecular epidemiology, identification of reassortant gene segments, and identification of specific mutations and their correlation with biological properties. Because of the large amount of sequence data available, multiple influenza specific websites have been developed to help manage the data, such as Influenza Research Database (IRD, https://www.fludb.org/brc/home.spg?decorator=influenza) and Global Initiative on Sharing All Influenza Data (GISAID, https://www.gisaid.org/). Studies of gene sequences have highlighted gaps in AI surveillance based on gaps in phylogenetic trees between newly identified viruses and those identified previously.

Pathotype

Based on pathogenicity (i.e., the ability to produce disease), AI viruses from poultry are classified into two pathotypes: (1) HP, and (2) LP (i.e., non-HP). H5 and H7 LPAI viruses became notifiable in 2006 because these subtypes have the potential to mutate to HPAI when allowed to circulate in poultry populations (181). The OIE Terrestrial Code in 2019 lists "notifiable" avian influenza as follows (182):

1) HPAI viruses have an IVPI in six-week-old chickens greater than 1.2 or, as an alternative, cause at least 75% mortality in four- to eight-week-old chickens infected intravenously. H5 and H7 viruses which do not have an IVPI of greater than 1.2 or cause less than 75% mortality in an intravenous lethality test should be sequenced to determine whether multiple basic amino acids are present at the cleavage site of the haemagglutinin molecule (HA0); if the amino acid motif is similar to that observed for other HPAI isolates, the isolate being tested should be considered as HPAI virus;
2) LPAI viruses are all influenza A viruses of H5 and H7 subtypes that are not HPAI viruses.

The non-H5 and non-H7 LPAI viruses, for which there is no formal requirement to report to OIE unless they are causing a severe disease, still may be reportable to national and state/provincial authorities. However, based on pathobiological criteria (e.g., disease, lesions, and signalment), all LPAI viruses are indistinguishable irrespective of the H and N subtype. Pathogenicity classification is based on *in vivo* testing in chickens, but similar outcomes are often obtained for related birds in the order Galliformes (8, 202). However, most AI viruses that are HP for chickens have been LP for domestic ducks except for some strains of H5Nx Gs/GD lineage HPAI virus, which are also highly lethal for young domestic ducks, but not always highly lethal in older ducks (196). Pathogenicity test results are highly predictive only for the host used in the test.

Being an H5 or H7 subtype is not a predictor of HP; i.e., only some of H5 and H7 LPAI viruses have mutated to the HP phenotype. By contrast, all naturally occurring H1–4, H6, and H8–16 AI viruses have been of low virulence (i.e.,, LP) for chickens when challenged by the respiratory route but some are still capable of causing disease.

Laboratory Host Systems

The preferred method for isolation and propagation of AI viruses has been 9–11-day-old embryonating chicken eggs inoculated via the chorioallantoic sac (CAS) (296), but with some isolates, inoculation by the yolk sac route or inoculation onto the chorioallantoic membrane resulted in isolation where the CAS route failed (155). In embryonating chicken eggs, AI viruses generally grow to high titers and have a cleaved HA (75). Most inactivated vaccines have been produced by cultivation in embryonating chicken eggs.

Avian influenza viruses replicate in a limited number of cell culture systems (75). Primary cultures of chicken embryo fibroblasts (CEF) or kidney cells are most commonly used for plaque assays and virus neutralization tests. Madin–Darby canine kidney cell cultures have also been used. However, in CEF and some other cells, LPAI viruses require the addition of exogenous trypsin to the medium or agar overlay in order to cleave the HA and produce infectious virus (75). Absence of exogenous trypsin will produce plaques less than 1 mm in size or no plaques depending on the virus strain. The HPAI viruses

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do not require the addition of exogenous trypsin for cleavage of HA and the subsequent production of infectious virus.

The chicken has been the most frequently used animal in laboratory studies to determine pathogenicity and study pathogenesis. Other commonly used laboratory species include the turkey, domestic duck (*Anas platyrhynchos*), house mouse (*Mus musculus*), guinea pig (*Cavia porcellus*), and ferret (*Mustela putorius furo*). The mouse, guinea pig, and ferret have been used as models to assess the risk of interspecies transmission of AI viruses to humans (69), and the other hosts provide assessment of infections in natural host species.

Pathogenicity

Clinical Groups in the Field

Although only two pathotypes of AI viruses can be demonstrated in the laboratory (HP and LP), natural infection by AI viruses results in a wide range of clinical outcomes which are dependent on virus strain, host species, host age, host immunity, coinfection with other pathogens, and environmental factors. From mortality patterns, clinical signs, and lesions in the field, AI can be categorized into four clinical groups: (1) highly virulent, (2) moderately virulent, (3) mildly virulent, and (4) avirulent. First, the highly virulent clinical group results from infection by HP H5 or H7 AI viruses usually in chickens or closely related gallinaceous birds and is expressed as a severe, highly fatal, systemic disease that affects most organ systems. Morbidity and mortality approach 100%. Experimentally, the HPAI viruses alone reproduce the lesions and high mortality rates seen in the field (283). Second, the moderately virulent clinical group results from infection by LPAI viruses, potentially of any HA or NA subtype, usually with coinfection by secondary pathogens or accompanied by other stress factors (42, 301). The mortality rates vary but range from 5–97% with the highest mortality occurring in young birds, reproductively active hens, or severely stressed birds (36, 42). Lesions usually have been in the respiratory tract, reproductive organs, kidney, or pancreas (42, 117, 345). Some of these cases may have involved concurrent infection with bacteria that secreted proteases which cleaved the HA of LPAI viruses thus exacerbating the AI virus infection (238). Third, the mildly virulent clinical group results from infection by LPAI virus alone, producing low mortality and mild respiratory disease or drops in egg production. Mortality is usually less than 5%, and is typically in older birds. Fourth, the avirulent clinical group results from infections by LPAI viruses without any increased mortality or clinical signs. This has been most frequent with infections by LPAI viruses in wild birds of orders Anseriformes and Charadriiformes (282). In poultry, this has been seen following the introduction of a poorly host-adapted LPAI virus from wild birds. Such an example would be the first cases of AI in range turkeys following exposure to wild waterfowl AI viruses that resulted in seroconversion detected at slaughter without any previously noted clinical signs (282). H7N9 LPAI viruses that emerged in China in 2013 produced largely asymptomatic infection in chickens. Occasionally HP and LP viruses may appear together and this causes confusion in the field and the laboratory. In individual field situations, the clinical outcomes can be a mixture of the four clinical groups. For example, during the changing of a H5 or H7 LP to HPAI virus, gross lesions consistent with highly virulent AI will be seen in some dead birds, but the mortality rates will be low, similar to mildly or moderately virulent AI because some birds will have already seroconverted before the mutation to HPAI.

Effect of the Hemagglutinin Protein on Pathogenicity

The HA gene is the primary but not the only determinant of high pathogenicity in chickens; a proper constellation of all eight gene segments is required for the maximal expression of virulence potential (31). In brief, the cleavage of the HA protein into the HA1 and HA2 proteins is essential for the virus to be infectious and produce multiple replication cycles. With LPAI viruses, the HA protein is released from the host cell uncleaved and the virion is not infectious. The HA protein can be cleaved by trypsin-like proteases found in restricted anatomical sites, such as respiratory and intestinal tracts, which accounts for the restricted replication and lower virulence. The difference between the cleavage site of LPAI and HPAI viruses is the number of amino acids, with basic amino acids being important, in the HA1 near the cleavage site that determines whether trypsin-like proteases or furin-like proteases can cleave the protein. The LPAI viruses generally have only two non-consecutive basic amino acids at the carboxy-terminus of the HA1, which is only cleavable by trypsin-like proteases. In contrast, H5 and H7 HPAI viruses have either multiple basic amino acids or an insertion of amino acids at the carboxy-terminal of the HA1 protein that allows proteolytic cleavage by furin-like proteases that are ubiquitous in many cells throughout the body (253). For HPAI virus, the HA is cleaved inside the cell before virus assembly and is infectious when it is released from the host cell. This effectively increases the cell tropism of the virus leading to virus replication in numerous visceral organs, the nervous system, and the cardiovascular system leading to systemic disease with high mortality (Table 6.5). Until recently it was uncommon for HPAI viruses to circulate in wild birds, but first recognized in 2005 was poultry adapted viruses of the Goose/Guangdong lineage (H5Nx) that transmitted from poultry back to wild birds and persisted in the wild bird population with variable pathology.

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Table 6.5 Examples of genetic mechanisms for low pathogenicity (LP) to high pathogenicity (HP) change based on deduced amino acid sequence of hemagglutinin (HA) proteolytic cleavage sites in H5 and H7 AI viruses (modified from [218]).

				Mechanism[a]					
Influenza virus	**Subtype**	**Pathotype**	**Amino acid sequence**	**1**	**2**	**3**	**4**	**5**	**References**
Typical H5 LPAI	H5	LP	PQ.....RETR*GLF						(218)
A/Turkey/England/91	H5N1	HP	PQ....RKRKTR*GLF	X	X				(218)
A/Chicken/PA/1370/83	H5N2	HP	PQ.....KKKR*GLF	X				X	(218)
A/Tern/South Africa/61	H5N9	HP	PQRETRRQKR*GLF	X		X			(218)
A/Chicken/Puebla/8623-607/94	H5N2	HP	PQ....RKRKTR*GLF	X	X				(94, 118)
A/Chicken/Queretaro/14588-19/95	H5N2	HP	PQRKRKRKTR*GLF	X	X				(94)
Typical H7 LPAI	H7	LP	PEIP.....KTR*GLF						(218)
A/Chicken/Victoria/85	H7N7	HP	PEIP.....KKREKR*GLF			X			(218)
A/Turkey/Italy/4580/99	H7N1	HP	PEIPKG....SRVRR*GLF			X			(42)
A/Chicken/Chile/176822/02	H7N3	HP	PEKPKTCSPLSRCRETR*GLF[b]				X		(263)
A/Chicken/Canada/AVFV2/04	H7N3	HP	PENPK...QAYRKRMTR*GLF[c]				X		(199)

[a] Mechanisms: (1) substitutions of non-basic with basic amino acids, (2) insertions of multiple basic amino acids from codons duplicated from hemagglutinin cleavage site, (3) short inserts of basic and non-basic amino acids from unknown source, (4) non-homologous recombination with inserts which lengthen the proteolytic cleavage site, (5) loss of the shielding glycosylation site at residue 13.
[b] 30 nucleotides from nucleoprotein of same virus gene coding 10 amino acid insert.
[c] 21 nucleotides from matrix of same virus gene coding 7 amino acid insert.

LPAI viruses of the H5 and H7 subtype can mutate under some circumstances (typically during replication in gallinaceous hosts) to the HPAI form by changes of the HA cleavage site including: (1) substitutions of non-basic with basic amino acids, (2) insertions of multiple basic amino acids from codons duplicated at the hemagglutinin cleavage site, (3) short inserts of basic and non-basic amino acids from unknown source, or (4) non-homologous recombination with inserts which lengthen the proteolytic cleavage site but which may or may not contain additional basic amino acids (Table 6.5) (42, 118, 201). One additional factor, the presence or absence of a glycosylation site at the amino terminal end of the HA1 protein, has been shown to influence HA cleavage. This glycosylation site when present, because it is physically located close to the HA cleavage site, can shield the cleavage site so that furin-like proteases can't function, and the viruses may have a LPAI phenotype even with cleavage compatible with other HPAI viruses (Table 6.5) (94). The specific sequence of the HA cleavage site for H5 and H7 AI viruses are predictive of low or high pathogenicity in chickens (Table 6.5). Experimental insertion of multiple basic amino acids into HA cleavage sites of some non-H5 or non-H7 subtypes have produced viruses capable of causing systemic infection in experimentally challenged poultry (325).

In vitro tests have been used to predict pathogenicity potential. The ability to produce plaques in tissue culture, such as chicken embryo fibroblast cultures, without trypsin supplementation correlates with furin cleavage of HA and HP in chicken *in vivo* tests, but LPAI viruses require the addition of exogenous trypsin to cleave the HA and produce large plaques (31).

An issue separate from HA cleavability is receptor binding between the receptor-binding site of the HA and the receptor on the host cells. Avian influenza viruses typically have preferential binding to N-acetylneuraminic acid-α2,3-galactose linkage on sialoligosaccharide (α2,3 linkage) receptors of avian cells (122). However, the binding avidity varies greatly depending on the structure of the oligosaccharide. This is a poorly understood phenomenon but impacts host specificity (host-adaption) and cell, tissue, and organ tropism within the host. Changes in the receptor-binding site of the HA have been shown to change the host range of an influenza virus (175).

Mechanisms of Cellular Pathobiology

Based on morphologic and biochemical evidence, AI viruses exert pathological effect on avian cells by two mechanisms: necrosis or apoptosis (115, 211, 258). Necrosis has been identified in many cell types including renal tubule cells, pancreatic acinar epithelium, cardiac myocytes, adrenal cortical cells, and pulmonary epithelial cells in chickens (258). Necrosis has been associated with intense virus replication and demonstration of abundant AI viral nucleoprotein in the nucleus and cytoplasm (271). Apoptotic cell death has been demonstrated

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in various cell culture systems and involved several cytokines including interferon-beta and transforming growth factor-beta (115, 211, 212, 310). *In vivo*, apoptotic cell death has been identified most often in lymphocytes, especially in the absence of direct AI viral replication (258). However, apoptosis has been demonstrated in neurons, respiratory epithelium, and pulmonary alveolar cells of mice infected with mouse-adapted influenza viruses (172, 173). In chicken embryos, apoptosis and necrosis may share similar biochemical features and indicate that differentiation morphologically and biochemically between them is not always easy nor clear (88).

Pathobiology and Epizootiology

Incidence and Distribution

Avian influenza viruses have a worldwide distribution with reports of isolations from Africa, Asia, Australia, Europe, and North and South America, and serologic evidence of infection in penguins from Antarctic (75, 170, 247, 283). Proceedings of the International Symposia on AI have tabulated LP and HPAI outbreaks and incidences since 1981 (16, 72, 74, 265, 267, 268, 280, 287, 292).

Avian influenza viruses have been isolated from free-flying aquatic birds, especially of the orders Anseriformes (ducks and geese) and Charadriiformes (shorebirds, gulls, terns, and auks), which are considered the natural reservoirs of all AI viruses (250). In these species, AI virus infections usually cause no disease (LPAI viruses) with the exception of high mortality in common terns of South Africa during 1961 and infections and mortality in a variety of wild birds with H5Nx Gs/GD lineage HPAI virus that originated from Asia in 1996 (Table 6.3). Dabbling ducks, especially mallards (*Anas platyrhynchos*) in the Eastern flyway and northern shoveler (*Anas clypeata*) in the Pacific flyway of North America, have the highest reported isolation rates of AI viruses with over 30% of juvenile ducks being infected prior to migration in the late summer (111, 113). This frequency decreases during migration with the lowest rate (0.4–3.1%) being seen in ducks on the wintering grounds (249). However, the frequency of infection in native, nonmigratory ducks increases when migratory ducks arrive in the wintering grounds (108, 249). The H3, H4, H6, N2, N6, and N8 subtypes have been dominant among AI viruses isolated from free-flying ducks (113, 140, 209, 235, 250). For shorebirds (order: Charadriiformes), the greatest number of isolations have been in the spring with a second peak during the fall migration (128). The dominant AI virus subtypes have been H3, H9, H11, H13, N2, N4, N8, and N9 (96, 128, 140). Waders have been shown to play a less important role in avian influenza infections in Europe compared to Anatidae (174). Most combinations of the 16 HA and 9 NA subtypes have been reported in free-flying birds. Avian influenza viruses are infrequently isolated from wild terrestrial birds because they occupy ecosystems that do not favor maintenance of AI viruses (250). However, H5Nx Gs/GD lineage HPAI viruses have been found in some wild terrestrial birds including species used for religious release ceremonies (98). Outbreaks of Gs/GD lineage HPAI have been reported in crows in a number of countries and over 60 wild bird species, mainly waterbirds, but some predators, scavengers, and passerine species were found to be infected in the 2016–2017 H5N8/N5 outbreak in Europe (76).

Avian influenza viruses have been isolated sporadically from domestic poultry, most frequently chickens, turkeys, and ducks and captive wild birds held as caged pets, or in quarantine stations, private collections/reserves, and zoological parks (10, 11). However, incidence and distribution varies greatly with geographic region, species, age of bird, time of year, and the environmental or agricultural system occupied. Zoological collections have been affected in multiple countries by Gs/GD lineage H5Nx HPAI viruses especially during intercontinental waves of transmission such as the one in 2016–2017.

Turkeys and other gallinaceous birds (including chickens) are not natural reservoirs of AI viruses (200, 259). Humans have altered the natural ecosystems of birds through captivity, domestication, industrial agriculture, national and international commerce, and nontraditional raising practices (282, 293). This has created new niches for AI viruses and variations in the incidence and distribution of AI infections. Five distinct man-made ecosystems have been identified that have impacted AI virus ecology (282, 293): (1) integrated indoor commercial poultry; (2) range-raised commercial poultry, especially domestic ducks in paddy rice field rearing system; (3) LPM; (4) village, backyard, and hobby flocks; and (5) bird collection and trading systems. The frequency of AI infections within each system has varied.

In most integrated commercial poultry systems in developed countries, AI has been a rare occurrence considering greater than 55 billion chickens are reared each year (297). However, when AI infections do occur, they sometimes spread rapidly throughout the integrated system from farm-to-farm resulting in epidemics of HPAI (Table 6.3) or LPAI. The H9N2 LPAI, from several distinct lineages in particular has become endemic and widespread in Asia, the Middle East, Africa, and Europe (97, 151, 176). Other subtypes have also become endemic in poultry, including H3 and H6, in Asia. As of 2017, H5Nx Gs/GD lineage HPAI was endemic in Bangladesh, China, eastern India, Egypt, Nigeria, Myanmar, Indonesia, Cambodia, and Viet Nam, but has caused epizootics in over 83 countries in Asia (including the Middle East), Africa, Europe, and North America. In the endemic countries, the virus is being maintained in the complex

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poultry sector comprising a mix of millions of small-scale poultry flocks mixed with larger commercial farms, many with deficient biosecurity practices, many free-ranging ducks, the sale of millions of live poultry every day, often moved by unregulated traders through poorly regulated markets and traders' yards. This combination of factors has provided sufficient susceptible poultry to allow H5Nx Gs/GD lineage viruses to remain endemic (84, 180). Passive surveillance systems in these countries are weak with few incentives for farmers or traders to report HPAI-compatible events (270).

Historically, LPAI outbreaks in the United States occurred in the fall in range-reared turkeys in Minnesota (USA) during the 1970s and 1980s (99, 105). However, eliminating range rearing prevented exposure to migratory waterfowl infected with LPAI virus thus eliminating seasonal LPAI outbreaks in turkeys (105). However, migratory waterfowl exposure alone does not adequately explain year-to-year variations in seasonal LPAI outbreaks in turkeys. Individual virus strain and host species impact interspecies transmission of AI viruses from migratory waterfowl to poultry and among poultry species (294). For example, the H7N2 LPAI virus in Virginia during 2002 produced a higher proportion of affected turkey than chicken farms and, in experimental laboratory tests, this virus was more infectious for turkeys than chickens as evident by requiring 100–250 times less virus to infect turkeys than chickens (317). Differences in susceptibility of poultry and wild waterfowl species have also been identified with H5 Gs/GD lineage and other HPAI and LPAI viruses (7, 28, 196, 197, 246, 317).

The LPM systems have some of the highest AI virus infection rates (226) but the levels can be reduced through appropriate management practices including source control. Historically, poorly controlled movement and lack of biosecurity caused AI to become endemic in some poultry populations, especially between 1900–1930 in Europe and some areas of Asia (257). This holds true today. Surveys of poultry in LPM of Hong Kong, New York, and other large cities has indicated LPAI viruses can become endemic in these agricultural systems (216, 224, 226, 315, 332). LPMs in Hong Kong were found to have a high prevalence of H5N1 Gs/GD lineage HPAI virus in December 1997 just prior to depopulation and AI viruses (H5N1 HPAI and H9N2 LPAI) were detected regularly in LPM between 2001 and 2003. Following implementation of sanitary standards and other AI preventive measures including vaccination of birds on all farms supplying LPMs at the recommended ages, HPAI viruses are rarely found in Hong Kong markets today. LPMs were the site of a 1997 H5N2 AI outbreak in Italy, and the most likely source of LPAI virus that mutated to HPAI virus causing the 1983–1984 outbreak in the United States (44, 226, 258, 332). LPMs have been the main source of infection for humans in China with H7N9 avian influenza viruses. Permanent market closures have reduced the effect of these viruses but tend to shift the problem to other areas (52). Mandatory vaccination against H7N9 avian influenza was implemented for poultry in China in 2017. This was followed by a reduction in the levels of market contamination and human cases compared to previous years.

Most influenza infections in domestic poultry have been the result of avian-origin influenza A viruses. However, H1N1, H1N2, and H3N2 swine influenza A viruses have infected turkeys, especially turkey breeders, causing severe and prolonged decreases in egg production with considerable economic losses (75, 168, 260, 311), and the 2009 human H1N1 pandemic influenza virus has also infected some turkey flocks (162).

Natural and Experimental Hosts

Avian influenza viruses have been shown to naturally infect a wide variety of wild and domestic birds, especially free-living birds occupying aquatic habitats. Some AI infections have involved wild terrestrial birds, but these birds do not represent a major source or reservoir of AI viruses (250), but potentially play an important role in local transmission of H5 Gs/GD lineage HPAI virus (227, 228). In brief, AI viruses have been isolated from more than 90 species of free-living birds representing 13 different orders: Anseriformes (ducks, geese, and swans), Charadriiformes (e.g., shorebirds [turnstones and sandpipers], gulls, terns, puffins, and guillemots), Ciconiiformes (herons and ibis), Columbiformes (doves), Falconiformes (raptors), Galliformes (partridge and pheasant), Gaviiformes (loons), Gruiformes (coots and moorhen), Passeriformes (perching birds—e.g., mynahs, finches, and weaverbirds), Pelecaniformes (cormorant), Piciformes (woodpecker), Podicipediformes (grebe), and Procellariiformes (shearwater) (248, 251). This represents 61% of known avian families, but the actual number of naturally infected species is most likely much greater (11).

In man-made ecosystems (agriculture, caged, hobby flocks, and exhibition systems), infections have been reported in Psittaciformes (parrots, cockatoos, and parakeets), Casuariiformes (emu), Struthioniformes (ostrich), Rheiformes (rhea), and most domesticated Galliformes and Anseriformes. The latter two groups include chickens, turkeys, Japanese quail (*Coturnix japonica*), helmeted guineafowl (*Numida meleagris*), Bobwhite quail (*Colinus virginianus*), pheasants (various species), chukar partridges (*Alectoris chukar*), geese (*Anser anser domesticus*), and ducks (mallards [*Anas platyrhynchos domesticus*] and Muscovy [*Cairina moschata domesticus*]) (75). Birds of the orders Psittaciformes probably are infected after capture and during mixing with infected birds at holding sites or in quarantine (75). Some

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infections of free-living Passeriformes (perching birds—starlings and sparrows) have been associated with outbreaks on poultry farms where they may have acquired infections from close contact with poultry (154, 171). Experimentally, sparrows have been shown to be capable of transmitting infection back to poultry (98).

LPAI viruses have caused epidemics or sporadic cases of respiratory disease in mink, seals, whales, and other sea mammals (326). H5N1 Gs/GD lineage HPAI virus have been reported to cause sporadic infections in donkeys, large felids (tigers, leopards, lions) domestic dogs, house cats, mink, red foxes, Owston's palm civets, a stone martin, and pigs (326). Most of these cases in carnivores involved close contact with or consumption of infected birds. Cases of natural infections by AI viruses in humans have been reported (see "Public Health Significance") and there is serological evidence of more widespread infection.

In experimental studies, specific strains of AI viruses have been shown to infect pigs, ferrets, rats, rabbits, guinea pigs, mice, dogs, foxes, cats, mink, nonhuman primates, and humans (22, 70, 75, 112, 135, 142, 225).

Transmission and Carriers

Avian influenza virus is shed from the nares, mouth, conjunctiva, and cloaca of infected birds into the environment because of virus replication in the respiratory, intestinal, renal, and/or reproductive organs. HPAI viruses can also be detected in epidermis including feathers, feather follicles, and glands such as preen gland resulting in environmental contamination (65, 202). In intranasally inoculated 3–4-week-old chickens, peak levels of HPAI virus recovery have been greatest from the oropharynx (swabs—$10^{4.2–7.7}$ mean chicken embryo infective doses EID_{50}/mL of respiratory secretions), and peak levels from the cloaca have been lower (swabs—$10^{2.5–4.5}$ EID_{50}/gm of feces) (285, 289); LPAI viruses typically produce lower oropharynx (swabs—$10^{1.1–5.5}$ EID_{50}/mL) and cloacal (swabs—$10^{1.0–4.3}$ EID_{50}/mL) titers (289). With HPAI viruses, high virus levels in tissues of infected birds make consumption of carcasses through predation or cannibalism another source of virus transmission to susceptible birds. Titers in meat vary with virus strain, bird species, and clinical stage of infection: (1) titers from dead chickens infected with 1983 H5N2 HPAI Pennsylvania virus had $10^{2.2–3.2}$ EID_{50}/gm of meat while 2003 H5N1 HPAI S. Korean virus had $10^{5.5–8.0}$ EID_{50}/gm of meat, and (2) H5N1 Gs/Gd lineage HPAI viruses produced different titers in clinically normal ($10^{2.0–3.4}$ EID_{50}/gm) or sick ($10^{4.0–6.0}$ EID_{50}/gm) domestic ducks (289, 312).

The virus is transmitted by direct contact between infected and susceptible birds or indirect contact through aerosol, droplets, or exposure to virus-contaminated fomites (29, 75). Transmission through virus from the oropharyngeal replication, because of the high virus concentrations, are likely through water contamination and direct contact, but the large volume of lower concentration AI virus in infected feces makes fomites a major mode of transport. Thus, AI viruses are readily transported to other premises by people (contaminated shoes and clothing) and equipment shared in production, live-haul, or live-bird marketing (75). Airborne virus is generated during slaughter of infected poultry in LPM settings and in an experimental model of a LPM in which the virus was transmitted to poultry and ferrets located 80 centimeters away from the infected birds and mock slaughter area (29, 344).

Influenza viruses exhibit varying degrees of adaptation to individual host species with intraspecies transmission possible (282, 293). Interspecies transmission occurs, especially between closely related host species in the same taxonomic family, such as chickens, turkeys, guinea fowl, and quail of the order Galliformes, family Phasianidae. Interspecies transmission can occur across different orders within the same class such as with free-flying duck-(order: Anseriformes)-to-turkey (order: Galliformes), but this is less frequent than occurs with closely related host species (282). Furthermore, interspecies transmission between different phylogenetic classes is even less frequent as has occurred rarely with chicken-to-human (282). One exception to the preceding rule has been the ease and frequency of transfer of swine H1N1 and H3N2 and pH1N1 viruses to turkeys when the two species were raised in close geographic proximity (168, 261, 282, 311). Obviously, many factors such as geographic restriction of host distribution, intermixing of species, age and density of birds, weather, and temperature also impacted the ability of the AI virus to move within and between host species and affected the overall incidence of infections (282). Outbreaks in 2014–2015 in North America demonstrated that transmission of Gs/GD-lineage H5N2 virus could occur between wild birds and domestic poultry, although how specifically the virus was transmitted was not clearly defined.

Potential sources of infection for the initial introduction of the influenza virus into commercial poultry flocks (i.e.,, primary infections) include: (1) other domestic and confined poultry, (2) migratory waterfowl and other wild birds, (3) domestic pigs, and (4) companion or pet birds (10, 11). The relative risk associated with each of these sources varies depending on the likelihood of direct or indirect contact with poultry. First, the LPM system poses a significant risk to the introduction of LPAI and HPAI viruses into the commercial integrated poultry systems. In the LPMs in the Northeast United States, the movement of poultry between markets or movement of contaminated trucks from markets back to farms was considered a risk factor for moving the virus (39). However for at least this market system, surveillance supported the virus being maintained in the market system and not from infection of the supplier farms.

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For commercial poultry where biosecurity should be at a higher level, how a virus is introduced on to the farm is often unclear. Theoretically, transmission could occur by airborne dissemination as proposed in some AI outbreaks (63). High volume air sampling during the 1983–1984 H5N2 and 2014–2015 H5N2 HPAI outbreaks in the United States yielded viable influenza virus in a few samples within 45 and 70 meters of an infected house (37, 214, 314). These findings and the initial location of outbreaks in houses near to ventilation points suggests airborne transmission may have some role in interflock dissemination of AI virus, including on dust particles (32). Nevertheless mechanical movement of virus on fomites including equipment, clothing, or shoes (37) is more likely to be responsible. Of especially high risk is movement of dead infected birds from farms through a shared rendering system or from the farm for burial without adequate sealing and decontamination of transport vehicles (32). However, in many outbreaks of AI, local transmission within control areas occurs but the precise pathway for virus introduction is not determined (93).

Second, introduction of AI viruses (especially LPAI viruses) from wild birds, especially waterfowl, has been well documented (99). The source is suspected to be contaminated feces from the ducks either through direct contact with poultry or indirectly through contamination of feed or water (95). The transmission potential of AI viruses from wild waterfowl emphasizes the need for producers of commercial poultry to separate domestic and wild bird populations and to treat surface water before using in a poultry house (75). In 2014–2015, plowing of grain fields adjacent to poultry houses and frequented by migratory waterfowl was associated with risk of introduction (334). Third, turkeys can be infected by introduction of pH1N1, H1N1, H1N2, or H3N2 and, potentially other subtypes of swine-origin influenza viruses, either by fomites or via humans infected with swine-origin influenza viruses (75). Fourth, AI viruses have been recovered from caged birds, usually during quarantine, but transmission for this source to poultry has not been documented, but caged bird transmission to poultry has occurred with Newcastle disease virus (75). To minimize the risk of introduction and dissemination of AI viruses, producers should raise only one species of bird in an individual operation, have an all-in-all-out production system, or add new birds only after testing and quarantine and practice a high degree of biosecurity, which is not the case in many places where Gs/GD lineage H5Nx HPAI viruses are endemic.

Secondary dissemination of AI viruses during an outbreak can be by fomite transmission, movement of infected poultry, or in some situations possibly airborne dissemination. Wild birds may play a major role in initial introduction of AI viruses in domestic poultry, but once established or adapted in commercial or LPM poultry, wild birds have had a limited or no role in secondary dissemination (114, 178). However, with the H5 Gs/GD lineage HPAI viruses, wild birds have been infected and played an important role in intercontinental spread of the virus to Europe and the Middle East in the winter of 2005–2006 (H5N1, Clade 2.2); Europe and the Middle East in 2009–2010 (H5N1, Clade 2.3.2.1); Europe and North America in 2014–2015 (H5Nx, Clade 2.3.4.4A); Europe, the Middle East, and Africa in 2014–2015 (H5Nx, Clade 2.3.2.1C); and 2016–2017 (H5Nx, Clade 2.3.4.4B) (153). Wild birds have also been responsible for introduction of virus to South Korea and Japan on multiple occasions since 2003, including 2016–2017 (H5N6, Clade 2.3.4.4C) (85, 150, 152, 153). This lineage of virus has persisted at a low level in wild birds and has been associated with other outbreaks since then. However, at this time it appears that certain H5Nx Gs/GD lineage HPAI viruses have the capacity to persist in wild bird populations with periodic transmission to gallinaceous poultry and this phenomenon has not been previously observed with other HPAI viruses (87).

Although horizontal transmission of AI viruses commonly occurs, proof of vertical transmission is lacking (75). However, natural and experimental HPAI virus infection of hens has resulted in virus recovery from the eggshell surface and the internal contents of the eggs (19, 41, 134). However, AI viruses are embryo lethal, and hatching of internally contaminated eggs has not been demonstrated, but eggs from infected flocks should still be destroyed (19). Cleaning of fecal material and disinfection of egg shells may be necessary to prevent hatchery-associated dissemination of AI viruses. Most LPAI and HPAI viruses cause reduction or cessation, respectively, of egg production further limiting the potential for vertical transmission of AI virus.

Successful experimental routes of exposure include aerogenous, intranasal, intrasinus, intratracheal, oral, conjunctival, intramuscular, intraperitoneal, intracaudal air sac, intravenous, intracloacal, and intracranial administration of the various viruses (75).

In experimental studies, AI virus has been shown to replicate and be excreted with virus shedding peaks at 2–5 days and a rapid decline of detectable virus when antibodies can be detected which is usually around 7 days after infection. However, the shedding pattern is not the same for all bird species, and rare cases of prolonged infection have been reported. Rare cases of detection of virus after 7 days is uncommon but has been noted in ducks for up to 30 days (329), chickens for up to 36 days (272), pheasants up to 45 days (119), and turkeys for up to 72 days (71, 116). However, on a population basis, AI virus can be maintained for much longer time periods within a population of birds on a premise and in one case re-emerged after a significantly stressful event. An H7N2 LPAI virus in Pennsylvania during 1997–1998 was recovered

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from dead chickens collected from the normal mortality of a layer flock six months after the initial AI infection and in another flock eight weeks after the induction of a molt, which the latter is a stressful event (345). Once a large flock is infected, it should be considered a potential source of virus until depopulated unless measures like vaccination are taken to eliminate the virus from the exposed flock (79, 107, 290). In wild waterfowl, AI viruses are maintained by passage in susceptible birds throughout the year, with peak prevalence in migratory waterfowl being in juvenile birds prior to fall migration likely because the juveniles are naïve to infection and congregate in larger numbers which increases the opportunities for transmission (75, 111). Prevalence of AI virus in migratory waterfowl is low as they arrive in the wintering grounds, but upon arrival, they can infect susceptible resident waterfowl which starts their own cycle of infection (108, 249). Thus, resident ducks contribute to the generation of virus during the winter and this source might reinfect migrating waterfowl prior to the spring migration. No information is available on the duration of infection in a flock of infected domestic ducks.

Incubation Period

The incubation periods for the various diseases caused by these viruses range from as short as a few hours in intravenously inoculated birds to 3 days in naturally-infected individual birds and up to 14 days in a flock (75). The incubation period is dependent on the dose of virus, the route of exposure, the species exposed, and the ability to detect clinical signs (75). In timed studies, intranasally inoculated chickens with H5N1 Gs/GD lineage HPAI virus from Mongolia developed clinical signs within 24 hours (61). However, this criterion may not be applicable to all AI viruses, especially LPAI viruses. Many infections by LPAI viruses do not cause clinical disease in all ages and all species of birds. "Infectious period", as defined as the time from exposure or detection of the virus to when the virus is no longer detected, may be more applicable for control and eradication purposes, especially in dealing with H5/H7 LPAI viruses. In biological terms, a more accurate way to assess incubation period would be from the time of exposure to virus shedding.

Clinical Signs

The pathotype of AI virus (LP or HP) has a major impact on the clinical manifestation of the disease. However, clinical signs of disease are extremely variable and depend on other factors including host species, age, sex, concurrent infections, acquired immunity, and environmental factors (75).

Low Pathogenicity Avian Influenza Viruses

Most infections by LPAI viruses in wild birds produce no clinical signs. However, in experimental studies in mallard ducks, LPAI virus infections suppressed T-cell function and produced a one-week depression in egg production (145, 147).

In domestic poultry (chickens and turkeys), clinical signs reflect abnormalities in the respiratory, digestive, urinary, and reproductive organs. The most frequent signs represent infection of the respiratory tract and include mild to severe respiratory signs such as coughing, sneezing, rales, rattles, and excessive lacrimation. In layers and breeders, hens may exhibit increased broodiness and decreased egg production, which in some cases may never recover back to preinfection levels. In addition, domestic poultry will exhibit generalized clinical signs including huddling, ruffled feathers, listlessness, decreased activity, lethargy, decreased feed and water consumption, and occasionally diarrhea. Emaciation has been reported but is infrequent because AI is an acute, not a chronic disease. Secondary infections can exacerbate clinical disease and increase mortalities.

In ratites, LPAI viruses produced similar respiratory signs to those in gallinaceous poultry and in some cases green diarrhea or green "urine" (190).

High Pathogenicity Avian Influenza Viruses

In wild and domestic waterfowl, most HPAI viruses replicate to a limited degree and produce few clinical signs. The major exception to this rule are some H5Nx Gs/GD lineage HPAI viruses which can infect and cause clinical disease including neurological signs, depression, anorexia, and sudden death (149, 316). Occasional sporadic, isolated cases of mortality have been reported in wild birds with other HPAI viruses. One unusual outbreak in wild birds occurred in 1961 with H5N3 HPAI outbreak in common terns in South Africa, which produced sudden death without any other clinical signs, and was localized to the tern population without involvement of gallinaceous birds (24).

In domestic chickens, turkeys, and related galliformes, clinical signs reflect virus replication and damage to multiple visceral organs, and cardiovascular and nervous systems. However, clinical manifestations vary depending on the extent of damage to specific organs and tissues (i.e., not all clinical signs are present in every bird). In most cases in chickens and turkeys, the disease is fulminating with some birds being found dead prior to observation of any clinical signs. If the disease is less fulminating and birds survive for 3–7 days, individual birds may exhibit nervous disorders such as tremors of the head and neck, inability to stand, torticollis, opisthotonus, and other unusual positions of head and appendages. The poultry houses may be unusually quiet because of decreased activity and reduction in normal vocalizations

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of the birds. Listlessness is common as are significant declines in feed and water consumption. Precipitous drops in egg production occur in breeders and layers with typical declines including total cessation of egg production within six days. Respiratory signs are less prominent than with LPAI viruses but can include rales, sneezing, and coughing. Other poultry have similar clinical signs but may live longer and have evidence of neurologic disorders such as paresis, paralysis, vestibular degradation (torticollis and nystagmus), and general behavior aberrations (202). Corneal opacity has been observed in domestic ducks infected with H5N1 Gs/GD lineage HPAI viruses (342).

In ostriches (*Struthio camelus*), reduced activity and appetite, listlessness, ruffled feathers, sneezing, hemorrhagic diarrhea, and open mouth breathing have been reported (43, 46, 54, 160). In addition, some birds were uncoordinated, exhibited torticollis, and had paralysis of the wings and tremors of the head and neck. However, signs observed depend on the virulence of the virus, for example an H5N2 AI virus in South Africa caused subclinical infection in most ostriches (313). Immune status, management, population density, and other causes of stress in ostriches are regarded as the ultimate determinants of the severity of avian influenza in this species (3).

Morbidity and Mortality

In chickens, turkeys, and related gallinaceous birds, morbidity and mortality rates are as variable as the signs and are dependent on virus pathogenicity and the host as well as age, environmental conditions, and concurrent infections (75). For the LPAI viruses, high morbidity and low mortality rates are typical. Mortality rates are usually less than 5% unless accompanied by secondary pathogens or if the disease is in young birds. For example, in the 1999 Italian H7N1 LPAI outbreak, mortality rates as high as 97% were observed in turkey poults less than four weeks of age when accompanied by secondary pathogens (42).

With the HPAI viruses, morbidity and mortality rates are high (50–89%) and can reach 100% in some flocks. Typically, the virus spreads rapidly among poultry housed on the floor with peak mortality (70–100%) occurring in 3–5 days of first clinical signs, but in poultry housed in cages, the virus spreads slower through the house with peak mortality taking 10–15 days. With the H5Nx Gs/GD lineage HPAI viruses, the mean death times in experimental studies (intranasal inoculation) are usually much shorter for chickens and turkeys than for other gallinaceous birds (205), but in domestic ducks, the mortality was dependent on virus strain and the age of the ducks with no illness or death from 1997–2001 H5N1 Gs/GD lineage HPAI viruses. Some viruses from 2001–2012 caused high mortality in 2-week-old ducklings and no mortality in 5–6 week old ducklings (194). This experimental variation based on age provides one explanation of why mortality rates in domestic ducks and geese in the field have been low (229, 341). Ducks also seem to have a different tolerance to even systemic infection, where experimentally a South Korean H5N1 Gs/GD lineage virus infected and caused systemic disease with high levels of virus in muscle tissue, but no clinical signs were observed (316). Nevertheless, high mortality can still occur in ducks and geese. For example over 1,000 deaths were reported in a flock of 8,200 four-week-old fattening ducks infected with a Gs/GD lineage H5N6 HPAI virus in Netherlands in December 2017 (25). High mortality was also reported in geese infected with Gs/GD lineage H5 HPAI viruses in Taiwan in 2015 (51).

In ostriches, LP and HPAI viruses usually produce moderate morbidity and low mortality rates but this depends on the strain of virus (2, 43). Typically, the morbidity and mortality have been highest in young birds (less than 3 months) with mortality of 30% being seen (43), but mortality rates as high as 80% have been reported for LPAI viruses in chicks less than one month of age (13)

Pathology

Numerous reviews have been published on the pathology of AI viruses (10, 11, 117, 123, 142, 169, 195, 205, 256, 257, 295). Details of field outbreaks and experimental studies have been published and are summarized in this section (5, 16, 18, 42, 43, 45, 46, 56, 70, 75, 125, 136, 137, 141, 145–147, 167, 190, 193, 200, 202–204, 206, 221, 234, 236, 258, 271–279, 283, 316). The lesions in wild birds have been reported for some Gs/GD lineage H5 HPAI viruses, but such information is beyond the scope of this chapter.

Gross

Gross lesions have been extremely variable with regard to their location and severity, depending greatly on the host species, pathogenicity of the infecting virus, and presence of secondary pathogens. Most frequently, descriptions of gross lesions have been provided for naturally occurring or experimental infections in chickens and turkeys.

Low Pathogenicity Avian Influenza Viruses. In gallinaceous poultry, the most frequent lesions are in the respiratory tract, especially sinuses, and are characterized as catarrhal, fibrinous, serofibrinous, mucopurulent, or fibrinopurulent inflammation. The tracheal mucosa can be edematous with congestion and occasionally hemorrhages. Tracheal exudates may vary from serous to caseous, with occasional occlusion of airways and resulting asphyxiation. Fibrinous

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to fibrinopurulent air sacculitis may be present. The fibrinopurulent inflammation usually is accompanied by secondary bacterial infections. The infraorbital sinuses may be swollen with muco-to-mucopurulent nasal discharge. Fibrinopurulent bronchopneumonia can result when accompanied by secondary pathogens such as *Pasteurella multocida* or *Escherichia coli*.

Catarrhal to fibrinous inflammation may be noted in the air sacs and coelomic cavity ("peritoneal cavity"), and "egg yolk peritonitis" may be observed. Catarrhal-to-fibrinous enteritis may be observed in the ceca and/or intestine, especially in turkeys. Inflammatory exudates may be found in the oviducts of laying birds, and the last few eggs laid will have reductions in calcium deposition within the eggshells. Resulting eggs may be misshapen and fragile with loss of pigmentation. Ovaries will undergo regression, beginning with hemorrhage in the large follicles and progressing to colliquation. The oviduct may be edematous and contain catarrhal-to-fibrinous luminal exudates before undergoing involution. In a few natural cases in laying hens and in intravenous inoculated chickens, swollen kidneys occurred and were accompanied by visceral urate deposition ("visceral gout").

Sporadically, other lesions have been reported including a firm pancreas with pale mottling and hemorrhage, usually in turkeys.

In domestic ducks and geese, LPAI viruses may produce lesions in the respiratory tract such as sinusitis, conjunctivitis, and other respiratory lesions. Coinfections with bacteria are common.

In rheas (*Rhea americana*) and emus (*Dromaius novaehollandiae*), LPAI virus infection produced ocular discharge; fibrinous sinusitis, tracheitis, and air sacculitis; interstitial pneumonia; congested visceral organs; hemorrhage in trachea; and occasional fibrinous perihepatitis and pericarditis.

Highly Pathogenic Avian Influenza Viruses. In gallinaceous poultry, HPAI viruses produce a variety of edematous, hemorrhagic, and necrotic lesions in visceral organs and the skin. Although, when death is peracute, no gross lesions may be observed. In chickens, swelling of the head, face, upper neck, and feet may be observed which results from subcutaneous edema and may be accompanied by petechial-to-ecchymotic hemorrhages (see Figures 6.2, 6.3, and 6.4). Periorbital edema may be seen (Figure 6.3). Necrotic foci, hemorrhage, and cyanosis of the non-feathered skin have been reported, especially wattles and combs. Lesions in visceral organs vary with virus strain but most consistently are represented by hemorrhages on serosal or mucosal surfaces and foci of necrosis within parenchyma of visceral organs. Especially prominent are hemorrhages on the epicardium (Figure 6.5), in pectoral muscles, and



Figure 6.2 Multifocal necrosis and hemorrhage of comb and wattles, H5N2 HPAI virus, 7 days postinfection (DPI). (USDA, M. Brugh) (*For color detail, please see the color section*.)



Figure 6.3 Severe edema of comb and wattles with accompanying periorbital edema and necrosis of epidermis at base of the comb, H5N2 HPAI virus, 7 days postinfection (DPI). (USDA, D. Swayne) (*For color detail, please see the color section*.)



Figure 6.4 Severe subcutaneous hemorrhages of feet, 3-week-old chicken, intranasal exposure to A/Hong Kong/156/1997 (H5N1), 5 days postinfection (DPI). (USDA, D. Swayne) (*For color detail, please see the color section*.)

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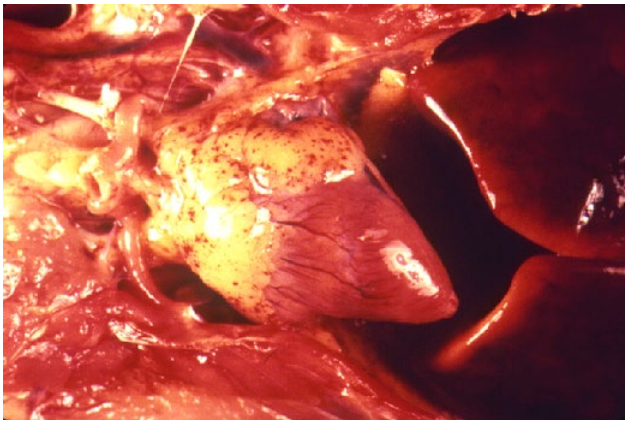


Figure 6.5 Petechial hemorrhages in epicardial fat, H5N2 HPAI virus, 4 days postinfection (DPI). (USDA, M. Brugh) (*For color detail, please see the color section.*)

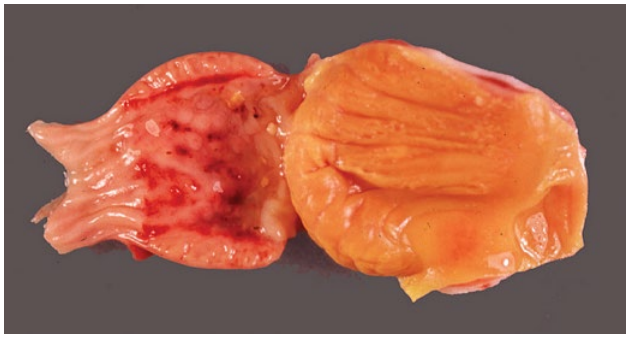


Figure 6.6 Mucosal petechial hemorrhages surrounding proventricular glands, 3-week-old chicken, intravenous exposure to A/Hong Kong/156/1997 (H5N1), 2 days postinfection (DPI). (USDA, D. Swayne) (*For color detail, please see the color section.*)

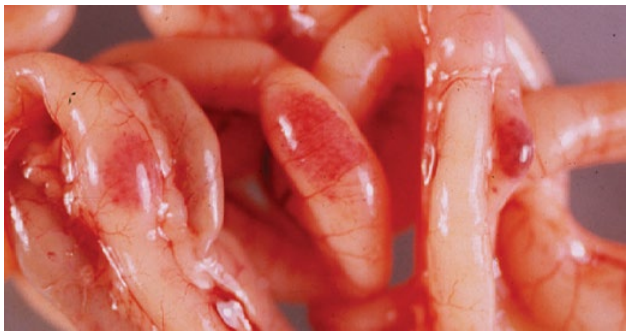


Figure 6.7 Hemorrhage in lymphoid tissue of Peyer's patches and Meckel's diverticulum of the jejunum, 3-week-old chicken, intravenous exposure to A/chicken/Hong Kong/27402/1997 (H5N1) HPAI virus, 1 days postinfection (DPI). (USDA, D. Swayne) (*For color detail, please see the color section.*)

in mucosa of the proventriculus and ventriculus (Figure 6.6). With the H5N1 Gs/GD lineage HPAI viruses, necrosis and hemorrhage in Peyer's patches of the small intestine were common as was reported with outbreaks of fowl plague in the early 1900s (Figure 6.7), and these viruses tend to produce more severe hemorrhage and edema in the lungs than other HPAI viruses (Figure 6.8).

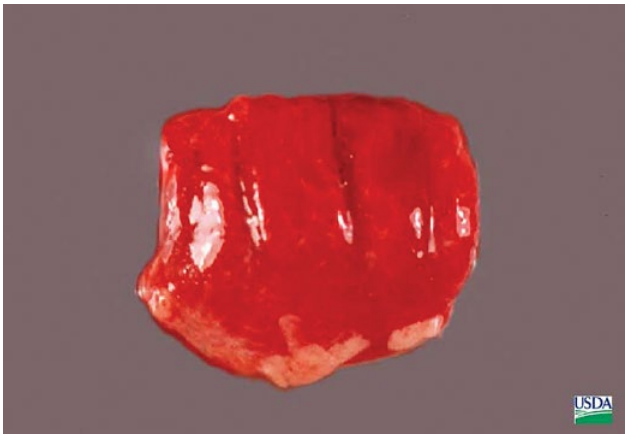


Figure 6.8 Severe pulmonary hemorrhage and edema, 3-week-old chicken, intravenous exposure to A/chicken/Hong Kong/156/1997 (H5N1) HPAI virus, 2 days postinfection (DPI). (USDA, D. Swayne) (*For color detail, please see the color section.*)



Figure 6.9 Hemorrhage and necrosis in the pancreas, 3-week-old turkey, intranasal exposure to A/chicken/Hong Kong/220/1997 (H5N1) HPAI virus, 2.5 days postinfection (DPI). (USDA, D. Swayne) (*For color detail, please see the color section.*)

With most HPAI viruses, necrotic foci are common in pancreas (Figure 6.9), spleen, and heart, and occasionally in liver and kidney. The kidney lesions may be accompanied by urate deposits. Lungs have focal ventral-to-diffuse interstitial pneumonia with edema. The lungs can be congested or hemorrhagic. The cloacal bursa and thymus are usually atrophic. Splenomegaly is frequent in gallinaceous birds infected with H5N1 Gs/GD lineage HPAI virus.

In ostriches, HPAI viruses produced edema of head and neck, severe hemorrhagic enteritis, enlarged and firm pancreas, mild-to-severe air sacculitis, hepatitis, peritonitis, renomegaly, and splenomegaly. Lesions are more severe and frequent in young birds.

Microscopic

Low Pathogenicity Avian Influenza Viruses. In poultry, LPAI viruses produce pneumonia varying in character from ventromedial, fibrinocellular-to-peribronchiolar

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lymphocytic. In severe cases, the pneumonia may be diffuse with air capillary edema. Heterophilic-to-lymphocytic tracheitis and bronchitis have been common. On intravenous (IV) or intranasal inoculation and in field cases in chickens, nephrosis and nephritis have been reported. However, this renal tropism is virus-strain specific and most consistently produced with IV inoculation. In turkeys, experimental and natural cases of pancreatitis with acinar necrosis have been seen, especially with the 1999 Italian H7N1 AI virus. Pancreatitis is less common in chickens than turkeys. Birds that die from LPAI have lymphocyte depletion and necrosis or apoptosis of lymphocytes in the cloacal bursa, thymus, and spleen, whereas other tissues such as trachea and nasal cavity have lymphocyte accumulations. Viral antigen is rarely seen in lymphocytes but is commonly demonstrated in necrotic respiratory epithelium, renal tubule epithelium, and pancreatic acinar epithelium. The former is primarily in IN-inoculated chickens.

In rheas (*Rhea americana*), the LPAI viruses produced heterophilic-to-pyogranulomatous sinusitis, bronchitis, and pneumonia with necrosis of respiratory epithelium. In ostriches, lesions of splenic and hepatic necrosis, enteritis, and sinusitis were seen.

Highly Pathogenic Avian Influenza Viruses. Lesions in natural outbreaks have been reported and have been reproduced in experimental studies with chickens. Histologic lesions are most consistent in tissues having gross lesions. Specific histopathologic descriptions for experimental studies vary with individual viruses as a result of variations in inoculum doses, strain of chicken, route of inoculation, and passage history. Basically, the histologic lesions consist of multiorgan necrosis and/or inflammation. The most consistent and most severely affected tissues are brain, heart, lung, pancreas, and primary and secondary lymphoid organs. Lymphocytic meningoencephalitis with focal gliosis, neuronal necrosis, and neuronophagia are common, but edema and hemorrhage may be seen. Focal degeneration to multifocal-diffuse coagulative necrosis of cardiac myocytes has been reported, usually with accompanying lymphohistiocytic inflammation. Lesions in the brain and heart have abundant associated influenza virus proteins in neurons and myocytes, respectively. Other common lesions associated with AI virus replication include necrosis in skeletal myofibers, kidney tubules, vascular endothelial cells, corticotropic cells of adrenal, and pancreatic acinar cells. If the birds survive for 3–5 days, the quantity of necrosis is reduced and the intensity of lymphohistiocytic inflammation is increased. In lymphoid tissue, necrosis, apoptosis, and depletion are common in cloacal bursa, thymus, and spleen, but AI viral antigen is rarely seen in lymphocytes. The lesions in respiratory tract vary widely from minimal to severe. The non-feathered skin contains numerous microthrombi within dermal and hypodermal capillaries and small blood vessels. This is accompanied by vasculitis, perivascular-to-generalized edema, subcutaneous edema, and necrosis of capillary endothelium. The epidermis has various stages of vesicle formation progressing to full-thickness necrosis. Virus can be demonstrated in feather shaft and follicle epithelium.

In gallinaceous species other than chickens and turkeys, lesions are similar to above, but in general, since the birds survive longer than chickens or turkeys, the necrosis and inflammation are more common and prominent in tissues.

In ostriches, HPAI viruses produced coagulative necrosis in spleen, kidney, and liver. Fibrinoid necrosis was common in the arterioles of the brain and spleen. The pancreas had necrosis of acinar cells with mild mononuclear cell inflammation and fibrosis. Foci of malacia and neuronophagia were present in brains, and necrotic and hemorrhagic lesions were present in the intestine.

Pathogenesis of the Infectious Process

In poultry, the process begins by inhalation or ingestion of infectious LPAI or HPAI virions. Because trypsin-like enzymes in respiratory and intestinal epithelial cells allow cleavage of the surface hemagglutinin, multiple replication cycles occur in respiratory and/or intestinal tracts with either type of virus. In gallinaceous poultry, the nasal cavity is a major site of initial replication.

With HPAI viruses, after initial replication in respiratory epithelium, the virions invade the submucosa, entering capillaries. The virus replicates within endothelial cells and spreads via the vascular or lymphatic systems to infect and replicate in a variety of cell types in visceral organs, brain, and skin. Alternatively, the virus may become systemic before having extensive replication in vascular endothelial cells. The virus is present in the plasma, and red and white blood cell fractions. Macrophages appear to play a role in systemic virus spread. The presence of a HA proteolytic cleavage site that can be cut by ubiquitous furin-like cellular enzymes is responsible for this pantropic replication. Clinical signs and death are due to multiple organ failure. Damage caused by AI viruses is the result of one of four processes: (1) direct virus replication in cells, tissues, and organs; (2) indirect effects from production of cellular mediators such as cytokines; (3) ischemia from vascular thrombosis, and (4) cardiovascular collapse from coagulopathy or disseminated intravascular coagulation.

For the LPAI viruses, replication usually is limited to the respiratory or intestinal tracts. Illness or death is most often from respiratory damage, especially if accompanied by secondary bacterial infections. Sporadically in some species, LPAI viruses spread systemically, replicating

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and causing damage in kidney tubules, pancreatic acinar epithelium, oviduct, and other organs with epithelial cells having trypsin-like enzymes.

Pathogenesis of the infection process is less well understood in non-gallinaceous birds.

Immunity

Active

Humoral Immunity. Infection with AI viruses as well as immunization with vaccines elicits a humoral antibody response at the systemic and potentially mucosal levels (259). This includes a systemic IgM response by 5 days post infection (DPI), followed shortly by an IgY response. The mucosal immune response is poorly characterized (259). The intensity of the antibody response varies with bird species. Leghorn-type chickens appear to have the highest antibody response followed by pheasant, turkeys, quail, and ducks (110, 259)

Antibodies against both the surface proteins, HA and NA, are neutralizing and protective, but antibodies to the HA are superior to NA for protection (208, 259, 308). The level of protection against mucosal infection and subsequent shedding of challenge virus may depend on the degree of antigenic (or protein sequence) similarity between HA of vaccine and challenge virus (281, 285, 286). Duration of protection is unknown, but in layers, experimental protection against clinical signs and death has been demonstrated to greater than 30 weeks following a single immunization (38). Birds that have recovered from field exposure are protected from the same HA and NA subtypes. Under field conditions, most meat producing poultry, such as waterfowl, turkeys, and chickens require a minimum of two vaccinations to maintain adequate protection, and long-lived poultry such as breeders and layers, may require three or more vaccinations throughout their life (77, 297). Single vaccination may only be effective for short-lived, highly immune competent broiler chickens that lack maternal antibodies to the AI virus of concern.

Cellular Immunity. As previously described, humoral immunity to the HA gene has a clear role for protection from virulent challenge, and killed adjuvanted vaccines typically provide high levels of HA specific antibody. However, the humoral immune response is just one part of the immune response, and cell-mediated immunity, mucosal immunity, and innate immunity are all involved in the control of AI virus infections or live viral vectored vaccination. The effector response to cell mediated immunity is thought to primarily be cytotoxic T cells, usually CD8 cells, that identify infected cells and mediate their deaths to abort the virus replication cycle. The level of protection is dependent on the number of circulating T cells that can recognize influenza-specific peptides, but the level of reactive influenza-specific T cells quickly declines after infection or vaccination leaving only a small number of memory T cells. The memory T cells when activated will replicate to increase the number of killer T cells, but this ramping up process takes several days, which by itself is insufficient to protect from highly virulent viruses like HPAI than can kill in two or three days (259). Experimental evidence of heterologous subtype infection by live H1N1 virus providing partial protection, likely due to one or more internal proteins, from H5N2 HPAI challenge have been described (179). Cell-mediated immunity from live virus vectored vaccines is also thought to play an important role in protection from virulent challenge because the HVT-AI and fowlpox-AI vaccines produce low levels of HA specific antibody, but vaccinated birds are often protected after challenge (269). Unfortunately there are not diagnostic tests that can easily measure the cellular immune response.

Passive

Partial protection by maternal antibodies to homologous HA or NA have been reported experimentally (64, 159). Such antibodies can provide protection from virulent challenge during a short period post-hatch, depending on concentration of such antibodies in progeny. Maternal antibodies also interfere with active immunity in progeny, when using either live or inactivated AI vaccines (64, 164).

Diagnosis

A definitive diagnosis of AI is established by: (1) direct detection of AI viral proteins or nucleic acid in specimens such as tissues, swabs, cell cultures, or embryonating eggs; or (2) isolation and identification of AI virus. A presumptive diagnosis can be made by detecting antibodies to AI virus. During outbreaks of HPAI, mortality rates, clinical signs, and lesions may be useful as part of the case definition in deciding which farms to quarantine and possibly for depopulation of birds for eradication purposes.

Sample Selection and Storage

Avian influenza viruses are commonly recovered from tracheal, oropharyngeal, or cloacal swabs of either live or dead birds, because most HPAI and LPAI viruses replicate in the respiratory and intestinal tracts. The swabs should be placed in a sterile transport medium containing high levels of antibiotics to reduce bacterial growth or immediately placed on wet ice for prompt shipment to the laboratory (296). Tissues, secretions, or excretions from these tracts are appropriate for virus isolation or

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detection. Tissues can be collected and placed into sterile plastic tubes or bags and also kept on wet ice until transported to the laboratory. In the examination of organs for virus, efforts should be made to collect and store internal organs separately from the respiratory and intestinal tract tissues because isolation of virus from internal organs may be an indication of systematic spread and is most often associated with HPAI viruses. In the case of systemic infections produced by HPAI viruses, virtually every organ can yield virus because of the high titer viremia or direct replication in parenchymal cells.

If the samples for virus detection can be tested within 48 hours after collection, they may be kept at 4°C; however, if the samples must be held for additional time, storage at –70°C or colder is recommended. Every freeze–thaw cycle will reduce the amount of infectious virus in the sample, so proper planning in the laboratory is essential. Before testing for virus, tissues should be ground as a 5–10% suspension in the transport medium and clarified by low-speed centrifugation.

Direct Detection of AI Viral Proteins or Nucleic Acids

The direct demonstration of influenza virus RNA or viral proteins in samples from animals is routinely used as a diagnostic screening test, used both in the laboratory and as rapid "point-of-care" tests in the field. Several commercial and laboratory specific antigen detection kits are available for detection of influenza A nucleoprotein and have been used to detect influenza viral antigen in avian specimens and allantoic fluid of inoculated embryonating chicken eggs (26, 53, 62, 138, 237, 338,). These antigen capture immunoassays vary in sensitivity with the best tests being 3–4 log less sensitive than virus isolation or real-time reverse transcriptase polymerase chain reaction (rRT-PCR) (338). The antigen capture immunoassays are recommended for use on dead or sick birds and not for random surveillance of healthy birds because of the sensitivity issue (239). Polyclonal and monoclonal antibodies are useful for localizing viral antigen in tissues by immunofluorescent or immunoperoxidase staining methods (232, 234, 321).

Reverse transcriptase polymerase chain reaction (RT-PCR) and real-time RT-PCR (rRT-PCR) methods are routinely used for field case diagnosis of AI (6, 244, 340). The rRT-PCR has a three-hour test time, and sensitivity and specificity comparable to virus isolation procedures, which has accelerated influenza diagnosis and field monitoring (91). In the United States, screening of tracheal or oropharyngeal samples from gallinaceous birds are done using a matrix gene rRT-PCR test and if positive, the samples are reflexively tested with H5 and H7 subtype specific rRT-PCR tests (244). For waterfowl and wild birds, both tracheal and cloacal swabs testing is recommended because some AI viruses are more enterotropic and respiratory screening alone may have decreased sensitivity (57, 124, 264, 309). With almost every study of wild bird sampling, a discrepancy is observed between rRT-PCR virus detection and virus isolation in embryonating chicken eggs with the rRT-PCR typically identifying more positive samples.

Virus Isolation

Methods for the isolation and identification of influenza viruses have been described in detail (181, 296). Chicken embryos, 9–11 days old, are inoculated via the allantoic cavity with approximately 0.2 mL of sample. In some cases, yolk sac inoculation has yielded viruses when allantoic cavity inoculation has failed (155, 337).

The death of inoculated embryos within 24 hours after inoculation usually results from bacterial contamination or inoculation injury, and these eggs should be discarded. A few viruses may grow rapidly and kill the embryos by 48 hours; however, in most cases the embryos will not die before this time. After 72 hours, or at death, the eggs should be removed from the incubator, chilled, and allantoic fluids collected. The presence of virus is demonstrated by hemagglutinating activity using chicken erythrocytes.

Generally, if virus is present in a sample, there will be sufficient growth in the first passage to result in hemagglutination, and repeated passage is unnecessary. Repeated passage of samples, blind passages, increases the risk of cross-contamination in the laboratory.

Virus Identification

Allantoic fluid positive for hemagglutination using standardized methods is used for virus identification (181, 296). It is important to determine whether the hemagglutinating activity detected in the allantoic fluid is due to influenza virus or other hemagglutinating viruses, such as paramyxoviruses including Newcastle disease virus (NDV). Thus, the isolate is tested in HI assays against Newcastle disease and other antiserum. If negative, the virus then is tested for the presence of the type A specific antigen to establish that an influenza A virus is present. The type-specific NP (nucleoprotein) or matrix protein may be detected by the double immunodiffusion test (17, 68), the single-radial-hemolysis test (68), or commercial antigen capture immunoassay. Monoclonal antibodies that react with the nucleoprotein or matrix proteins have proven useful in identifying these antigens in ELISA (328).

The next step in the identification procedure is to determine the antigenic subtype of the surface antigens, HA and NA. The NA subtype is identified by a micro-NI assay with antisera prepared against the nine known NAs (181, 296). The HA is identified in the HI test (181, 296) using a panel of polyclonal antisera prepared against whole virus representing the 16 distinct HA subtypes. Subtyping is facilitated by using antisera against the HA

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alone (i.e., not the whole virus) or against reassortant viruses with heterologous NAs; this helps avoid steric inhibition due to antibodies against the NA (129, 131). An influenza virus with a new HA would not be detected in tests using antisera to the known HA subtypes. Therefore, it is essential to confirm that the unknown hemagglutinating agent is an influenza virus using the type-specific test described previously.

Alternatively, screening is often performed directly with RT-PCR to confirm the presence of a type A influenza virus, and positive samples are reflexively tested for H5 or H7 because of the greater significance of isolation of these subtypes. Primers specific for many other subtypes are also available, although many are not validated. However, with the cost of sequencing continuing to drop, the direct sequencing of influenza viruses is becoming more common which allows for direct HA and NA subtyping. Rapid sequencing and analysis procedures are likely to gain in importance as routine diagnostic tools (66).

Final identification is most commonly accomplished by state, federal, or OIE influenza reference laboratories.

Serology

Serologic tests are used to demonstrate the presence of AI-specific antibodies, which may be detected as early as five days after infection. Several techniques are used for serologic surveillance and diagnosis. In serologic surveillance programs, a double immunodiffusion test (agar gel immunodiffusion or AGID) and ELISA assays are used to detect anti-NP antibody (IgM and IgY, respectively) because they are type A-specific antigens shared by all influenza A viruses (4, 23, 86, 166, 220, 241, 343). Several ELISAs are commercially available including indirect ELISAs specific for chickens and turkeys or competitive ELISAs that can be used with any species. Although the competitive tests can be used with any species, these tests have only been validated with a limited number of species, and true sensitivity and specificity are not known for all avian species. In general the ELISA tests are more sensitive than AGID or HI, but they also appear to be more prone to false positive results as well (161). ELISA tests are now commonly used for screening purposes, and positive results are confirmed by AGID or HI tests. Once influenza is detected by immunodiffusion or ELISA, HI tests can be used to determine the HA subtype. However, specific HI tests may be used as the primary test when specific strains are endemic.

In serologic assays, there is considerable variation in the immune response among the various avian species. For example, antibodies to the NP are generally prominent in turkeys and pheasants but may be undetectable in ducks (233). In addition, antibodies may be induced in ducks, as well as other species, which fail to be detected in conventional HI tests performed with intact virus (132, 157).

The sera of many species contain nonspecific inhibitors that may interfere with the specificity of the HI and other tests. Because these inhibitors are especially active against certain viruses, they present a practical problem in serologic testing and the identification of viruses. Therefore, sera should be treated to reduce or destroy such activity, although it should be recognized that some treatments may lower specific antibody levels. The two most commonly used treatments for these inhibitors have been receptor destroying enzyme (RDE) and potassium periodate (50, 68). In addition to the non-specific inhibitors of hemagglutination, sera from other avian species, such as turkey and goose, may cause nonspecific agglutination of the chicken erythrocytes used in the HI test. This may mask low levels of HI activity. Such hemagglutinating activity can be removed by pretreatment of the serum with chicken erythrocytes (177). This problem may sometimes be avoided by using erythrocytes in the HI test of the same species as the serum being tested. Receptor destroying enzyme is not normally needed for chicken sera.

Differential Diagnosis

Because of the broad spectrum of signs and lesions reported with infections by AI viruses in several species, a definitive diagnosis must be made by virologic and serologic methods. For HPAI viruses, other causes of high mortality must be excluded such as velogenic Newcastle disease, septicemic fowl cholera, heat exhaustion, water deprivation, and some toxins. For LPAI viruses, other causes of respiratory disease and drops in egg production must be investigated such as lentogenic NDV, avian metapneumovirus and other paramyxoviruses, infectious laryngotracheitis, infectious bronchitis, chlamydia, mycoplasma, and various bacteria. Concurrent infections with other viruses or other bacteria have been commonly observed (75).

Intervention Strategies

Management Procedures

Interventions for AI include measures designed to: (1) prevent introduction of virus, (2) reduce the likelihood of infection of birds once virus is introduced, (3) prevent movement of virus from a premise with infected birds to another premise and, if possible, (4) eliminate/eradicate the virus. These outcomes are accomplished using combinations of five specific, interrelated components: (1) biosecurity (including modifications to the way poultry are reared and sold, movement management, and cleaning and disinfection), (2) active and passive surveillance (and associated diagnostic services), (3) elimination of

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infection in poultry (mainly through stamping out), (4) decreasing host susceptibility (mainly through vaccination), and (5) education including risk communication. Adoption of control and preventive measures relies on extensive farmer and trader education/risk communications. Implementation of control measures is facilitated by availability of indemnities for losses associated with destroyed poultry and other property. The measures can be applied at any level—regions, countries, parts of countries, farms, and markets.

First, preventing introduction of virus to regions or countries relies on implementation of strict import controls on poultry and poultry products. Rules for import of poultry and poultry products described in the OIE Terrestrial Code (181) markedly reduce the likelihood of transmission but are not applied consistently in all places. Illegal trade in live poultry has been an important mode of introduction of virus (e.g., cross-border trade in spent hens to Viet Nam). Introduction of both LPAI and HPAI through wild birds is more problematic with the latter best illustrated by the long-distance movement of Gs/GD lineage H5 viruses since 2003 across four continents (Africa, Asia, Europe, and North America). Some intensive poultry farming areas are located in high risk zones for wild bird introductions of virus (e.g., Fraser Valley in British Columbia and parts of northern Italy) demonstrating the importance of location in risk of introductions.

At the farm level, biosecurity measures are used to prevent entry of AI viruses. All well-managed, intensive commercial farms have biosecurity measures in place, but flaws in biosecurity become evident at times when high concentrations of virus are present in an area and outbreaks occur (93, 334). As a rule well-managed commercial broiler farms appear to be better protected than chicken layer and turkey farms based on experiences from the United States and this probably relates to the nature of biosecurity measures in place. Preventing introduction of AI viruses to LPM depends on strict source control. In some places (e.g., Hong Kong), reducing the number of species sold and compulsory vaccination for all birds destined for LPM has assisted in reducing this risk.

Second, to reduce the likelihood of infection of poultry once virus enters a farm or market, biosecurity measures play an important role. Vaccination has been added as an additional measure in some places to increase resistance to infection. Appropriate monitoring and surveillance systems are required to detect incursions and subsequent infections of poultry (and humans).

Third, measures taken to prevent transmission between farms once poultry are infected include stamping out, biosecurity measures (including movement management), and vaccination. Vaccination has been used both as an emergency measure during outbreaks (78) and as a means of reducing levels of virus circulation in endemically infected countries. It has been used successfully to reduce the likelihood of human exposure to zoonotic avian influenza viruses especially in places where birds are sold live in markets (H7N9 in China, H5Nx in multiple countries).

Fourth, measures taken to eliminate/eradicate the virus requires the highest level of practice of biosecurity, active and passive surveillance, and elimination of infection in poultry. Decreasing host susceptibility through vaccination can be helpful if potent, antigenically matched vaccine is available and can be properly applied and monitored in the field. Education including risk communication are critical to achieve any of the four outcomes. The disinfection of farms and manure and carcass management are important to break the transmission chain.

The success of preventing virus introduction, reducing infection of birds, preventing movement of virus from an affected premise, and eliminating/eradicating the virus is dependent upon how many of the components are used and how thoroughly they are or can be practiced in the field, which in turn depends on the quality of veterinary and animal production services and the nature of the poultry industries in the area where the disease occurs (84). The goals for individual LPAI and HPAI control and preventive strategies may differ depending on the country, subtype of the virus, economic situation, and risk to public health.

There is no "single" control/preventive strategy for AI. A combination of measures must be used. In higher-income countries, most HPAI outbreaks have been eradicated within three months to a year using stamping-out programs, coupled with movement controls and tracing, but in some lower- income countries, complex poultry production and marketing systems and weak veterinary infrastructure have made eradication unachievable.

In these situations, management of the disease to a low infection rate has been the only realistic option. By comparison, control and preventive measures applied for LPAI have varied greatly among individual countries, and even between states and provinces within a single country (290). Notably good control programs in the United States have emerged from Minnesota (102, 207) and Pennsylvania (63) which have been successful in eradicating LPAI viruses on multiple occasions. Recommendations and responsibilities for containing influenza outbreaks have been described (89). The designation of H5/H7 LPAI as reportable to OIE (2015–2019) has increased the international use of stamping out in dealing with these two AI subtypes as a means to prevent emergence of HPAI viruses. It has also resulted in disproportionate restrictions applied by some countries as non-tariff trade barriers on imports from affected countries even in the face of localized outbreaks.

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Biosecurity Including Movement Management

Biosecurity is the first line of defense (see Chapter 1), and consists of exclusion measures to keep the virus out of virus-free premises and containment to prevent virus from spreading once cases occur (293). Every farm should have a biosecurity plan that examines all potential pathways for entry of AI virus followed by development of appropriate measures to minimize the risk for each pathway. This can include both facilities (such as fences and bird proofing of houses) and management procedures (use of cleaning and disinfection, segregation of newly introduced poultry, procedures for handling dead birds, preventing vehicles' entry to areas close to poultry houses, etc.) overseen by a designated biosecurity manager (often the company veterinarian).

The most likely source of virus for poultry is other infected birds, so the basic means for the prevention of infection of poultry with influenza viruses is the separation of susceptible birds from infected birds and their secretions and excretions. Transmission can occur when susceptible and infected birds are in close contact or when infectious material from infected birds is introduced into the environment of susceptible birds. Such introductions are associated with the movement of cages, equipment, footwear and clothing, vehicles, insemination equipment, and so on. The presence of virus in fecal material and respiratory secretions is a likely means for movement of the virus either by ingestion, contact with mucus membranes, or inhalation. Contaminated poultry manure is a high risk source for virus transmission between flocks. Certain things have been identified that contribute to spread once AI has been introduced into commercial flocks: moving contaminated equipment and crews, partial flock marketing, marketing an actively infected flock, shared rendering pick-up of daily mortality, moving the birds, and inadequate cleaning and disinfection (104). Poultry raised outdoors or which have outdoor access have been infected following exposure to wild birds, primarily infected ducks and shorebirds. Measures have been devised to reduce the likelihood of these birds coming into contact with wild birds, but in some countries production systems are such that contact between domestic and wild waterfowl cannot be avoided. In some countries, LPM and traders' facilities are important reservoirs of influenza virus and pose a risk for introduction to commercial poultry if adequate biosecurity is not practiced. Swine may serve as a source of H1 and H3 swine influenza viruses to turkeys where the virus is transmitted by fomites or by infected people or pigs (75).

Biosecurity practices limit spread of influenza by preventing contamination; controlling the movement of birds or their products, people, and equipment; or reducing the amount of the virus (e.g., cleaning and disinfection) (100, 293). Persons who have direct contact with birds or their manure have been the cause of most virus transmission events between houses or premises, but airborne transmission has likely served as a source to some farms in association with certain depopulation and cleaning activities during the peak of infection (32, 63, 215). It was also suspected in some cases to be associated with wild birds. Equipment that comes in direct contact with birds or their manure should not be moved from farm-to-farm without adequate cleaning and disinfection, and it is important to keep the traffic area near the poultry house free from contamination by manure. Visitors on farms should not be allowed or should be strictly controlled with changes of footwear mandatory, disinfection of footwear, and cleaning of clothing. Farm-to-farm spread of influenza virus must first be brought under control before the disease can be eradicated.

Movement management is also used to limit the spread of virus to other farms and markets. However, in many low- and medium-income countries controls imposed on poultry movements are weak, poultry farms are located too close together and a considerable part of the demand for poultry occurs through improperly managed LPM. Live poultry markets and associated traders' facilities can become sites of viral propagation and persistence and have been sites for transmission to humans of zoonotic AI viruses. Virus transmission from markets back to farms can occur through fomites and movement of infected live poultry. As with farms, every LPM should have a set of measures in place to reduce the likelihood of entry of virus and subsequent transmission to and between birds held in the market (83).

Special biosecurity procedures must be used when depopulating or marketing LPAI infected or dangerous contact flocks, including movement only after virus detection is found to be negative; re-routing trucks away from other farms; and sealing, cleaning, and disinfecting depopulation trucks before they leave farms. In addition, special biosecure practices are needed in repopulating within an infected zone or compartment during the recovery phase to prevent resurgence of the virus.

Experiences with Gs/Gd lineage H5Nx HPAI and H7N9 avian influenza have demonstrated that some production and marketing practices need to be modified for successful control and prevention of infection. These have included measures such as compulsory vaccination of poultry destined for LPM (80), segregation of species and introduction of market rest days, in which all poultry remaining unsold in a market are killed at the end of a trading day, and no new poultry are introduced until the market has been thoroughly cleaned and disinfected. Some cities have shifted from sale of live poultry in markets to sale of chilled carcasses from central slaughterhouses. These measures can shift the problem from areas where markets are closed to other areas (52).

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In many parts of the world, but in particular lower-income countries, biosecurity systems for chicken production on many farms have not kept pace with the expanding demand for poultry products in large urban centers (e.g., Jakarta, Cairo, Dhaka). Management procedure such as partial harvesting of broiler flocks by traders to meet market demands for small birds increases the risk of virus incursion to farms. Biosecurity measures on commercial duck and geese farms in many countries are also weak, especially for those enterprises rearing birds outdoors on ponds, watercourses, and fields.

Numerous guides have been developed on biosecurity for farmers (320) but implementation of the measures remains variable especially when resources are limited. Communication and education campaigns have been used to increase uptake of these measures with variable results. Farm accreditation programs applied by many commercial farms in high-income countries require adherence to strict biosecurity standards.

Diagnostics and Surveillance

Accurate and rapid diagnosis of AI is a prerequisite to early and successful control. The speed with which AI is controlled in newly infected places is largely dependent upon how quickly the first case or cases are detected, the existing biosecurity measures, and how quickly control strategies are implemented, especially if virus elimination is the goal.

Samples submitted through passive surveillance are critical to differentiate LPAI virus as the cause of respiratory disease or drops in egg production from causes of endemic diseases with similar signs. Similarly, HPAI virus must be differentiated from other causes of high mortality events. However, many farmers in low-income countries do not submit samples from these cases. Active surveillance is essential for detecting infection in species such as domestic ducks that can be infected subclinically, in LPMs, or to detect infection with LPAI viruses that cause mild or no clinical disease in gallinaceous birds. Determining where the virus is located within a country, zone, or compartment, is best accomplished through either serological testing of birds for antibodies and/or random testing of daily mortality for the presence of AI virus. Market-based virological surveillance has been used widely in Asia. Surveillance is also crucial for ongoing evaluation of the success of control strategies and for use in decision-making as a prelude to improving control strategies. Serological testing has been used to certify a country, zone, or compartment as AI free, or during an AI outbreak to determine the extent of the infected zone for quarantine purposes. However, serology for HPAI in non-vaccinated chickens is of low value in detecting infections given the high rates of mortality associated with these viruses.

Avian influenza surveillance systems have improved over the past 15 years following considerable investment as a response to Gs/GD lineage H5Nx HPAI viruses. Nevertheless, in many lower-income countries, passive surveillance systems, based on reporting of AI-compatible events by farmers to veterinary authorities, remain weak. Active surveillance has improved but in countries where AI viruses remain endemic it detects only a small proportion of affected farms and consignments of poultry. Surveillance systems rely on laboratories that have in place appropriate quality management systems that provide high levels of confidence in test results. Field-based rapid tests, including some based on antigen detection using immunological reactions and others based on rapid nucleic acid extraction, amplification, and testing, are being deployed in parts of Asia.

Elimination of Infection in Poultry

After identification of infected flocks, elimination of infection in the flocks, their eggs, and manure is required to prevent onward transmission to other premises. For HPAI, this has been typically accomplished through depopulation/stamping out and disposal of carcasses, eggs, and manure by a method such as composting, incineration, rendering, or landfill burial. For LPAI, orderly marketing of birds after recovery from infection has been an acceptable means for elimination, and eggs can be marketed if properly cleaned. Stamping out is the method applied most frequently once HPAI virus is detected in farms or markets that were previously not known to be infected. It is compulsory in the United States once infection with H5 or H7 HPAI virus is detected. Virus elimination using stamping out is possible if all cases are detected early, which, in turn, depends on a well-developed surveillance and tracing system and appropriate veterinary capacity. Nevertheless, even when successful, the cost associated with stamping out can be very high especially if multiple large farms are involved. Outbreaks involving the destruction of 20 million or more birds have become more frequent in the past 17 years. For example, the 2015 Gs/GD lineage H5N2 HPAI outbreak in the upper midwest of the United States resulted in the death or destruction of over 50 million poultry. The virus was eliminated from poultry but the direct cost was estimated at $USD 850 million (304).

In some places infection with AI viruses is endemic and virus elimination using stamping out has not been achieved due to a number of constraints including the nature of the poultry industry. For example, in Viet Nam in 2004 over 45 million head of poultry were culled or died of infection, but the Gs/GD lineage H5N1 virus was not eliminated. Endemic AI viruses that have not been amenable to stamping out include H5N2 LPAI and H7N3 HPAI viruses (Mexico), H9N2 LPAI viruses (many countries in Asia, northern Africa, and the Middle East),

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H7N9 LP and HPAI viruses (China), and Gs/GD lineage H5Nx HPAI viruses (Egypt, Nigeria, China, Viet Nam, Cambodia, Indonesia, South Asia including Bangladesh).

Most influenza virus shedding occurs during the first two weeks of infection and usually by four weeks after the initiation of the infection, virus cannot be detected by sampling. Sero-positive flocks have not been associated with a high risk of transmission if maintained under biosecure practices. However, there should be no contact with recovered flocks because the length of time birds within a population shed virus is not clearly defined. Because the economic losses due to influenza may be severe, the control program should not unnecessarily penalize the growers. Indemnities by federal governments may be necessary for control and eradication of both HPAI and H5/H7 LPAI. Methods used for depopulation are rapidly evolving and include the use of foam (14).

Indemnities and Compensation. In all high-income countries farmers are paid indemnities/compensation for the loss of poultry as a result of stamping out for AI control. Compensation may be provided for other items destroyed such as feed but usually does not cover consequential losses. This acts as a form of insurance and may provide an incentive to report disease outbreaks early especially given the explosive nature of HPAI and the different rates offered in some countries for healthy and sick poultry. Formal insurance schemes that cover losses from AI are available in some high-income countries. Experiences in low- and middle-income countries where compensation is available but reporting of AI-compatible events remains weak strongly suggest that other economic and social drivers of behavior determine whether reporting occurs.

Decreasing Host Susceptibility

If poultry are at risk to AI virus exposure, increasing the resistance of birds to infection may be necessary to break the infection cycle. Theoretically, this might be achieved by genetic selection for resistant bird strains or breeds, but to date, only minor genetic resistance to LPAI virus infectivity has been attributed to differences in chicken breed (276), but genetic resistance to HPAI virus infection and prevention of lethality in chickens is minimal and is not linked to MHC genes (120). However, transgenic chickens with inserted short-hairpin RNA that inhibits and blocks influenza viral polymerase resulted in reduced transmission of the virus, but did not prevent infection and death when inoculated directly into the chickens (158). Another method to increase resistance is through active or passive immunity to the AI viral HA or NA. This is predominantly done through vaccination, but antibody and immune cell transfer can be protective.

Education and Risk Communication

One critical aspect in prevention and control is to work with poultry and allied industry personnel to put in place appropriate measures to minimize the risk of virus introduction and spread. This relies on understanding pathways for virus introduction and spread. An individual's control of risky behaviors greatly reduces the spread of AI virus by controlling fomite or aerosol movement of the virus thus preventing AI virus movement on the farm and between farms. Much has been learned from major outbreaks about the manner in which AI virus is introduced to farms and the role played by farm management practices and this has been converted to guidance for farmers and traders. Risk communications have been a cornerstone of efforts to assist in containing H5Nx Gs/GD lineage HPAI although this will not eliminate all high risk practices (327).

Vaccination

Various vaccine technologies have been developed and have shown efficacy in experimental studies, mostly in chickens and turkeys, to provide protection from LPAI and HPAI viruses (288). The most frequently licensed AI vaccine technology has been inactivated whole AI virus adjuvanted vaccines, typically made using LPAI field outbreak strains or reverse genetic (rg) generated AI vaccine strains, followed by chemical inactivation and oil emulsification (291, 306). Live recombinant fowl poxvirus, herpesvirus of turkeys, and Newcastle disease vaccines with AI H5 gene inserts (rFPV-AIV-H5, rHVT-AIV-H5, and rNDV-AIV-H5, respectively) have been licensed and are used in a few countries (303). Recently, an RNA particle vaccine, based on Venezuelan equine encephalitis virus replicon particles (213), containing H5 Clade 2.3.4.4 Gs/GD HA insert has been licensed in the United States and is included along with a rHVT-AIV-H5 and a rgH5N1 inactivated oil-emulsified vaccines in the US poultry emergency vaccine bank (30, 303). A recombinant duck enteritis virus vaccine shows potential for improving H5 AI control in domestic duck populations (156).

Vaccines have been used in a variety of poultry and other avian species, and their effectiveness in preventing clinical signs and mortality and reducing viral shedding is well documented. However, protection is virus HA subtype specific. Birds are susceptible to infection with influenza A viruses belonging to any of the 16 HA subtypes, and universal vaccines that protect against all 16 subtypes are not available. Thus it is not practical to use preventive vaccination against all possible HA subtypes. However, if a particular HA subtype is at risk for introduction or after an outbreak occurs and the HA subtype of the virus is identified, vaccination may be a useful tool in a control and preventive program (101). At this time

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protective vaccines based on conserved AI proteins (such as nucleoprotein, matrix proteins, or polymerase proteins) are not available but experimental studies are continuing (60, 198, 242).

Numerous experimental studies (9, 15, 27, 35, 38, 40, 127, 130, 254, 255, 298, 331, 336) have demonstrated that AI vaccines are capable of inducing antibody and providing protection against mortality, morbidity, and declines in egg production. In addition, properly administered vaccines increase resistance to AI virus infection, reduce the number of birds infected and shedding virus, greatly reduce the titer of challenge virus shed, prevent experimental contact transmission, and reduce group transmission evident by reducing the R_0 below 1 (323). Carefully controlled use of vaccines in an H5 and H7 LPAI outbreak may reduce the chance of the emergence of HPAI viruses by reducing the amount of virus replication. Most frequently, vaccines are administered by subcutaneous administration. The rFPV-AIV-H5 and rHVT-AIV-H5 vaccines are given by subcutaneous or wing web (rFPV-AIV-H5 only) inoculation at one day post-hatch in chickens only, although some work on *in ovo* use has been proposed. Post-hatch, the rFPV-AIV-H5 and rHVT-AIV-H5 vaccines cannot be used in chickens that have received a prior poxvirus or Marek's disease herpesvirus (MDV) vaccine or have been infected by a field strain of fowl poxvirus or MDV respectively, otherwise production of AI active immunity will be inhibited (82, 284). The rNDV-AIV-H5 is administered by spray or eye drop. Both rFPV-AIV-H5 and rNDV-AIV-H5 have been used primarily as a priming vaccine at one day of age followed by a boost 10–21 days later with inactivated AI vaccine. The rHVT-AIV-H5 has been purported in specific pathogen free (SPF) chicken studies to give lifetime immunity, but in the field, a boost with inactivated vaccine may be needed to give long-term immunity in long-lived chickens and is used in this manner in Egypt (133). Maternal antibodies to NDV vector or AI virus interfere with active immunity from the recombinant vaccines. In the United States, only USDA licensed AI vaccines are allowed. The state veterinarian can approve field use of H1–4, H6, and H8–16 vaccines, but the field use of H5 or H7 AI vaccines also requires approval by USDA.

Vaccination has a number of potential disadvantages, all of which can be managed. At present, uptake of vaccination is inhibited by overemphasis of the disadvantages with insufficient consideration of the advantages provided by vaccination, the epidemiological situation in places where vaccination could be of value, and the mitigation measures that can be applied to overcome the disadvantages.

When vaccination is used as a preemptive measure in high risk places that want to remain virus free, appropriate surveillance systems must be in place to detect viral transmission into vaccinated flocks (e.g., Hong Kong). This condition also applies when vaccination is used as a component of a virus elimination program. Various methods are available to do this including virological surveillance using samples from routine dead bird monitoring, environmental samples (e.g., drinking water samples), and unvaccinated sentinel birds, or serological surveillance that rely on tests that specifically detect antibodies from field virus infection and are not induced by vaccination (DIVA tests). Tests based on detection of virus give information on the state of infection at the time of sampling.

A range of serological tests could be used. For example, with birds vaccinated with a heterologous NA vaccine, detection of anti-NA antibodies against the NA of the field AI virus (47) is indicative of infection in vaccinated birds. If using recombinant vaccines that express only the AI virus hemagglutinin (such as rFPV-AI-H5, rHVT-AIV-H5, or rNDV-AIV-H5, and boost with RNA-particle vaccine), detection of antibodies against AI virus NP or M proteins (AGID or ELISA) indicate infection in vaccinated chickens. Vaccinated flocks cannot be considered influenza virus-free without adequate surveillance. Serological tests provide information on exposures that occurred two or more weeks previously, can be complicated by immune responses to other cocirculating influenza viruses and are relatively insensitive. Vaccinated flocks must be identified and monitored for the presence of AI virus until slaughtered using cost-effective approaches (266).

Additional considerations that influence decisions on vaccination for H5 or H7 LPAI viruses have been discussed (20, 102, 106). Previously, the lack of a government indemnity program for H5 and H7 LPAI in the United States resulted in some industry segments (e.g., egg layers) being subject to severe economic damage from these viruses. In some low-income countries, by withholding vaccine availability, regulatory agencies may have provided the producer with an incentive to intentionally expose his/her flock during the pullet stage or early growout stage to reduce the economic impact of H5 and H7 LPAI on egg production or air sac condemnations, respectively. Intentional exposure is likely to contribute to the spread of the disease. Controlled, effective vaccine use will reduce the population of susceptible poultry and reduce the quantity of virus shed if infection occurs. Recent examples where inactivated H5 or H7 vaccine has been used as an aid in controlling H5 and H7 LPAI include Minnesota (106, 290), Utah (92, 103), Italy (81), Portugal (297), Mexico and other Central American countries (297), and Connecticut (290). Ten billion doses of H5 and H7 vaccine were used against LPAI from 2002–2010 (301). For HPAI, vaccine use was first implemented in Mexico (H5N2) and Pakistan (H7N3) in 1995 (297). Large scale vaccination against Gs/GD lineage of H5 HPAI began in 2002 with Hong Kong and has

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surpassed 113 billion accumulative doses by 2010 and the rate of 25 billion doses per year continues today. For 2002–2010, the majority of vaccine was used in national routine vaccination programs in China (91%), Egypt (4.6%), Indonesia (2.3%), and Viet Nam (1.4%) and the remaining 0.7% was used in targeted vaccination programs divided among 11 countries/regions (Cote d'Ivoire, France, Hong Kong, Israel, Kazakhstan, North Korea, Mongolia, The Netherlands, Pakistan, Russia, and Sudan). Inactivated oil-emulsified vaccine accounts for 95.5% of vaccine used and 4.5% are recombinant vaccines. In 2018, large-scale vaccination was practiced officially in China (H5 and H7), Egypt (H5), Indonesia (H5), Vietnam (H5), Bangladesh (H5), and Mexico (H5 and H7). It is also being used unofficially in a number of other Asian countries. A national US HPAI control program has been implemented covering all aspects of outbreak control (318) including emergency vaccine use which to date has not been used.

It is clear that opportunities to develop a variety of effective vaccines exist, but the main issue is the logistics for proper administration in the field in order to induce effective population immunity. The ensuing debate (20) centers on the role they should play in controlling influenza viruses of varying pathogenicity in different domestic bird populations in different geographic regions. Based on the multitude of influenza A viruses in wild bird populations, it is reasonable to expect that these viruses will continue to cause serious disease problems when introduced into the LPM, rural or village poultry, and commercial poultry industries. Therefore, judicious use of vaccines may be appropriate to reduce influenza transmission and decrease susceptibility of poultry to the viruses, so eradication methods can be implemented before the disease spreads and becomes endemic. Preemptive vaccination is also applicable in high risk places that have experienced repeated outbreaks of HPAI following wild bird introduction. It is also being used in places where virus has become endemic. In these places other control/preventive measures should be introduced such as improvements to farm biosecurity, market hygiene, and movement management. In such cases monitoring and surveillance programs need to be implemented to detect antigenic variant viruses to ensure that appropriate, closely matched, vaccine antigens are incorporated into vaccines. Vaccination programs should be targeted at high risk populations, and regular reviews on the need for vaccination as well as the target population are conducted. This approach is used in a number of countries where elimination of H5N1 HPAI by classical methods proved impossible and remains a distant goal. Live virus vaccines that cannot transmit but which can be applied in an emergency by mass means (i.e., spray or drinking water) may be of benefit in outbreak control in the future given the difficulties in applying vaccination that relies on individual bird inoculation in the face of an outbreak.

Treatment

Presently, no practical, specific treatment exists for AI virus infections in commercial poultry. Amantadine has been shown experimentally to be effective in reducing mortality (21, 67, 75, 144, 330), but the drug is not approved for food animals, and its use rapidly gives rise to amantadine-resistant viruses which compromises public health (19, 330). Supportive care and antibiotic treatment have been employed to reduce the effects of concurrent bacterial infections. The use of human anti-influenza drugs such as M2 inhibitors (amantadine and rimantadine) and NA inhibitors (oseltamivir and zanamivir) is strongly discouraged because of the potential to generate resistance that can result in a loss of these drugs for public health use. Treatment of infected birds is not recommended.

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7

Infectious Bursal Disease

Nicolas Eterradossi and Yehia M. Saif

Summary

Agent, Infection, and Disease. Infectious bursal disease (IBD) is an immunosuppressive disease of young chickens of worldwide prevalence. It is caused by IBD virus (IBDV), a double-stranded RNA birnavirus which is highly resistant to harsh environmental conditions. Two serotypes of the virus (serotypes 1 and 2) have been described and antigenic variants of both serotypes have been recognized. However, only viruses of serotype 1 are pathogenic and multiple serotype 1 pathotypes have been described. The disease could result in high morbidity and mortality. In addition, the immunosuppressive effect of the disease lowers the bird's resistance to other infections and reduces responsiveness to commonly used vaccines.

Diagnosis. Clinical signs are not pathognomonic, but gross and microscopic lesions are highly suggestive of the disease. Gross lesions mostly observed in the bursa of Fabricius include enlargement, change in color, and hemorrhages followed by atrophy. Other gross lesions may include hemorrhages in the breast and leg muscles. Microscopic lesions of the bursa include necrosis of lymphocytes followed by appearance of heterophils. Furthermore, microscopic examination of IBDV-infected bursal sections often reveals hemorrhagic and cystic cavities. The virus can be propagated in embryonated chicken eggs and a variety of primary and established cell lines. However, virus isolation is not practical for routine diagnosis. Bursal homogenates are the preferred material for virus detection and currently RT-PCR is the preferred test for virus/RNA detection. Commercial ELISA kits are available for antibody detection but virus neutralization is the only test that can distinguish the serotypes and their variants.

Intervention. Vaccination is used successfully to prevent the disease. Breeder flocks are intensively vaccinated with live and inactivated vaccines to passively transfer high levels of maternal antibodies to progeny. Vaccination schedules of progeny vary depending on the type of bird and vaccine availability. Commercially available vaccines include live attenuated, viral vectored recombinant, and inactivated vaccines.

Introduction

Infectious bursal disease (IBD) is an acute, highly contagious viral infection of young chickens that have lymphoid tissue, especially the cloacal bursa (bursa of Fabricius) as its primary target. IBD was first described in 1962 (41) and was referred to as "avian nephrosis" because of the extreme kidney damage found in birds that succumbed to infection. Since the first outbreaks occurred in the area of Gumboro, Delaware, "Gumboro disease" is still a frequently used synonym for this disease. The economic importance of this disease is manifested in two ways. First, some virus strains may cause up to 60% mortality in chickens 3-weeks-of-age and older. The second, and more important, manifestation is a severe, prolonged immunosuppression of chickens when infected at an early age. Sequelae that have been associated with immunosuppression induced by the virus include gangrenous dermatitis, inclusion body hepatitis-anemia syndrome, *Escherichia coli* infections, and vaccination failures. Protection of young chicks from early infection is paramount. This is usually accomplished by a combination of transfer of maternal antibodies, and active immunization of the newly hatched chick. The virus does not affect humans and has no public health significance.

History

Early studies to identify the etiologic agent of IBD (avian nephrosis) (41) were clouded by the presence of infectious bronchitis virus (IBV) with nephropathogenic tendencies in the kidneys of field cases (280). Subsequent studies with IBD succeeded in isolating an agent in embryonating eggs (281). The isolate was referred to as "infectious bursal agent" and was identified as the true

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cause of IBD and the term infectious bursal disease was proposed as the name of the disease causing specific pathognomonic lesions of the cloacal bursa (99).

In 1972, IBD virus (IBDV) infections at an early age were recognized as immunosuppressive (8), a finding that greatly increased the interest in their control. The existence of a second serotype was reported in 1980 (174). Control of IBDV infections has been complicated by the recognition of "variant" strains of serotype 1 IBDV, which were found in the Delmarva poultry producing area, USA (223, 224). These strains were breaking through maternal immunity against "classic" strains, and they also differed from classic strains in their biological properties (219). These variants, or subtypes, were either already present in nature but unrecognized or were new mutants that have arisen, possibly due to immune pressure. In the late 1980s, very virulent strains of IBDV (vvIBDV) were isolated in the Netherlands (32). The vvIBDV strains quickly spread to Africa, Asia, and South America (53) and were reported in the United States in late 2008 (246). The vvIBDV has not been reported from Australia or New Zealand.

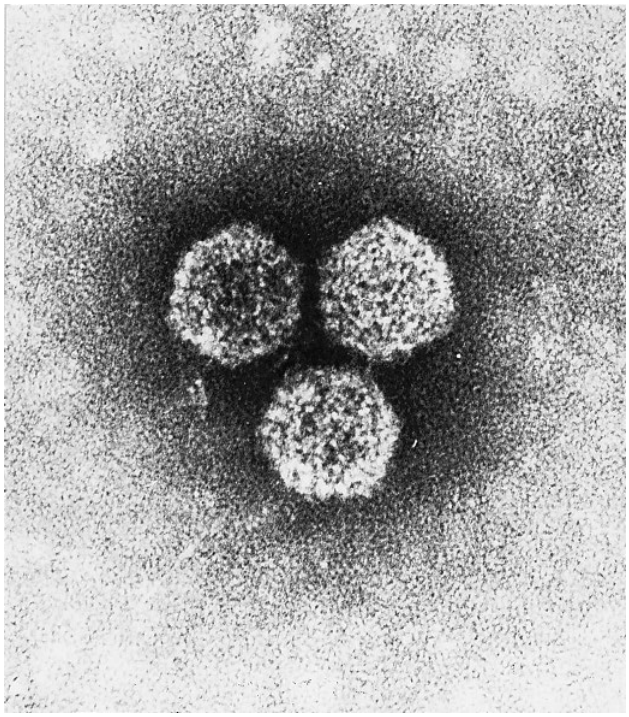


Figure 7.1 Electron micrograph of negatively stained infectious bursal disease (IBD) viral particles. ×200,000. (D.E. Reed.)

Etiology

Classification

Infectious bursal disease virus is a member of the Birnaviridae family (28, 56, 192), named for the bisegmented, double-stranded RNA nature of the genome of its members (164, 192, 245). The family has 4 genera including; *Aqua birnavirus*, whose type species is infectious pancreatic necrosis virus (IPNV) of fish, molluscs, and crustaceans; *Blosnavirus* whose type species is blotch snakehead virus (BSNV), *Avibirnavirus* whose type species is IBDV which infects birds; and *Entomobirnavirus* whose type species is Drosophila X virus which infects insects (52). Another poultry Birnavirus, chicken proventricular necrosis virus, has currently not been placed into a genus (84).

Morphology

The virus is a single-shelled, non-enveloped virion with icosahedral symmetry and a diameter varying from 55–65 nm (96, 199, 298) (Figure 7.1).

Buoyant density of complete particles in cesium chloride gradients has been reported to range from 1.31–1.34 g/mL. Lower density values were reported for incomplete virus particles (for a review, see (133)).

The capsid symmetry is askew, with a triangulation number of T = 13 and a typical laevo icosahedral geometry (213).

Chemical Composition

The dsRNA of the IBDV genome has two segments designated A and B (17, 56, 119, 192). The nucleotide sequence of the whole genome of both serotype 1 and serotype 2 IBDV isolates has been determined (185). Infectious bursal disease virus is one of the rare polyploid RNA viruses: each virus particle may incorporate up to four dsRNA segments. Incorporation of less than four genome segments creates incomplete particles with reduced buoyant densities that show up as multiple bands in cesium chloride gradients (162).

Five viral proteins designated VP1, VP2, VP3, VP4, and VP5 are recognized (17, 54, 56, 182, 199, 257) with approximate molecular weights of 97 kDa, 41 kDa, 32 kDa, 28 kDa, and 21 kDa, respectively. Additional proteins, such as VPX or pVP2, have been observed and have a precursor-product relationship ([54], see reference [133] for a detailed review of IBDV proteins). VP2, VP3, and VP1 are the structural proteins of IBDV. In serotype 1 viruses, they constitute 51%, 40%, and 3% of the virus proteins, respectively (56). Once believed to make as much as 6% of IBDV structural proteins (56), VP4 has now been recognized as a nonstructural protein that may be copurified with virus particles in cesium chloride gradients (81). In addition to the structural viral proteins, mature virus particles also harbor at their surface four small peptides that are formed when VP2 is progressively matured (46).

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VP1 is the viral RNA dependent RNA polymerase (RdRp) and exhibits an original organization as compared with other viral RdRps (78, 272). It is present in virus capsids both as a genome-linked and as a free protein (191). VP2 is the capsid protein. It forms 260 trimers, which are the basic units of the virus shell, forming a crystal structure (42, 75, 145). VP3, the other major structural protein, interacts with all other components of the virus particles, and plays a critical role in both virion morphogenesis, encapsidation, and replication (34, 74, 153, 249). VP3 is not exposed at the surface of the virion, but is tightly associated with dsRNA into filamentous structures (163) and supports viral polymerase activity (74, 169). Finally, interactions involving VP3 C terminal amino acids are critical for a correct assembly of VP2 into capsids with the proper symmetry (34, 170). VP4 is a viral protease (104, 195) that exhibits an unusual Ser-Lys catalytic dyad (21, 147). VP4 plays a major role in the maturation of capsid protein VP2, by progressively trimming several peptides at the VP2 carboxy-terminal extremity during virus assembly (147). However the complete processing of pVP2 into mature and correctly assembled VP2 also involves an autoproteolytic activity of pVP2 residue 431 (105). The crystal structure of a birnavirus protease has been determined in blotched snakehead virus (67). VP5 has a regulatory function in virus release and dissemination, as well as an anti-apoptotic function at the early stages of infection (151, 154, 184, 275). Two of the peptides that arise from the maturation process of pVP2 are crucial determinants that control the geometry of the virion assembly process (33, 46). One of these peptides, pep46, also has a destabilizing effect on cellular membranes (33, 46).

The small segment of the IBDV genome (B, approximately 2.9 kbp) codes for VP1, whereas the large segment (A, approximately 3.3 kbp) encodes the VP5 protein and, in another and partially overlapping reading frame, a 110 kDa polyprotein that will yield VP2, VP4, and VP3 upon co-translational cleavage by VP4 (11, 104, 180). In both genome segments, the coding regions are flanked by short 5' and 3' untranslated regions (79 to 111 nucleotides long) (185). The secondary structure of the 3' untranslated region appears to be critical for efficient replication (23).

VP2 is the protective immunogen of IBDV (18, 64). Two antigenic domains were identified in the VP2/pVP2 protein (12). One domain is conformation-independent, located at the carboxy-terminal end of VP2/VPX and elicits non-neutralizing monoclonal antibodies (12, 18, 65). Some of these are group-specific (18), others are strain specific (268). The second major VP2 antigenic domain is conformation-dependent and is encoded by the mid-third of the VP2 gene (12). Due to the higher frequency of amino acid changes in this region, it is known as "VP2 variable domain" (16). It groups serotype- or strain-specific epitopes that elicit neutralizing and passively protective antibodies (65, 241). The largest panels of neutralizing monoclonal antibodies (mAb) (62, 65, 241, 265, 290) detect as many as 6 VP2-located neutralizing epitopes, which colocate in at least three overlapping antigenic sites. Further analysis of IBDV strains with different mAb reactivities identified hot spots for antigenically significant amino acid changes (60, 91, 140, 228, 263, 265). These are located within stretches of hydrophilic amino acids in the VP2 sequence: aa 212–224 and 314–324 are known as "VP2 major hydrophilic peaks" or "hydrophilic peaks A and B," respectively (228), whereas aa 248–252 and 279–290 are designated as "VP2 minor hydrophilic peaks 1 and 2," respectively (265). Structural studies demonstrated that these "peaks" correspond to the loops located in the most exposed part of the projection domain of VP2 and to amino acids displayed at the most external surface of the virus particle (42, 148).

VP3 elicits non-neutralizing and nonprotective antibodies (18, 65). Up to four VP3-located antigenic domains have been identified (126, 165, 290, 297). All contain epitopes common to both serotypes (group-specific epitopes), whereas two of these domains also contain serotype-specific epitopes (165).

Although some progress has been made, the molecular basis for pathogenicity of the virus has not been determined yet. The development of reverse genetics systems (186, 214) made it possible to manipulate the virus genome. Using this approach, segment A was demonstrated to form the genetic basis for bursal tropism in serotype 1 IBDV (296). Swapping the VP2 gene between vvIBDV and attenuated IBDV strains and characterizing *in vivo* the resulting recombinant viruses showed that VP2 is not the sole determinant of virulence (24). However, introduction into VP2 of a pathogenic virus the amino acid changes required for adaptation to cell culture resulted in attenuation (27, 149, 181, 269). Epidemiological and experimental evidence suggests that both genome segments might be required for the expression of the vvIBDV or pathogenic phenotypes (22, 101, 106, 143, 144, 152). It has been suggested that reassortment phenomena might be involved in the emergence of vvIBDV (29, 101). Reassortant IBDV strains with segment A of a vvIBDV and segment B derived from another serotype 1 (143) or serotype 2 (124) virus have been described. They have a reduced pathogenicity as compared with typical vvIBDV. Some phylogenetic studies have suggested that intrasegment homologous recombination events between different IBDV strains may occur (90, 100).

Virus Replication

Virus replication of IBDV has been previously reviewed (133, 194). In brief, the virus was shown to attach to chicken embryo kidney cells maximally 75 minutes after

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inoculation (157). The multiplication cycle in chicken embryo cells is 10–36 hours, and the latent period is 4–6 hours (17, 119, 157, 199). In Vero and BGM-70 cells, a longer (48-hour) multiplication cycle was described (115, 134, 158).

Serotype 1 and serotype 2 IBDV may use several receptors, either common to both serotypes or serotype specific, on different cell types (200). However, IBDV strains adapted or not to Vero cells were shown to bind to the same receptor. This suggests that some barriers to the propagation of vvIBDV in cell culture may exist beyond the attachment step (293). The receptor for virulent IBDV is an N-glycosylated membrane protein expressed in the IgM-bearing immature B lymphocytes (203). The λ light chain of surface IgM (161), chicken heat-shock protein 90 α (150, 294) and the α4β1 integrin heterodimer (51) have been identified as parts of the IBDV receptor in the avian DT40, avian DF1, and murine 3T3 cell lines, respectively. The α4β1 integrin interacts with an integrin-binding motif conserved in the most exposed domain of VP2 in all IBDV strains (51).

The internalization of the bound IBDV particles occurs by a clathrin-independent endocytosis mechanism, dependent on several cellular factors (292). Ca^{2+} ions have a stabilizing effect on the conformation of the virus particle (75). Their low concentration in the endosomal environment triggers the loss of the compact structure of the virus particle (145) and promotes the release of pep46 (33, 46, 72). Pep46 further destabilizes the endosomal membrane by creating pores into it (71), which is a process critical for IBDV infectivity (72).

The mechanism of viral RNA synthesis has not been clearly determined. A dsRNA-dependent RNA polymerase, VP1, was described (244, 272), and genome-linked proteins have been demonstrated, indicating that the virus replicates its nucleic acid by a strand displacement mechanism (244). A baculovirus expressed IBDV RdRp specifically used the 3' untranslated region of an IBDV positive strand template to initiate the synthesis of a complementary strand by a "copy-back" mechanism (272). RNA polymerase activity could be demonstrated without the pretreatment of the virus, indicating that transcription and replication occurred following cell penetration without the uncoating of the virus (244). Interaction between VP1 and a VP1-binding motif of VP3 induces a conformational change that activates VP1 (74). It has been hypothesized that non-polyadenylated mRNAs are extruded through pores possibly located at the 5-fold symmetrical axis in IBDV capsid (42).

The synthesis of host proteins is not shut off in chicken embryo fibroblasts (CEFs) infected with IBDV (17). In chicken bursal lymphoid cells grown *in vitro*, the viral polypeptides were detected in the cells and their culture media at 90 minutes and 6 hours postinoculation (PI), respectively (188). An interaction exists between VP1 and eukaryotic translation initiation factor 4AII; this association suggests that VP1 could also be involved in the translation of IBDV RNA (250). The lack of accumulation of the polyprotein in infected cells suggests its co-translational cleavage (188). The model currently proposed for the assembly of IBDV particles involves most virus proteins: VP1 most probably first interacts with virus RNA, as described in IPNV, another birnavirus (55). VP3 then interacts with itself, pVP2, VP1, and the viral genome, thus playing a critical chaperone role in virion morphogenesis and encapsidation (34, 153, 249). The final maturation of pVP2 by serial cleavage of its last carboxy-terminal peptides occurs within the virus capsid (33, 35).

Virus particles accumulate within the cytoplasm of infected cells (167). This could be favored by the fact that VP5 prevents apoptosis at the early stages of infection, by interfering with the caspases and NF-KB pathways (151). However, VP5 (291) and/or VP2 (68) have also been reported to induce apoptosis in infected cells. The VP5-mediated formation of pores in the membrane of the infected cells (154) would contribute to virus release.

Susceptibility to Physical and Chemical Agents

The virus is highly stable and resists treatment with ether and chloroform, was inactivated at pH 12 but not by pH 2 (19). The virus was unaffected by exposure for 1 hour at 30 °C to 0.5% phenol and 0.125% thimerosal. There was a marked reduction in virus infectivity when exposed to 0.5% formalin for 6 hours. The virus was also treated with various concentrations of three disinfectants (an iodine complex, a phenolic derivative, and a quaternary ammonium compound) for a period of 2 minutes at 23 °C. Only the iodine complex had any deleterious effects. A 0.5% chloramine killed the virus after 10 minutes (141). High concentrations of a compound that releases methyl isothiocyanate as a fumigant were able to inactivate IBDV in contaminated litter after one hour contact (76) Invert soaps with 0.05% sodium hydroxide either inactivated or strongly inhibited the virus (232). IBDV in bursal homogenates heated at 70°, 75°, and 80 °C exhibited a rapid initial drop in virus titer followed with a gradual decline. A drop of 1 log 10 at 70°, 75°, and 80 °C took 18.8, 11.4, and 3.0 minutes, respectively (7). Chicken parts or chicken products inoculated with the virus and then cooked to an internal temperature of 71° and 74 °C, respectively, allowed recovery of viable virus (168). IBDV was fully inactivated in infected tissues after 14 days composting, with 8.8 days above 55 °C (83). Gamma-irradiation at 3.0 kiloGrays, the maximum level approved by FDA, did not significantly reduce the titer of the vaccine or pathogenic IBDV strains (124).

The hardy nature of this virus is one reason for its persistent survival in poultry houses even when thorough cleaning and disinfection procedures are followed.

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Strain Classification

A variety of phenotypic and molecular genetic procedures have been developed to classify isolates of IBDV. Classification systems based on phenotypic traits, such as serotyping, have been used successfully since the discovery of the virus. Serotyping of IBDV isolates using polyclonal antibodies in cross virus-neutralization (VN) tests has correlated well with protection studies. Molecular genetic procedures are useful for diagnostic and epidemiologic studies, but are not satisfactory for virus classification because of the endless possibilities of different genotypes.

Antigenicity

Two serotypes of IBDV, designated as 1 and 2, are recognized (118, 174, 176). IBDV serotypes 1 and 2 share only 30% antigenic relatedness (174). Antigenic relatedness of only 33% between 2 strains of serotype 2 was reported (176), indicating an antigenic diversity similar to that of serotype 1 viruses.

The two serotypes are differentiated by virus-neutralization (VN) tests, but not by fluorescent antibody tests or enzyme-linked immunosorbent assay (ELISA). Immunization against serotype 2 does not protect against serotype 1. The reverse situation cannot be tested because no virulent serotype 2 viruses are available for challenge (109). The first isolates of serotype 2 (118) originated from turkeys, but later studies showed that serotype 2 viruses could also be isolated from chickens (110), and antibodies to serotype 2 IBDVs are common in both chickens and turkeys (117, 224).

Variant viruses of serotype 1 were described (223, 224). Vaccine strains available at the time did not elicit full protection against the variants, which are antigenically different from the original serotype 1 isolates (currently, designated classic viruses). Six subtypes were distinguished by cross-neutralization among 13 serotype 1 vaccine or field strains. One of the subtypes included all of the variant isolates (116). Using mAbs, it was shown that a major antigenic shift in serotype 1 viruses may have occurred in the field (241, 243). Sequencing studies identified several amino acid changes in "VP2 hydrophilic peaks" that correlated with the antigenic changes observed in the variant viruses (91, 140, 263). Australian (227) and European (59, 148) strains evocative of variant viruses were also identified but they differ genetically and antigenically from North-American variant viruses. The impact of antigenic variation on cross-protection has been less documented for these Australian and European viruses.

The vvIBDV strains that were first described in Europe (32) were shown to be antigenically similar to the classic serotype 1 viruses (3, 26, 61, 261, 264).

In summary, there are currently three well-documented antigenic types. These are classic (often called standard) and variant serotype 1 and serotype 2 viruses. Subtypes of the three antigenic types have also been described.

Immunogenicity or Protective Types

Cross-challenge studies with IBDV has yielded results similar to those obtained by cross VN studies used for the antigenic classification (108). There are currently two serotype 1 protective types, classic and variant groups. Serotype 2 viruses do not protect against challenge with serotype 1 viruses. Classic serotype 1 viruses protect against themselves and provide partial protection against serotype 1 variants. Variant strains of serotype 1 protect against themselves and the classic viruses.

Molecular Genetic Types and Gene Sequencing

Molecular genetic techniques are used to group different isolates of IBDV (114). These techniques have become popular because of their sensitivity, the time they save, the ability to use them on crude samples or inactivated samples, and they do not require replication of the virus. The most commonly used procedure is the reverse transcriptase-polymerase chain reaction/restriction enzyme fragment length polymorphisms (RT-PCR/RFLP), mostly applied to the characterization of the genomic region encoding "VP2 variable domain". A RT-PCR/RFLP approach has also been implemented for the molecular grouping of IBDV strains according to their segment B restriction profile (256). Currently described molecular groups do not correspond to antigenic or protective groups, and one has to be careful in interpreting the significance of this classification.

A more thorough molecular characterization can be achieved by sequencing the virus genome and studying the phylogenetic relationships of the studied isolate with reference viruses. Care should also be taken that for an optimum assessment of genetic relatedness both genome segments should be characterized (123, 143). As the genetic basis for virulence has not been defined yet, attempts to infer the phenotype from genetic data should still be considered as tentative.

Laboratory Host Systems

Chicken Embryos

Initially, most workers had difficulty in isolating virus or, if successful, in serially transferring virus using chicken embryos (141). Continued studies (99) uncovered three factors that could explain these difficulties: (1) embryonating eggs that originated from flocks recovered from the disease were highly resistant to growth of the virus, (2) in early virus passage, the allantoamnionic fluid (AAF) had very low virus content and the chorioallantoic membrane (CAM) and embryo each had a much higher and nearly equal virus content, (3) comparison of the allantoic sac, yolk sac, and CAM as routes of inoculation showed the allantoic sac to be the

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least desirable, yielding embryo-infective dose—50% (EID_{50}) virus titers of 1.5–2.0 log_{10}—lower than those obtained after inoculation by the CAM route. The yolk sac route gave titers that were intermediate.

An embryo adapted virus was shown to reach a peak virus concentration in the embryo 72 hours PI (99).

Injection of the virus into 10-day-old embryonating eggs results in embryo mortality from days 3–5 PI. Gross lesions observed in the embryo were edematous distention of the abdominal region; cutaneous congestion and petechial hemorrhages, particularly along feather tracts; occasional hemorrhages on toe joints and in the cerebral region; mottled-appearing necrosis and ecchymotic hemorrhages in the liver (latter stages); pale "parboiled" appearance of the heart; congestion and some mottled necrosis of kidneys; extreme congestion of lungs; and pale spleen, occasionally with small necrotic foci. The CAM had no plaques, but small hemorrhagic areas were observed at times. Lesions induced in embryos by IBDV variants differ from those induced by classic isolates. Splenomegaly and liver necrosis are characteristic of the lesions induced by the variants, but there is little mortality (219). The vvIBDV strains induce similar lesions in embryos as the classic strains, with the CAM being the most sensitive route for infecting chicken embryos with vvIBDV, but the yolk sac route was a good alternative (251). It is the authors' experience that serotype 2 viruses usually do not induce typical lesions when inoculated via the CAM route into embryonating chicken eggs, with the only signs being feeble, pale, and yellowish embryos.

Cell Culture

Many strains of IBDV have been adapted to cell cultures of chicken embryo origin, and cytopathic effects have been observed, allowing for plaque assay or microtiter techniques. Chicken embryo fibroblasts proved suitable to propagate egg-adapted strains of IBDV (218). Chicken embryo fibroblasts proved more sensitive than either embryonating eggs or suckling mice. Cell cultures from the chicken embryo cloacal bursa were also used, followed by passage in embryo kidney cells and subsequent propagation in CEF, to develop an attenuated live virus vaccine from a wild-type virus (157). In addition to cells of chicken origin, the virus has been grown in turkey and duck embryo cells (175).

Several continuous cell lines of avian origin have also been used to propagate IBDV strains. These include the avian fibroblastic lines DF1 (274) and QT35 (43), a chicken macrophage cell line (131), the avian leukosis virus-induced DT40 lymphoma cell line (253), and the B-lymphoblastoid cell line LSCC-BK3 (93). The two latter lines were reported to successfully propagate pathogenic IBDV strains that had not been previously adapted to CEF (253, 260). The DT40 cell line may become persistently infected with IBDV (50).

Mammalian cell lines have also been used to propagate IBDV. These were derived from rabbit kidneys (RK-13) (218), murine embryonic cells (BALB/c 3T3) (51), monkey kidneys (Vero, MA104) (158), and baby grivet monkey kidney cells (BGM-70) (115). In one study, the Vero, MA104, and BGM-70 cell lines could all propagate several IBDV strains of both serotypes including serotype 1 variants but cytopathic effects were most pronounced in the BGM-70 cells (115). The growth curve of one strain tested in BGM-70 cells was similar to that in CEFs, and VN titers in BGM-70 cultures compared well with those in CEFs.

Normal chicken lymphocytes were the first host cells that propagated virulent IBDV (and in a lymphoblastoid B cell line derived from an avian leukosis virus-induced tumor) and showed that IgM-bearing B lymphocytes were the probable target cells of IBDV (94, 196). Enriched Ig-bearing cells showed IBDV replicated preferentially in a population of proliferating cells and that susceptibility did not correlate with expression of immunoglobulins on their surface (187). In a recent study, chicken mesenchymal stem cells isolated from the bone marrow of normal chickens were shown to be susceptible to IBDV infection (132).

Isolation of IBDV from field cases may be difficult, with clinical experience showing isolation and serial propagation of the virus in cell cultures of chicken embryo origin with difficulty (174). In one study, turkey strains (5 of 5) were readily adapted to CEF cells after 3 to 10 blind passages. Only 2 of 9 chicken strains could be adapted to CEF cells; the other 7 strains could be grown only in chicken embryo cloacal bursa cells, even after 20 bursal cell passages (146).

BGM-70 cells were used successfully for isolation of IBDV from the cloacal bursas of naturally infected chickens (225). Usually, a cytopathic effect was detected after two or three blind passages. Passage of the virus six times in BGM-70 cells or CEF resulted in loss of pathogenicity, but similar passages in chicken embryos did not affect the pathogenicity of the virus (88). Later, it was reported (4) that adaptation of the virus to BGM-70 cells resulted in a significant reduction in the ability of the virus to replicate in the cloacal bursa.

One aspect that should be considered concerning *in vitro* replication of the virus is the possibility of development of defective particles. Serial passages of undiluted virus in chicken embryo cells resulted in fluctuations in infectivity and the development of a stable small-plaque-forming virus that interfered with the replication of the classic virus and favored the generation of defective particles (189). The defective particles had lost the large segment of dsRNA.

Compared to classic and variant strains of serotype 1, adaptation of the vvIBDV viruses to cell culture has been difficult (3). LSCC-BK3 cells were shown to be superior to BGM-70 cells and CEF in an infectivity assay, however

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IBDV had to be detected by antigen capture in their supernatant (260).

Pathogenicity

Chickens are the only animals known to develop clinical disease with distinct lesions when exposed to IBDV. Care should be taken, when comparing experiments aimed at assessing the pathogenicity of different IBDV isolates, that these experiments do include relevant strains with well characterized pathogenicity (controls). Major variables to be standardized in comparative trials are the breed or genetic lineage, age and immune status of the challenged chickens, the route of inoculation of the challenge virus, the possible presence of contaminating viruses in the inoculum, and, most importantly, the virus dose (2). Field viruses exhibit different degrees of pathogenicity in chickens. In the authors' experience based on the experimental reproduction of acute IBD in specific pathogen free (SPF) white leghorn chickens, "variant" IBDV induces little if any clinical signs and mortality, but marked bursal lesions. Classical IBDV induces approximately 10–50% mortality with typical signs and lesions, and vvIBDV induces approximately 50–100% mortality with typical signs and lesions (2). Comparative studies show that it may prove difficult to define cut off values, and that putative vvIBDV, when identified by the genetic sequence of their segment A only, may greatly vary in pathogenicity (123, 138, 266). Attenuated live vaccine viruses also have varying pathogenic potential in chickens.

Neither clinical signs nor gross or microscopic lesions were observed in chickens inoculated with serotype 2 isolates (109), irrespective of the species (chicken or turkey) from which the virus had been isolated. In turkey poults inoculated at 1- to 8-days-of-age, an isolate of serotype 2 from turkeys failed to cause disease or lesions in the cloacal bursa, thymus, or spleen (121); however, the virus was infectious, and the poults responded serologically to the infection. Experimental infection in 1-day-old poults with isolates representing serotypes 1 and 2 that originated from turkeys showed virus-infected cells by immunofluorescence in several tissues of infected birds, but no clinical disease resulted. Only slight gross changes were observed, and no histologic differences were seen between infected and non-infected birds (201). In general, the distribution of fluorescing (infected) cells from these tissues seemed to indicate that the majority were not lymphocytes. The number of plasma cells in the Harderian gland was reduced at 28-days-of-age. As indicated earlier, the effect of the host system on pathogenicity of the virus may be profound (88, 258). In recent studies, the OH strain of serotype 2 virus that was back passaged five times in chicken embryos was shown to replicate in the embryos. Nonetheless, that virus was not pathogenic for 2-week-old SPF chickens or turkeys (5).

Pathobiology and Epidemiology

Incidence and Distribution

Infections with serotype 1 IBDV are of worldwide distribution, occurring in all major poultry producing areas. The incidence of infection in these areas is high; essentially, all flocks are exposed to the virus during the early stages of life, either by natural exposure or vaccination. Because of vaccination programs carried out by most producers, all chickens eventually become seropositive to IBDV. Clinical cases are rare in the United States because infections are either modified by antibodies or are due to variant strains that do not cause clinical disease. These variant strains seem to be the predominating viruses that exist in the United States, but isolated outbreaks due to vvIBDV occurred in late 2008 in California (125). Classical viruses and a local type of variants have been reported in Australia (227). In Europe, Africa, Asia, and South America, the vvIBDV strains seem to predominate.

In the United States, it was shown that antibodies to serotype 2 IBDV were widespread in chicken (117, 224) and turkey flocks (13, 37, 118), indicating the common prevalence of the infection.

Natural and Experimental Hosts

Chickens and turkeys are the natural hosts of the virus. A serotype 1 virus was isolated from two 8-week-old ostrich chicks that had lymphocyte depletion in the cloacal bursa, spleen, and/or thymus (283). Serotype 1 isolates were obtained from healthy (174) or dead (128) waterfowl. The latter viruses were genetically related to vvIBDV and pathogenic for chickens (128). Other vvIBDV were isolated from a dead magpie (128) in Korea. Viruses genetically related to vvIBDV were detected by RT-PCR from apparently healthy pigeons and Guinea fowl in Eastern Africa (130). A serotype 2 IBDV was isolated from captive penguins that died without specific clinical signs (79). Challenge studies of pheasants, partridges, quails, and Guinea fowl with vvIBDV did not show any clinical signs or lesions in these species, however quail replicated the virus in their bursa, shed it for five days in their feces and developed neutralizing antibodies (267). This contrasts with a previous study that could not infect Coturnix quail with a chicken-origin virus (276) and IBDV-inoculated Guinea fowl did not develop lesions or antibody (204).

Several species of free-living and captive birds of prey were examined for antibodies to IBDV, and positive results were obtained from accipitrid birds (262). Antibodies were also detected in rooks, wild pheasants, and several rare avian species (31); in Antarctic penguins (73); in ducks, gulls, and shearwaters (278); and crows, gulls, and falcons (202).

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For many years, the chicken was considered the only species in which natural infections occurred. All breeds were affected, and many investigators observed that white leghorns exhibited the most severe lesions and clinical signs and had the highest mortality rate, but other studies showed no differences between heavy and light breeds (178).

The period of greatest susceptibility to clinical disease is 2- to 6-weeks-of-age. Susceptible chickens younger than two weeks do not exhibit clinical signs but have subclinical infections that are economically important as a result of severe immunosuppression.

The age-susceptibility of chickens to IBDV has been linked in several studies with the availability of a functional cloacal bursa, the target organ of the virus. Three-day-old chicks treated with cyclophosphamide were refractory to clinical signs and lesions when challenged at 4-weeks-of-age (63). Similar results were found with birds surgically bursectomized at 4-weeks-of-age. When they were challenged immediately or one week later, there was no clinical disease, whereas 100% of the control non-bursectomized chickens died (137). Bursectomized chickens challenged with virulent virus produced 1,000 times less virus than control birds, produced VN antibodies by day five, and had only discrete and transient necrosis of lymphatic tissues.

Histologic lesions in the cloacal bursa resemble an Arthus reaction (necrosis, hemorrhage, and large numbers of polymorphonuclear leukocytes). This reaction is a type of localized immunologic injury caused by antigen–antibody–complement complexes that induce chemotactic factors, which cause hemorrhage and leukocyte infiltration. At 72 hours PI, 2-week-old chickens have little complement compared with 8-week-old chickens. It was postulated that the reason why 2-week-old chickens did not develop Arthus-type lesions was a lack of sufficient complement (112, 236).

A role for mast cells in the pathogenesis of acute IBDV was reported (273). Mast cell populations, tryptase expression, and histamine release were all increased in the bursa of SPF chickens challenged with vvIBDV, whilst prevention of mast cell degranulation with ketotifen resulted in a dramatic reduction of both signs, gross and histological bursal lesions, and mortality.

It was suggested that increased clotting times in IBDV-infected chickens could contribute to the hemorrhagic lesions observed in an age-dependent way.

Naturally occurring infections of turkeys and ducks by serotype 2 and serotype 1 viruses, respectively, have been recorded (129, 174, 175, 206). But in one study, turkey sera collected before 1978 were negative suggesting that IBDV infections of turkeys were a relatively new occurrence (175).

Experimental IBDV infections of turkeys were subclinical in 3- to 6-week-old poults, producing microscopic lesions in the cloacal bursa. Virus-infected bursal cells were detected by immunofluorescence. Neutralizing antibodies were detected 12 days PI, and the virus could be reisolated after five serial passages in chicken embryos (77). Infection in 6- to 8-week-old IBDV-infected poults was subclinical, with no damage to the bursa being evident but an increase in VN antibodies was observed (276).

Transmission, Carriers, Vectors

Infectious bursal disease is highly contagious, and the virus is persistent in the environment of a poultry house. Houses from which infected birds were removed were still infective for other birds at 54 and 122 days later. They also demonstrated that water, feed, and droppings taken from infected pens were infectious after 52 days (20).

No evidence suggests that IBDV is transmitted through the egg or that a true carrier state exists in recovered birds. Resistance of the virus to heat and disinfectants is sufficient to account for virus survival in the environment between outbreaks. The lesser mealworm (*Alphitobius diaperinus*), taken from a house eight weeks after an outbreak, was infectious for susceptible chickens when fed as a ground suspension (239). In another study (173), the virus was isolated from several tissues of surface-sterilized lesser mealworm adults and larvae that were fed the virus earlier.

Infectious bursal disease virus was isolated from mosquitoes (*Aedes vexans*) that were trapped in an area where chickens were being raised in southern Ontario (102). The isolate was nonpathogenic for chickens. The IBDV antibodies were detected by the agar-gel precipitin (AGP) test in 6 of 23 tissue samples from rats found dead on four poultry farms that had histories of IBDV infection (205). There has been no further evidence to support that either mosquitoes or rats act as vectors or reservoirs of the virus.

A dog fed chickens that had died of acute IBD shed viable vvIBDV in its feces for up to two days after ingestion (207).

A study in 46 processing plants in the Eastern United States showed that 25% of the pools of cloacal bursae (collected in 42% of the studied plants) allowed molecular detection of nonvaccine IBDV strains and infectious IBDV was reisolated (122). Actual data on the frequency of contamination of commercial poultry products by IBDV are lacking, but increasing concern has been expressed that serotype 1 IBDV should be considered as potentially transmitted through the trade of poultry meat (40).

Incubation Period and Clinical Signs

The incubation period is short, and clinical signs of the disease are seen within 2–3 days after exposure.

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Morbidity and Mortality

In fully susceptible flocks, the disease appears suddenly, and there is a high morbidity rate, usually approaching 100%. Mortality may be nil but can be as high as 20–30%, exceptionally higher with vvIBDV, usually beginning on day 3 PI and peaking and receding in a period of 5–7 days. In the late 1980s, vvIBDV strains became a problem in Europe. Several of these isolates caused mortality rates of 90% (32) to 100% (264) in 4-week-old susceptible leghorn chickens. A 1970 isolate (52/70) (30) was compared with two vvIBDV isolates in a study; it caused 50% mortality compared with 90% for the vvIBDV strains (32). In another study, the vvIBDV isolates caused lower mortality, however percent mortality was again at least twice as much as caused by the 52/70 isolate (61).

Initial outbreaks on a farm are usually the most acute. Recurrent outbreaks in succeeding broods are less severe and frequently go undetected. Many infections are silent, owing to age of birds (less than three weeks old), infection with avirulent field strains, or infection in the presence of maternal antibody.

Pathology

Gross Lesions

Birds that succumb to the infection are dehydrated, with darkened discoloration of pectoral muscles. Frequently, hemorrhages are present in the thigh and pectoral muscles (Figure 7.2). There is increased mucus in the intestine, and renal changes (41) may be prominent in birds that die or are in advanced stages of the disease. Such lesions are most probably a consequence of severe dehydration. In birds killed and examined during the course of infection, the kidneys appear normal.

The cloacal bursa is the primary target organ of the virus. In a detailed study of bursal weights for 12 days PI, the sequence of changes should be understood when examining birds for diagnosis. On day 3 PI, the bursa begins to increase in size and weight because of edema and hyperemia (Figure 7.3). By day 4 PI, it usually is double its normal weight, and the size then begins to recede. By day 5 PI, the bursa returns to normal weight, but it continues to atrophy, and from day 8 PI forward, it is approximately one-third its original weight, or even less (36).

By day 2 or 3 PI, the cloacal bursa has a gelatinous, yellowish transudate covering the serosal surface. Longitudinal striations on the surface become prominent, and the normal white color turns to cream color. The transudate disappears as the bursa returns to its normal size.

Isolates of variant IBDV were reported generally not to induce an inflammatory response (223, 230), although one variant strain (IN) did so (87).

The infected bursa often shows necrotic foci and at times petechial or ecchymotic hemorrhages on the mucosal surface. Occasionally, extensive hemorrhage throughout the entire bursa has been observed (Figure 7.3); in these cases, birds may void blood in their droppings.

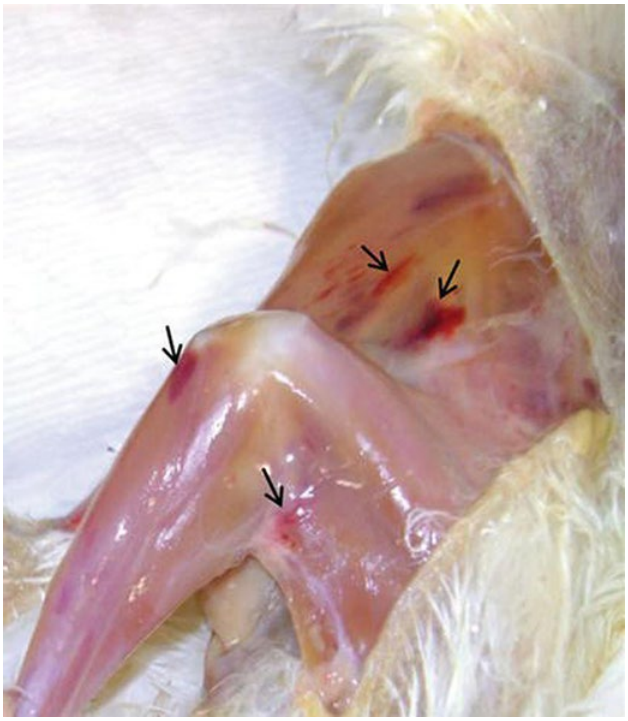


Figure 7.2 Hemorrhages (arrows) in thigh and breast muscles, at three days post-inoculation in two-week-old SPF chickens inoculated with 10^6 EID_{50} of bursa-derived classic IBDV (STC). (A. Rauf and Y.M. Saif.).

The spleen may be slightly enlarged and often has small gray foci uniformly dispersed on the surface (217). Occasionally, hemorrhages are observed in the mucosa at the juncture of the proventriculus and ventriculus (gizzard) and may cause melena (digestive content stained black with digested blood).

Compared with a moderately pathogenic strain of the virus, the vvIBDV strains caused a greater decrease in thymic weight index and more severe lesions in the cecal tonsils, thymus, spleen, and bone marrow, but bursal lesions were similar. It was also shown that pathogenicity correlated with lesion production in non-bursal lymphoid organs, suggesting that pathogenicity may be associated with antigen distribution in non-bursal lymphoid organs (252). In experimental studies, virus dose affects the clinical signs and lesions (1).

Microscopic Lesions

Microscopic lesions of IBD occur primarily in the lymphoid tissues (i.e., cloacal bursa, spleen, thymus, Harderian gland, and cecal tonsil). Changes were most severe in the cloacal bursa. As early as one day PI, there was degeneration and necrosis of lymphocytes in the medullary area of bursal follicles. Lymphocytes were soon replaced by heterophils, pyknotic debris, and hyperplastic

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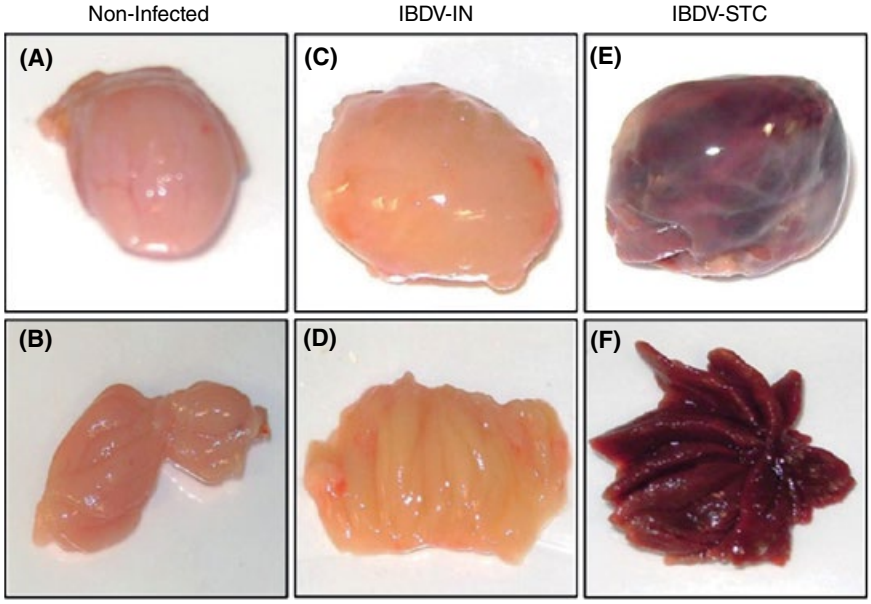


Figure 7.3 Cloacal bursa of two-week-old SPF chickens at three days post-inoculation (A–F). A–B. Non-infected control chicken. C–D. Chicken inoculated with 10^6 EID_{50} of bursa-derived variant (IN) IBDV. E-F. Chicken inoculated with 10^6EID_{50} of bursa-derived classic (STC) IBDV. Note characteristic pale yellow bursal color with yellowish transudate. (A. Rauf and Y.M. Saif.)

reticuloendothelial cells. Hemorrhages often appeared but were not a consistent lesion. All lymphoid follicles were affected by three or four days P.I. The increase in bursal weight seen at this time was caused by severe edema, hyperemia, and marked accumulation of heterophils. As the inflammatory reaction declined, cystic cavities developed in medullary areas of follicles; necrosis and phagocytosis of heterophils and plasma cells occurred; and there was a fibroplasia in interfollicular connective tissue. Proliferation of the bursal epithelial layer produced a glandular structure of columnar epithelial cells containing globules of mucin. During the suppurative stage, scattered foci of lymphocytes appeared but did not form healthy follicles during the observation period of 18 days PI (36, 92, 167, 211). Some of the histologic changes observed in the cloacal bursa are shown in Figure 7.4.

A sequential study (282) of the recovery of the cloacal bursa after neonatal infection by classical IBDV demonstrated that the initial depletion of B lymphocytes was maximal during the first week, and combined with a transient massive influx of T cells and macrophages until three days PI. From 1–8 weeks PI, two distinct types of bursal follicles were then observed: large reconstituted functional follicles, most likely reconstituted from endogenous bursal stem cells that survived IBDV infection, and small, poorly developed follicles lacking a discernible cortex and medulla. The structure of these small follicles suggested they were unable to produce functional peripheral B cells, a hypothesis confirmed by the lack of active antibody responses in birds lacking large reconstituted follicles. After their initial influx in the bursa, T cells declined in number along with viral clearance and persisted mostly in the small follicles during the recovery phase. Inflammatory foci persisted during the recovery phase, possibly centered on antigen presenting cells. Essentially similar but more severe and persistent histological lesions have been described following challenge with a vvIBDV, which also induced thymic lesions (279).

The spleen had hyperplasia of reticuloendothelial cells around the adenoid sheath arteries in early stages of infection. By day three, there was lymphoid necrosis in the germinal follicles and the periarteriolar lymphoid sheath. The spleen recovered from the infection rather rapidly, with no sustained damage to the germinal follicles.

The thymus and cecal tonsils exhibited some cellular reaction in the lymphoid tissues in the early stages of infection, but, as in the spleen, the damage was less extensive than in the bursa, and recovery was more rapid.

The Harderian gland may also be severely affected following infection of 1-day-old chicks with IBDV. Normally, the gland is infiltrated and populated with plasma cells as the chicken ages (57, 248). Infection with IBDV prevented this infiltration. From 1–week-of-age, the glands of infected chickens had populations of plasma cells 5–10-fold fewer than those of uninfected controls (57). In contrast, broilers inoculated with IBDV at 3-weeks-of-age had plasma cell necrosis in the Harderian gland 5–14 days PI, and the plasma cells were reduced by 51% at 7 days PI (58). Reduction in plasma cells, however, was transient, and the numbers were normal after 14 days.

Ultrastructural Changes

Sequential changes in the surface epithelium of the cloacal bursa of IBDV-infected chicks by scanning electron microscopy observed a reduction in number and size of microvilli on epithelial cells at 48 hours PI. There was gradual loss of the button follicles normally seen at the surface, and by 72 hours, most had involuted. By 96 hours, there were numerous erosions of the

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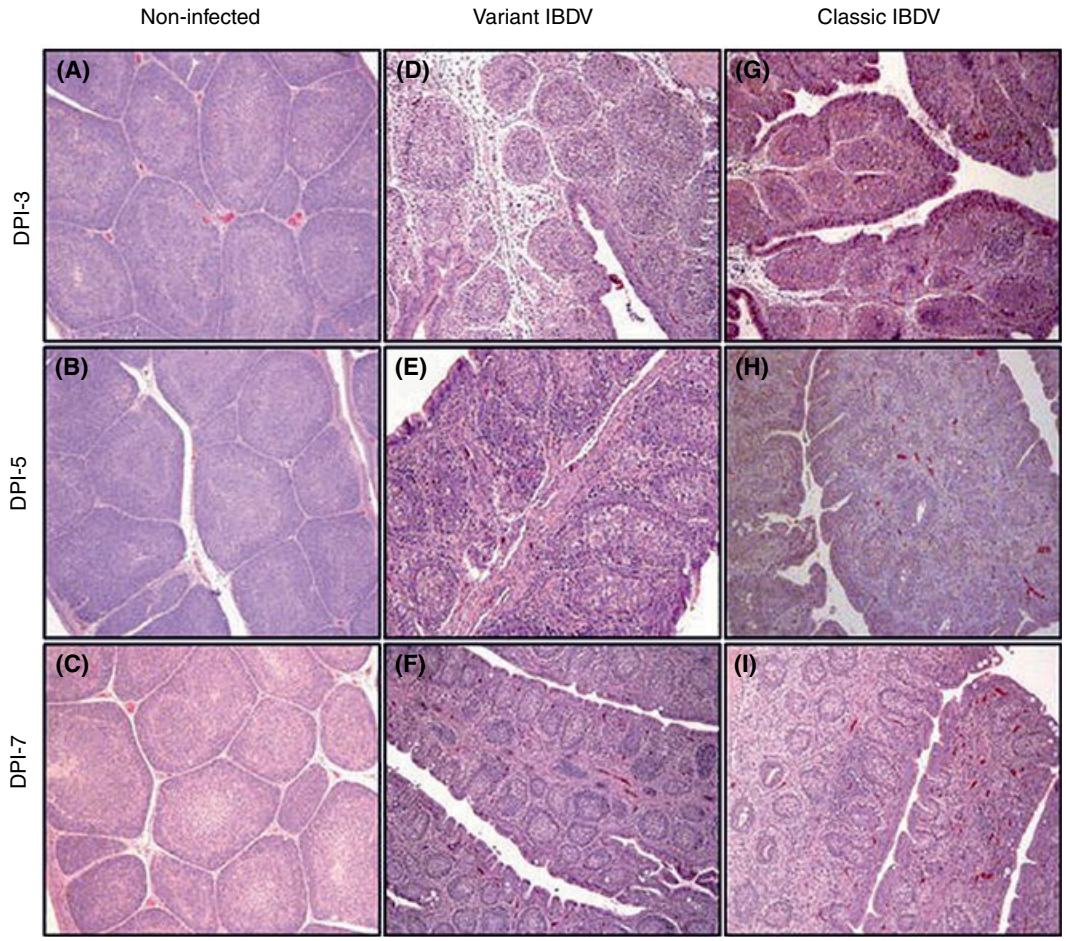


Figure 7.4 Histopathological lesions in the cloacal bursa at 3, 5, and 7 days post-inoculation (DPI). Normal lymphoid follicles in non-infected control chicken (A, B, and C; ×60). Mild to moderate follicular lymphocyte depletion in chickens inoculated with 10^4 EID_{50} of bursa-derived variant (IN) IBDV (D, E, and F; ×60). Severe follicular lymphocyte depletion in chickens inoculated with 10^4 EID_{50} of bursa-derived classic (STC) IBDV (G, H, and I; ×60). (A. Rauf and Y.M. Saif.) (*For color detail, please see the color section*.)

epithelial surface. The surface was intact by day 9 PI, but follicles were involuted, leaving deep pits (197).

Pathogenesis of the Infectious Process

Histologic evidence of infection in the cloacal bursa occurs within 24 hours (92). In sequential studies of tissues from orally infected chickens using immunofluorescence, viral antigen was detected in macrophages and lymphoid cells in the cecum at four hours PI; an hour later, virus was detected in lymphoid cells in the duodenum and jejunum (193). The virus is detected in the liver, at five hours PI. It then enters the bloodstream, where it is distributed to other tissues including the bursa; the bursal infection is followed by a second massive viremia, however virus peak titer in the non-lymphoid organs is several log10 lower than in the bursa and limited to the viremic period.

Infectious bursal disease virus was shown to persist in bursal tissues of experimentally inoculated SPF chickens for up to three weeks, but it persisted for shorter periods in the presence of maternal antibodies in commercial broilers (6).

Immunity

Viruses of both serotypes of IBDV share common group antigen(s) that can be detected by the fluorescent antibody test and ELISA (107, 118). Hence, it is not possible to distinguish serotypes or their antibodies by these tests. The common (group) antigens for both serotypes are on VP2 (40 kDa) and VP3 (32 kDa). VP2 also has serotype-specific group antigens that induce VN antibodies (12, 18). Antibodies against VP3 do not have any protective effect (18). *In vivo* studies (108, 120) corroborated this observation, because chickens having antibodies to serotype 2 viruses were not protected against serotype 1 viruses. The current thought is that VP2 has the major antigens that induce protection (12, 18).

Traditionally, serotype 1 viruses have been used for studies of the immune response to IBDV. All known

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isolates of serotype 2 were reported to be nonpathogenic in chickens and turkeys (109, 120, 121) or of low pathogenicity (39, 201, 210). The discovery of variant strains of serotype 1 has heightened interest in furthering the knowledge of the immune response to IBDV. It was interesting that variants were originally isolated from chickens that had VN antibodies to serotype 1 (223, 224). Inactivated vaccines and a live vaccine made from variant strains protected chickens from disease caused by either variant or classic strains, whereas inactivated vaccines made from classic strains did not protect, or only partially protected, against challenge with variant strains (108, 220).

Five different subtypes of serotype 1 IBDV were tested as inactivated vaccines against a variant strain of a different subtype (108). Of vaccines made with 10^8 but not 10^5 tissue-culture-infective doses, 50% were protective against a challenge dose of 10^2 EID_{50}. Even the higher vaccine dose did not protect against challenge with $10^{3.5}$ EID_{50}. Based on these results, it was suggested that all the subtypes of serotype 1 share a minor antigen(s) that elicits protective antibodies.

The contribution of humoral immunity to protection has been well documented as indicated by protection conferred by passive transfer of antibodies. Evidence is accumulating on the additive effect of cell-mediated immunity in protection from the disease (216, 231). The natural resistance of some breeds of chickens to the disease (86) was reported.

Active Immunity

Field exposure to the virus, or vaccination with either live or killed vaccines, stimulates active immunity. Antibody response may be measured by several methods. Antibody levels are normally high after field exposure or vaccination, and VN titers greater than 1:1000 are common. Adult birds are resistant to oral exposure to the virus but produce antibody after intramuscular or subcutaneous inoculation of IBDV (98). However, partial protection against IBD was achieved in chickens in the absence of detectable neutralizing antibodies, as a result of experimental immunization with a fowlpox recombinant virus that expresses the VP2 protein (15), which is an indication that cell-mediated immunity may also play a role in protecting against IBD.

Passive Immunity

Antibody transmitted from the hen via the yolk of the egg can protect chicks against early infections with IBDV, with resultant protection against the immunosuppressive effect of the virus. The half-life of maternal antibodies to IBDV is between three and five days (238). Therefore, if the titer of neutralizing antibodies in the progeny is known, the time that chicks will become susceptible can be predicted. After antibody titers fell below 1:100, chicks were 100% susceptible to infection, and titers from 1:100 to 1:600 gave approximately 40% protection against challenge (155). Titers must fall below 1:64 before chickens can be vaccinated effectively with an attenuated strain of IBDV (238). Use of killed vaccines in oil emulsions (including variant strains) to stimulate high levels of maternal immunity is extensively practiced in the field. Oil emulsion IBD vaccines can stimulate adequate maternal immunity to protect chicks for 4–5 weeks, and progeny from breeders vaccinated with live vaccines are protected for only 1–3 weeks (14, 155). As with many diseases, passively acquired immunity to IBDV can interfere with stimulation of an active immune response.

Immunosuppression

Suppression of the antibody response to Newcastle disease virus was greatest in chicks infected at 1-day-of-age (8, 66). There was moderate suppression when chicks were infected at 7 days, and negligible effects when infection was at 14 or 21 days (66), with decreased humoral antibody response to other vaccines as well (97). Not only was the response to vaccines suppressed, but chicks infected early with IBDV were more susceptible to inclusion body hepatitis (63), coccidiosis (9), Marek's disease (38, 229), hemorrhagic-aplastic anemia and gangrenous dermatitis (222), infectious laryngotracheitis (221), infectious bronchitis (209), chicken anemia agent (295), salmonellosis and colibacillosis (284), and to campylobacter infection and shedding (247).

A paradox associated with IBDV infections of chickens is that although there is immunosuppression against many antigens, the response against IBDV itself is normal, even in 1-day-old susceptible chickens (237). The mechanism is not clear.

The effect of IBD on cell-mediated immunity (CMI) responses is transient and less obvious than that on humoral responses. Suppression of CMI responsiveness, using the lymphoblast transformation assay (234), was found with maximal depression of cellular immunity at 6 weeks PI. A significant suppression of T cell response to the mitogen concanavalin A in poults was seen from 3 days to 4 weeks PI (201). A variant IBDV strain (A) had a significantly more severe effect on the CMI response than a classic strain (Edgar) when given to 1-day-old chicks, and the CMI was suppressed for 5 weeks (44). A similar transient suppression of the CMI was observed in chickens infected at 3-weeks-of-age.

Another lymphoid organ affected by the infection, the Harderian gland, is associated with the local immune system of the upper respiratory tract. IBDV infection of 1- to 5-day-old chicks produced a drastic reduction in plasma cell content of the Harderian gland that persisted for up to seven weeks (57, 209).

Chickens infected with IBDV at 1-day-of-age were completely deficient in serum immunoglobulin G and

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produced only a monomeric immunoglobulin M (IgM) (111, 112). The number of B cells in peripheral blood was decreased following infection with IBDV, but T cells were not appreciably affected (95, 235). The virus appears to replicate primarily in B lymphocytes of chickens (94, 111, 289). Infectious bursal disease virus has a predilection for actively proliferating cells (187), and it was suggested that the virus affected "immature," or precursor B lymphocytes to a greater extent than mature B lymphocytes (171).

Beside lymphocyte lysis, apoptosis is another mechanism of immunosuppression. Apoptosis is also a mechanism of lesion development and could occur in a variety of tissues and organs (8, 139, 254, 270, 271).

There is evidence of a role of T cells in immunopathogenesis (216, 231) resulting from tissue destruction enhancement mediated by cytokines.

Diagnosis

Acute clinical outbreaks of IBD in fully susceptible flocks are easily recognized, and a presumptive diagnosis can be readily made. The rapid onset, high morbidity, spiking mortality curve, and rapid recovery (5–7 days) from clinical signs are characteristics of this disease. Confirmation of the diagnosis can be made at necropsy by examination for characteristic grossly visible changes in the cloacal bursa. There are distinctive changes in size and color of the bursa during the course of infection (i.e., enlargement due to inflammatory changes followed by atrophy) (see Gross Lesions).

Isolation and Identification of the Causative Agent

The cloacal bursa is the tissue of choice for the isolation of IBDV because it contains the highest virus titers and persists longer. Other organs contain the virus, but at a lower concentration and for shorter periods.

The CAM of 9- to 11-day-old embryos was the most sensitive route for isolation of the virus (99). The virus subsequently could be adapted to the allantoic sac and yolk sac routes of inoculation. Death of infected embryos usually occurs in 3–5 days. Variant strains of IBD differ from classic viruses in that they induce splenomegaly and liver necrosis of embryos and produce little mortality (219). The embryonating egg is the most sensitive substrate for isolation of IBDV. In one study three of seven chicken isolates of IBDV failed to grow in CEF cells; however, they could be propagated in embryonating eggs (174).

Isolation and propagation of IBDV in cell culture was discussed previously in this chapter (see Laboratory Host Systems). Because the virus has been shown to replicate in B lymphocytes, either primary cells derived from the cloacal bursa or continuous cell lines of B cell origin would be used for the isolation of the virus. Some strains of virus are fastidious, and although they may replicate in embryonating eggs or B lymphocytes, they will not readily adapt to CEF cells or cells from other organs such as the kidney and liver (146, 174). The use of immunofluorescence and electron microscopy of infected embryos and cell cultures has proven to be of tremendous value for the early detection and identification of IBDV. Cell cultures containing 50% bursal lymphocytes and 50% CEF have been used to successfully isolate and serotype IBDV (156). The fibroblasts serve as a matrix for the lymphocytes, and the infected lymphocytes are detected by immunofluorescence. Identification of the virus by direct immunofluorescent staining of affected organs or direct examination by electron microscopy has proven to be an adjunct to the isolation and identification of IBDV (174). If antigen or virus is detected by these methods from field cases of disease, every effort should be made to isolate the virus using both embryonating eggs and cell-culture techniques. The isolation, antigenic analysis, and pathogenicity studies of viruses from field cases of IBD are needed continually so that changes in the wild virus population can be detected.

Nucleic acid probes (113) and antigen-capture enzyme immunoassays using monoclonal antibodies (242) to detect and differentiate IBDV directly in tissues may prove beneficial for rapid diagnosis and typing of field viruses. One study (89) compared antigen-capture enzyme immunoassay with cell cultures and determined that cell culture was more sensitive than antigen-capture and, in turn, that antigen-capture with polyclonal antibody was more sensitive than with monoclonal antibody. In a study using several procedures for detection of the virus in bursa of experimentally infected chickens, RT-PCR was the most sensitive test (5, 6). Other variations of the RT-PCR technique include multiplex RT-PCR (136) or real-time RT-PCR (179, 212) protocols aimed at detecting and differentiating the different strains of IBDV (classic, variant, and vvIBDV) directly from infected tissues. Provided an adequate dose effect curve is available, real-time RT-PCR may also be used to quantify the virus load in the studied sample (212).

Reverse transcription of the IBDV genome followed by its amplification by loop-mediated isothermal amplification (RT-LAMP) has been described as a possible alternative to RT-PCR for the molecular detection of IBDV, as RT-LAMP is less demanding in laboratory equipment than RT-PCR (288).

Smears of infected tissues on filter paper cards that can be soaked into some IBDV-inactivating chemical to limit biological hazards during handling, shipment, or storage, have been reported as practical and easily shipped samples allow subsequent molecular characterization of IBDV strains (172).

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Differential Diagnosis

The sudden onset, morbidity, ruffled feathers, and droopy appearance of the birds in initial disease outbreaks with occasional blood in droppings are suggestive of an acute outbreak of coccidiosis. The muscular hemorrhages and enlarged edematous or hemorrhagic cloacal bursas would, however, suggest IBD.

Birds that die from IBD may show an acute nephrosis. Because many other conditions may cause nephrosis and the inconsistency of kidney lesions, such lesions should not be sufficient cause for a diagnosis of IBD. Again, involvement of the cloacal bursa usually will distinguish IBD from other nephrosis-causing conditions. Water deprivation will cause kidney changes and possibly gray, atrophied bursas that closely resemble those associated with IBD infection. However, unless this occurs as a flock condition, such changes would be seen in relatively few birds. A history of the flock would be essential in aiding in the differential diagnosis of these cases.

Certain nephropathogenic strains of infectious bronchitis virus cause nephrosis (280). These cases can be differentiated from IBD by the fact that there are no changes in the cloacal bursa, and deaths usually are preceded by respiratory signs. The possibility that the two diseases may occur simultaneously in a flock should not be overlooked.

Bursal atrophy has been observed experimentally with some Marek's disease isolates (127), but the atrophy was observed 12 days PI and the histologic response was distinctly different from that found in IBD (see Chapter 15).

Experimental infections of 1-day-old, specific-pathogen-free (SPF) chickens with a type 8 avian adenovirus produced small bursas and atrophy of bursal follicles at 2 weeks PI (82). Several other organs such as the liver, spleen, pancreas, and kidneys were grossly affected, and intranuclear inclusion bodies were observed in the liver and pancreas.

Serology

The ELISA procedure is presently the most commonly used serological test for the evaluation of IBDV antibodies in poultry flocks. An indirect ELISA for measuring antibodies is more commonly used than VN because it is a rapid test with the results easily entered into computer software programs (171, 240, 255). With these programs, one can establish an antibody profile on breeder flocks that will indicate the flock immunity level and provide information for developing proper immunization programs for both breeder flocks and their progeny. To perform an antibody profile on a flock for the evaluation of the efficacy of vaccination programs, no less than 30 serum samples should be tested; many producers submit as many as 50–100 samples. The antibody profiles may be performed with serum collected either from the breeders or from 1-day-old progeny. If progeny sera are used, titers normally will be 60–80% lower than those in the breeders. It should be recognized that the indirect ELISA does not differentiate between antibodies to serotypes 1 and 2 (109) and that commercial kits may detect antibodies to both serotypes (10). It should also be kept in mind that ELISA kits may also vary in sensitivity and specificity (49), and that being a sensitive technique, ELISA may present both intra-laboratory and inter-laboratory variation (135). Thus, the introduction in the panel of tested sera of a reference sentinel serum with a known reactivity is advisable.

ELISA based on recombinant antigens (expressed IBDV proteins, alone or in combinations) have been developed and some have a good sensitivity and specificity (233). Improved ELISA reagents may prove useful to monitor the serological response to vector or subunit vaccines, for example, expressing only VP2 of IBDV, as the antibody response of the vaccinated birds will be more specific for this protein (142) than when replicating attenuated vaccines are used. Further, the use of subunit ELISA antigens specific for some IBDV proteins that are not present in the vectored or recombinant vaccines (e.g., using a VP3 ELISA antigen), would allow the implementation of a DIVA strategy, where birds vaccinated with the vectored or recombinant-subunit vaccines (e.g., without anti-VP3 antibodies) can be differentiated from infected animals (e.g., with anti-VP3 antibodies), according to the DIVA principle (190).

Prior to the use of the ELISA, the most common procedure for antibody detection was the constant virus-diluting serum VN test performed in a microtiter system (237). The VN test is the only serological test that will differentiate different serotypes of IBDV and it is still the method of choice to discern antigenic variations between isolates of this virus. The indicator virus used for VN can make a significant difference in test results due to the presence of several antigenic subtypes within a given serotype (116). Significant discrepancies in the determination of virus neutralizing titers in different laboratories are also not uncommon (177). Most chicken serums from the field have high levels of neutralizing antibody to a broad spectrum of antigenically diverse viruses owing to a combination of field exposure, vaccine exposure, and cross-reactivity from high levels of antibody.

The other method used for the detection of IBDV antibodies is the AGP test. In the United Kingdom, a quantitative AGP test is routinely used (45); however, as used in the United States, the test is not quantitative. This test does not detect serotypic differences; it measures primarily antibodies to group-specific soluble antigens.

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Intervention Strategies

Contact with infected birds and contaminated fomites readily causes spread of the infection. The relative stability of this virus to many physical and chemical agents increases the likelihood that it will be carried over from one flock to a succeeding flock. The sanitary precautions that are applied to prevent the spread of most poultry infections must be rigorously used in the case of IBD; this includes control of personal and material movements. In their study of the epidemiological factors associated with the spread of vvIBDV in Denmark, it was demonstrated that the highest risk for farms was when another case of IBD occurred during a short period of time or within a short range, a finding that emphasizes the role of local factors in the spread of IBDV (226). The possible involvement of other vectors (e.g., the lesser mealworm, mosquitos, dogs, and rats) has already been discussed; they could certainly pose extra problems for the control of this infection.

Management Procedures

Before the development of attenuated vaccine strains, intentional exposure of chicks to infection at an early age was used for controlling IBD. This was done on farms that had a history of the disease, and the chicks normally would have maternal antibodies for protection. Also, young chicks less than 2-weeks-of-age did not normally exhibit clinical signs of IBD. When the severe immunosuppressive effect of early IBD infections was discovered, the practice of controlled exposure with virulent strains became less appealing. On many farms, the cleanup and disinfection between broods is not thorough and, due to the stable nature of the virus, it easily persists and provides an early exposure by natural means.

Immunization

Immunization of chickens is the principal method used for the prevention of IBD in chickens and IBD vaccines have been reviewed (190). Especially important is the immunization of breeder flocks so as to confer parental immunity to their progeny. Such maternal antibodies protect the chick from early immunosuppressive infections. Maternal antibody will normally protect chicks for 1–3 weeks, but by boosting the immunity in breeder flocks with oil-adjuvanted vaccines, passive immunity may be extended to four or five weeks (14, 155).

The major problem with active immunization of young maternally immune chicks with attenuated IBDV vaccines is determining the proper time of vaccination. Of course, this varies with levels of maternal antibody, route of vaccination, level of exposure, and virulence of the vaccine virus. Environmental stresses and management may be factors to consider when developing a vaccination program that will be effective. Monitoring of antibody levels in a breeder flock or its progeny (flock profiling) can aid in determining the proper time to vaccinate. It should be mentioned that although they produce correlated antibody titers, the ELISA and VN tests may result in predicting different dates for vaccine susceptibility in progeny chicks (48). It is therefore advisable that the formula used for calculating the dates of vaccination be extensively evaluated.

Many choices of live vaccines are available based on virulence and antigenic diversity. Regarding virulence, vaccines that are available in the United States are classified as mild, mild intermediate, intermediate, intermediate plus, or "hot." Vaccines that contain variants, either in combination with classic strains or alone, are also available. Highly virulent (hot), intermediate, and avirulent strains break through maternal VN antibody titers of 1:500, 1:250, and less than 1:100, respectively (155, 238). Intermediate strains vary in their virulence and can induce bursal atrophy and immunosuppression in 1-day-old and 3-week-old SPF chickens (159). If maternal VN antibody titers are less than 1:1000, chicks may be vaccinated by injection with avirulent strains of virus. Some vaccine virus replicates in the thymus, spleen, and cloacal bursa where it persists for two weeks (160). After the maternal antibody is catabolized, there is a primary antibody response to the persisting vaccine virus. A vaccine made by mixing an intermediate plus vaccine strain with a measured amount of IBDV antibody before injection was described to immunize day-old chicks in the presence of maternal antibody but is not in current use (85).

Killed-virus vaccines are usually not practical or desirable for inducing a primary response in young chickens, however, injection of a fraction of a dose in broiler or pullet chicks between one and ten days of age has sometimes been reported (285, 286). Oil-adjuvant vaccines are most effective in chickens that have been "primed" with live virus either in the form of vaccine (287) or field exposure to the virus. Oil-adjuvant vaccines presently may contain both classic and variant strains of IBDV. Antibody profiling of breeder flocks is advised to assess effectiveness of vaccination and persistence of antibody.

In ovo vaccination of chickens for IBD and other agents at 18 days of incubation (70, 277) is a labor-saving technique and may provide a way for vaccines to circumvent the effects of maternal antibody and initiate a primary immune response. The injected material is a live IBD vaccine. *In ovo* injection of an intermediate IBD vaccine alone experimentally resulted in a faster recovery of bursal lesions, as compared with post-hatch vaccination, and had similar protection against challenge (215). *In ovo* and post-hatch vaccinations have been reviewed (198).

Finally, live recombinant virus vectors expressing IBDV immunogens have been reported. These include fowlpox

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virus (15), herpes virus of turkey (HVT) (47), Marek's disease virus (259), chicken adenovirus (CELO) virus (69), and Newcastle disease virus (103). Additional recombinant vaccine viruses include IBDV vaccines that have been modified to broaden their antigenic spectrum (183) or to allow the differentiation of vaccine induced antibodies (25). The currently commercially available recombinant vaccines are derived from HVT and induce an active anti-IBDV antibody response even in the face of high levels of neutralizing, maternally-derived antibodies (80).

A universal vaccination program cannot be offered because of the variability in maternal immunity, management, and operational conditions that exists. If high levels of maternal antibody are achieved and the field challenge is reduced, then vaccination of broilers may not be needed. Vaccination timing with attenuated and intermediate vaccines varies from as early as seven days to two or three weeks. If broilers are vaccinated at 1-day-of-age, the IBDV vaccine can be given by injection along with Marek's disease vaccine. Priming of breeder replacement chickens may be necessary, and many producers vaccinate with a live vaccine at 10–14-weeks-of-age. Killed oil-adjuvant vaccines commonly are administered at 16–18 weeks. Revaccination of breeders may be required if antibody profiling indicates a major drop in flock titers.

Treatment

No practical therapeutic or supportive treatment has been found to change the course of IBDV infection (208). Experimental immunotherapy where passively transferred antibody is injected intraperitoneally after challenge greatly reduced birds showing clinical signs, but this approach has not been tested in the field (166).

There are no reports in the literature concerning the use of some of the newer antiviral compounds and interferon inducers for the treatment of IBD. Ketotifen was reported experimentally to prevent the development of bursal damage, and reduce clinical signs and mortality induced by vvIBDV challenge when administered one hour before IBDV inoculation, but it is not licensed for food animal veterinary use (273).

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8

Chicken Infectious Anemia and Circovirus Infections in Commercial Flocks

Karel A. Schat and Vicky L. van Santen

Summary

Agent, Infection, and Disease. Chicken infectious anemia (CIA) is caused by CIA virus, which is classified as the only recognized species of the *Gyrovirus* genus of the Anelloviridae. Chicken infectious anemia virus (CIAV) has a single-stranded, circular DNA genome of approximately 2.3 kb coding for three viral proteins (VP). VP1 codes for the structural capsid protein, VP2 has multiple functions, and VP3 causes apoptosis. Chicken infectious anemia virus infections have been reported in commercial and backyard chickens in virtually all countries with poultry industries. All isolates belong to one serotype, but different genotypes have been described. Infection of newly-hatched chicks lacking maternal antibodies can result in severe thymus atrophy, replacement of hematopoietic cells by adipose tissue, anemia, and gangrenous dermatitis. Infection after maternal antibodies disappear causes a subclinical infection with immunosuppression. Specific-pathogen-free flocks can be positive for viral DNA in the absence of antibodies and virus is sometimes reactivated at or after the development of sexual maturity. Circovirus infections have been described in ducks, geese, and pigeons, often causing immunosuppression.

Diagnosis. Chicken infectious anemia virus is diagnosed using traditional or quantitative polymerase chain reaction, (q)PCR. Quantitative reverse transcription-PCR (qRT-PCR) is recommended to demonstrate CIAV replication. MSB-1 cells are used for virus isolation. ELISA kits are available for serology. PCR assays are used to diagnose circovirus infections in pigeons, ducks, and geese.

Intervention. Live attenuated CIAV vaccines are available for pullets to provide maternal immunity to newly-hatched chicks. Vaccination is recommended when pullet flocks lack or have an uneven seroconversion. Vaccines are not available for circovirus infections in pigeons, ducks, and geese.

Introduction

Chicken infectious anemia (CIA) was first recognized, and the causal agent isolated, in chicks by Yuasa et al. (338). The disease is characterized by aplastic anemia and generalized lymphoid atrophy with concomitant immunosuppression and is frequently complicated by secondary viral, bacterial, or fungal infections. Since the first description of this disease and subsequent isolation of chicken infectious anemia virus (CIAV) in cell culture in Japan (328), the virus has been isolated or detected in virtually all countries with poultry industries. CIAV appears to play a major role in the etiology of a number of multifactorial diseases associated with hemorrhagic syndromes and/or aplastic anemias. In addition to anemia and associated syndromes, subclinical CIAV infections, without anemia and increased mortality, are frequently observed in commercial flocks.

Other Circoviruses of Birds

During the last 20 years many new small viruses with single-stranded, covalently closed, circular DNA have been described in commercial poultry including chickens, ducks, geese, and pheasants. Originally these viruses were classified as Circoviridae and were subsequently divided into *Circovirus* and *Gyrovirus* genera (262). The *Circoviruses* include pigeon circovirus (PiCV), also referred to as columbid circovirus (CoCV), duck circovirus (DuCV), and goose circovirus (GoCV), while chicken (infectious) anemia virus (CAV or CIAV) was listed as the only virus in the *Gyrovirus genus* (262). This chapter provides a detailed description of CIAV in chickens followed by short descriptions of PiCV, DuCV, and GoCV.

The genome organization of *Gyroviruses* and *Circoviruses* is very different from each other. The *Gyroviruses* have a negative sense genome whereas the *Circoviruses* have an ambisense genome. The genome of the *Circoviruses* codes for a replication initiator protein

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(REP) and has a stem-loop structure, which is important for the initiation of DNA replication. *Gyroviruses* lack the stem-loop structure and the *rep* gene. Based on these and some other differences, the International Committee on Taxonomy of Viruses (ICTV) recently reclassified the *Gyrovirus* genus, with CIAV as the only recognized species, into the Anelloviridae family (7). In addition to the *Gyrovirus* genus, the Anelloviridae contain 11 genera of torque teno viruses, with the human torque teno virus (17) as the best-known example of the *Alphatorquevirus*. A key characteristic for the majority of the *Anelloviridae* is the presence of an apoptosis-inducing protein (51), which is essential for virus replication.

Additional viruses with characteristics of *Gyrovirus* have been described in chickens and humans. Avian *Gyrovirus* 2 (AGV2) was first identified by PCR in serum from a diseased backyard chicken in Brazil. It has a genome of 2383 nucleotides (nt) with a genome organization similar to CIAV and a nt identity of approximately 40% (219). AGV2 was subsequently found in The Netherlands, China, Hong Kong, South Africa, and the United States (2, 37, 55, 239, 321, 342). Sequence analysis indicates that at least three genotypes exist with 16% divergence between the groups (55). AGV2 has been identified in the feces of healthy commercial broilers (145). Its relevance as a pathogen for chickens has not been established. The finding that several vaccines produced in specific-pathogen-free (SPF) embryos or chicken embryo fibroblasts (CEF) were contaminated with AGV2 (288) is disconcerting. Additional AGV2-like viruses, including one named *Gyrovirus* 4, have also been detected by PCR in chicken meat and feces and in human skin and stool samples (e.g., 37, 145, 208, 223, 239, 342). It is not known if the positive samples from humans represent human pathogens or that the positive PCR reaction is the result of consuming virus-positive chicken meat.

Definition and Synonyms

The terminology for the causative agent has varied over the years. The agent was originally designated chicken anemia agent (CAA) (338) but after morphologic and biochemical characterization (75, 165, 265), it was renamed chicken anemia virus (CAV) (75, 185). The name CAV has been accepted by the ICTV (262). However, because the disease is commonly referred to as chicken infectious anemia, the causative virus is more logically referred to as chicken infectious anemia virus (CIAV) (302). This terminology will be used in this chapter.

CIA and closely associated syndromes have commonly been termed hemorrhagic syndrome (333), anemia-dermatitis (291), or blue wing disease (18, 67).

Economic Significance

Infection with CIAV has been confirmed as the cause of disease in chicken flocks between two and four weeks of age with syndromes suggestive of infectious anemia (228). In these flocks growth was retarded and mortality was generally between 10 and 20%, but occasionally it reached 60%. In chickens six or more weeks of age, the etiologic significance of CIAV infection associated with aplastic anemia-hemorrhagic syndromes (87, 196, 335) has not been definitely established.

Infection with CIAV constitutes a serious economic threat, especially to the broiler industry and the producers of SPF eggs. McIlroy et al. (156) reported a loss of net income of about 18.5% due to decreased weight at processing and increased mortality around three weeks of age in 15 broiler flocks. Approximately 30% of these broilers were derived from a common breeder flock, which was free of CIAV antibodies at 20 weeks of age, making the offspring susceptible to CIAV infection at a very early age. Interestingly, feed conversion ratios were not affected in the broilers with CIA. Davidson et al. (47) documented 14–24% reductions in weight of meat sold, as well as changes in feed conversion ratios, from CIAV-infected flocks exhibiting clinical signs characteristic of CIA.

Studies addressing the impact of subclinical CIAV infection on broiler flock performance have yielded conflicting results. In one study, subclinically infected flocks in Northern Ireland yielded a 13% lower net income than CIAV antibody-negative flocks, mostly due to decreased weight at processing and suboptimal feed conversion ratios (167). A Belgian study demonstrated a higher slaughterhouse condemnation rate in CIAV positive flocks compared to CIAV negative flocks, but was unable to detect differences in other performance criteria (50). However, others were unable to confirm the negative influence of subclinical infection on production in the United States (84) and Denmark (125). In a retrospective case-control study in the United States, although presence of CIAV was found to be a risk factor for disease (gangrenous dermatitis, coccidiosis, or respiratory disease), and disease was associated with production losses, the detection of CIAV alone was not associated with statistically significant decreases in performance or production losses (92). It is likely, however, that the impact of subclinical infection with CIAV is underestimated, especially because subclinical infection significantly reduces the development of antigen-specific cytotoxic T lymphocytes (CTL) (153) and also adversely affects macrophage functions (155).

The economic importance for the SPF industry is difficult to estimate, but seroconversion frequently occurs during the laying period (78, 322). As a consequence of seroconversion the flock is considered positive and the eggs are no longer SPF. The importance of this depends on legislation for vaccine production. The European

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Union requires the absence of CIAV from eggs used for the production of all poultry vaccines for administration in birds less than seven days of age. Eggs from CIAV-positive flocks may be used according to United States Department of Agriculture (USDA) guidelines. Contamination of various live poultry vaccines with CIAV has continued to be a problem in many parts of the world (14, 142, 151, 288). In most cases CIAV was detected only by PCR, but CIAV infectious to chickens has been demonstrated in Newcastle disease virus (NDV) and fowl pox vaccines (142). In one study, the levels of CIAV genome contamination in live poultry vaccines was assessed by quantitative PCR (qPCR) and found to be less than one CIAV genome copy per dose of vaccine (288). Vaccines for human use, such as measles and mumps, require the use of eggs free of CIAV in Australia, Europe, and the United States.

Public Health Significance

Although CIAV has been detected by PCR in chicken meat intended for human consumption and in human stool samples, there is no evidence that CIAV replicates in humans or is associated with human disease (37, 239, 342). Furthermore, results of serologic tests suggest that CIAV has no public health significance (302).

History

CIAV (Gifu-1 strain) was first isolated in 1979 in Japan by Yuasa et al. (338). However, the virus was present in chickens at least as early as 1970, when Jakowski et al. (117) described a condition of hematopoietic destruction in chickens with Marek's disease (MD). The ConnB isolate of CIAV was later isolated from an ampoule of tumor cells obtained from those chickens (247, 313). The demonstration of CIAV antibodies in archived sera indicates that CIAV was present in the United States as early as 1959 (272). Aplastic anemia syndromes, including inclusion-body hepatitis, were described many years before CIAV was detected. Their possible etiologic association with CIAV infection has been reviewed and is discussed in several papers dealing with CIA (160, 212, 294).

A major breakthrough was achieved in 1983 when Yuasa et al. (335) reported that virus could be propagated in certain chicken lymphoblastoid cell lines, for example, Marek's disease chicken cell (MDCC) MSB-1 (MSB-1), causing cytopathic effects (CPE). This enabled the development of *in vitro* serological assays such as indirect immunofluorescence assays (297, 332) and virus-neutralization (VN) tests (336). In addition virus could be easily purified from supernatant fluids of CIAV-infected cell cultures and characterized (75, 88, 110, 165, 265).

Virus identification was followed by studies that unraveled much of the pathogenesis and epizootiology of the infection. In the early 1990s, remarkable progress was made in research on the molecular biology of CIAV (reviewed in [225]). This resulted in the development of refined diagnostic methods and the potential for development of new types of vaccines (115, 131, 188).

Etiology

CIAV is currently classified as the only member recognized by the ICTV of the genus Gyrovirus of the *Anelloviridae* (7).

Morphology

CIAV virions consist of non-enveloped, icosahedral particles with an average diameter of 25–26.5 nm, as visualized in preparations negatively stained with 1% uranyl acetate (75, 165). In such preparations, two types of virus particles differing in their orientation on the grid are commonly detected. Type I particles exhibit three-fold rotational symmetry and show a pattern of one central hollow surrounded by six neighboring hollows with a center-to-center distance of 7.5 nm, forming a regular surface network (Figure 8.1B). Type II particles exhibit five-fold rotational symmetry and are characterized by 10 evenly spaced surface protrusions giving the impression of a "cog-wheel" structure (Figure 8.1A). The appearance of these particles suggested a regular T = 3 icosahedron with 32 morphologic subunits. However, modeling of unstained cryopreserved CIAV particles indicated a T = 1 lattice with 60 copies of VP1 in a capsid consisting of 12 pentagonal, trumpet-shaped capsomers. These protruding capsomers distinguish CIAV from Circoviridae, which have a smoother capsid surface (42).

Virions have a buoyant density in cesium chloride gradients variously reported as 1.33–1.34 g/mL (9, 265) (or between 1.35 and 1.37 g/mL (52, 63). The sedimentation coefficient of CIAV has an estimated value of 91 S in isokinetic sucrose gradients (9).

Chemical Composition

Viral DNA

The genome of CIAV consists of single-stranded, circular, covalently closed DNA (75, 265) of negative sense (185). Noteborn et al. (185) and Claessens et al. (38) published the first complete sequences of CIAV. Since then many more sequences have been published, and by May 7, 2019, 166 full CIAV genome sequences from around the world were available in GenBank. Nearly all CIAV genomes are 2,298 nt in length, and contain four 21-base tandem direct repeats (DR), with a 12-base insert between the second

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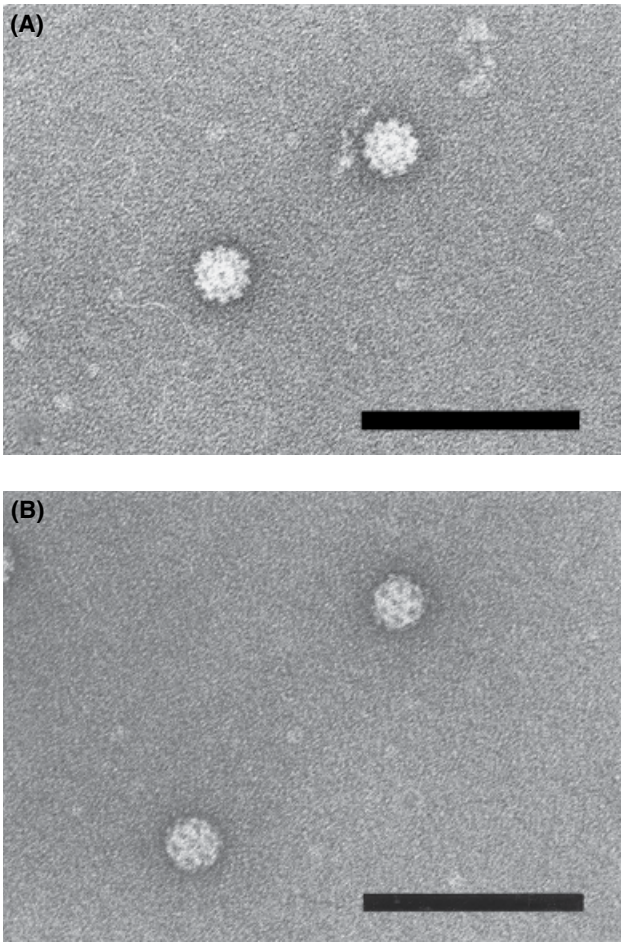


Figure 8.1 Electron micrographs of chicken CIAV. Different structural aspects of the CIAV capsids become apparent in negative-stained preparations. Two types of particle projections are obvious. (A) Particle projection type II characterized by 10 peripheral protrusions. ×250,000. Bar = 100 nm. (H. Gelderblom) (B) Projection type I showing CIAV capsids that exhibit six stain-filled morphologic units that surround one central hole.

and third DR. Todd et al. (263) reported that a fifth DR, upstream of the insert, was obtained after about 30 passages of the Cux-1 strain in MSB-1 cells, yielding a 2319 nt genome. The same DR insertion has also been identified in the genome of CIAV not passaged in culture, resulting in a 2316 nt genome (346). However, this is very rare among non-culture-passaged CIAV, found only once among the 124 full CIAV genome sequences in GenBank. In infected cells, both single-stranded and double-stranded DNA are present, but virions contain only the circular minus-strand DNA (209, 263).

All sequenced CIAV genomes have three partially overlapping open reading frames (ORFs) coding for proteins of 499 (VP1, ORF1, 52 kDa), 216 (VP2, ORF2, 24 kDa), and 121 (VP3, ORF3, 13 kDa) amino acids (AA), one promoter region, and one polyadenylation signal. ORF3 is located within ORF2, and ORF2 partly overlaps ORF1. This genomic organization distinguishes CIAV and other *Gyroviruses* from the Circoviridae, which transcribe mRNA from both strands of their replicative intermediates, making their genomes ambisense (182).

The promoter-enhancer region, consisting of the four (or very rarely five) 21 nt DR and the 12 nucleotide insert, is located upstream of ORF2 (190). The repeat units and the 12 nt insert contain recognition sites for different transcription factors (169, 174, 175, 185). Optimal transcription requires both the DR and the 12 nt insert. The presence of the fifth DR enhances transcriptional activity (190), whereas deletion of the first two DR reduces transcriptional activity by 40–50% (209). Disruption of the relative spacing of the DR region with other promoter elements and the start of transcription by insertion of a 7-bp linker decreases the rate of virus replication in culture (189). Noteborn (unpublished data cited in 189) found that CIAV DNA containing three DR but lacking the 12 nt insert was not able to produce infectious virus particles. Although the 12-bp insert binds the transcription factor SP1 (190), substitution of a different 12-bp sequence did not further impair replication of virus already impaired by insertion of a 7-bp linker between the DR region and the rest of the CIAV promoter (189). Some changes in the length of the 12-bp insert region resulted in decreased cytopathogenicity and rate of spread in culture, but the VN epitope in VP1 was still produced (189). The DRs contain sequences similar to the estrogen response element consensus half sites and compete with estrogen response elements for estrogen receptor binding in nuclear extracts (174). Expression from the CIAV promoter is higher in cells expressing estrogen receptor and is further increased by addition of estrogen (174), while binding of the transcription factor COUP-TF1 inhibits transcription (175). An additional negative regulator of transcription is the E-box-like sequence at the transcription initiation site, which binds delta-EF-1 (175).

Initially, only one unspliced, polycistronic mRNA of 2.1 kb, containing all three ORFs, and a minor transcript of approximately 4 kb were identified. Use of internal AUG start codons is required for the synthesis of VP1 and VP3 (186, 209). Subsequently, minor spliced mRNAs were identified in addition to the major unspliced mRNA by both Northern blotting and RT-PCR (129). One of the spliced mRNAs potentially encodes a protein that shares amino- and carboxy-terminal sequences with VP1, but lacks 197 AAs (positions 132–328) of VP1's 449 AAs. Other spliced mRNAs potentially encode novel proteins with frame-shifts. However, protein products of the spliced mRNAs have not been demonstrated.

Viral Proteins

A 499 AA viral protein (VP1) is the only protein detected in highly purified virus particles and forms the capsid (265). The N-terminal 40 AAs show a limited similarity

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to histone proteins, suggesting a DNA binding role (38, 169). The non-structural 216 AA VP2 probably acts as a scaffold protein during virion assembly, so that VP1 folds in the proper way (131, 188). The third viral protein, VP3 (121 AA) is associated with other viral proteins in nuclei in infected cells (32, 56, 187), but not with highly purified virus particles (22).

Studies using neutralizing monoclonal antibodies (mAb) in Western blots suggested that VP1 neutralizing epitope(s) are conformational in nature (22). Neutralizing antibodies were induced after inoculation of chickens with insect cells containing both VP1 and VP2, but not with cells containing only VP1 or only VP2 (131). A VN mAb reacted with baculovirus-produced VP1 only if VP2 was coproduced. These findings have been misinterpreted in the literature to mean that both VP1 and VP2 are targets for VN antibodies; VN antibodies target only VP1. In contrast, VN mAbs bound to VP1 produced in mammalian cells in the absence of VP2 (278). VN mAb binds to the native VP1 in virus capsids, which contain no VP2, but not to denatured VP1, lending further support to the role of VP2 as a scaffolding protein (188).

VP2 and VP3 are nonstructural proteins. VP2 is a multifunctional protein. In addition to its putative role as a scaffold protein enabling VP1 to attain its proper conformation, VP2 has serine/threonine and tyrosine protein phosphatase activities (207). VP2 can also induce apoptosis, as well as CPE characteristic of CIAV infection (127). VP3, also named apoptin, is a strong inducer of apoptosis in chicken thymocytes and chicken lymphoblastoid cell lines (187).

Virus Replication

Virions probably enter the cell by conventional adsorption and penetration. Low levels of the 2.1 kb polycistronic viral RNA transcript can be demonstrated at 8 hours postinfection of MSB-1 cells, with maximum levels attained at 48 hours (186, 209).

Viral DNA replication occurs via a double-stranded replicative form (RF), probably by the rolling-circle mechanism (266). The actual initiation site for DNA replication has not been identified, although Bassami et al. (15) reported the presence of a nonanucleotide motif that may be involved in the initiation of DNA replication. Transfection experiments with cloned tandemly-repeated CIAV RF suggest that homologous recombination can occur (266). This process can lead to double-stranded circular molecules that are identical to RF. The double-stranded RF may lead to the presence of latent episomal DNA and be responsible for the presence of viral DNA in gonadal tissues as reported by Cardona et al. (28). Todd (261) suggested that VP1 may have a role in DNA replication based on the presence of 3 AA motifs associated with rolling-circle replication of DNA.

VP3 can be detected at 6 hours postinfection (PI) in a few cells (267). VP2 is present at 12 hours postinfection (PI), while the capsid protein (VP1) was not detectable until 30 hours PI (56). More recently, VP1 has been detected as early as 12 hours PI in MSB-1 cells, but the time of appearance of VP2 and VP3 was not determined (278).

The protein phosphatase activity of VP2 is very important, but not absolutely required, for CIAV replication. CIAV with a mutation of the catalytic cysteine of VP2, which abolishes both serine/threonine and tyrosine phosphatase activities (207), exhibits impaired replication and cytopathogenicity in MSB-1 cells, reaching titers 10,000-fold lower than wild-type virus (206). Surprisingly, another VP2 catalytic site mutation that increases the tyrosine phosphatase activity and reduces serine/threonine phosphatase activity by only 30% impairs viral replication to a similar degree as the mutation abolishing both phosphatase activities (206). Other VP2 mutations, expected to have only subtle or no effects on protein phosphatase activity, resulted in varying degrees of impairment of CIAV replication (205). Unfortunately, the phosphatase activity of these other VP2 mutants was not reported. In addition to impaired replication, the VP2 mutants apparently exhibit reduced cytopathogenicity, which must be assessed subjectively, in culture. Interestingly, one VP2 mutation resulted in reduced cytopathogenicity without affecting viral replication efficiency, which suggests that viral replication functions and cytopathogenicity can be separated. In contrast to wild-type CIAV, in cells infected with VP2 mutants, VP3 is cytoplasmic rather than nuclear in location, suggesting a role for VP2 in VP3 trafficking and function. This is significant, because the nuclear location of VP3 correlates with its ability to cause apoptosis in transformed, but not primary cells, where VP3 remains cytoplasmic (45).

VP3 is essential for the virus replication cycle (213, 309). Unlike full-length (121 AAs) VP3, VP3 lacking AAs 59–88 or 80–112 is unable to fully complement replication of mutant CIAV lacking VP3 expression (213). Mutant CIAV expressing truncated VP3 lacking a nuclear localization signal within its C-terminal 24 AA replicates to 100-fold lower levels in culture and in chickens, and exhibits reduced pathogenicity in chickens (309). Phosphorylation of AA T108, which is necessary for inhibition of a nuclear export signal (211), is needed to induce apoptosis and virus replication in MSB-1 cells (133). Truncated VP3 lacking the last 11 AAs has greatly reduced ability to induce apoptosis in transfected MSB-1 cells (187).

Interestingly, VP3 was found to induce apoptosis in several malignant human lymphoblastoid cell lines (348) and human osteosarcoma cells (349), but not in normal human cells (44). This finding has been confirmed in over 70 cell types tested and extended to growth-transformed cells and cells from cancer-prone individuals exposed to UV irradiation (reviewed in 23). Animal

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experiments using adenovirus vectors expressing VP3 (apoptin) suggest that apoptin may be used to treat humans with cancer (210, 281). The potential of apoptin as an antitumor agent has stimulated investigation into the mechanism whereby it induces apoptosis in transformed cells (reviewed in 23). The specificity for tumor cells seems to depend on activation of apoptin by kinases expressed at higher levels in tumor cells. One mechanism whereby apoptin induces apoptosis that has been identified is by binding to the promoter for the anti-apoptic stress-response gene HSP70 and inhibiting its expression (324, 341).

VP3 has functions in the CIAV replication cycle in addition to induction of apoptosis. VP3 is absolutely required for viral DNA replication (213). Its function in production of infectious virus can be separated from its function in viral DNA replication by a CIAV mutant expressing VP3 that cannot be phosphorylated on AA 108. This mutant replicates viral DNA to the same levels as wild type virus, but produces 40-fold less infectious virus (213). The defect can be complemented with wild-type VP3. VP3 multimers form non-sequence-specific complexes with double- and single-stranded DNA and with RNA, with preference for ends in double-stranded DNA (139). Association with VP3 induces bends in DNA. These observations suggest possible mechanisms for VP3's role in viral DNA replication and suggest that it could also affect gene expression. Its sequence-specific binding to the HSP70 promoter does inhibit HSP70 expression, as noted above (324).

The replication of CIAV in very young chickens occurs primarily in hemocytoblasts in the bone marrow and T cell precursors in the cortex of the thymus (reviewed in 3, 177). Replication of the virus in the cortex of the thymus results in cell death by apoptosis (119) caused by VP3. Virus replication has also been demonstrated in other organs, where it is usually but not always associated with lymphocytes (241). Virus is also found at high levels in peripheral blood (310, 311). In chickens infected at three or six weeks of age, CIAV replicates in the thymic cortex, but CIAV positive cells are rare in the bone marrow (240).

Resistance to Chemical and Physical Agents

CIAV is extremely resistant to most treatments (160, 326, 338). Treatment of virus in liver suspensions with 50% phenol for five minutes inactivates CIAV but treatment with 5% phenol for two hours at 37 °C is ineffective. The virus is resistant to treatment with 50% ethyl ether for 18 hours and chloroform for 15 minutes. Treatment of liver suspensions with 0.1 N NaOH for two hours at 37 °C or 24 hours at 15 °C inactivates CIAV incompletely. Treatment with 1% glutaraldehyde for 10 minutes at room temperature, 0.4% β-propiolactone for 24 hours at 4 °C, or 5% formaldehyde for 24 hours at room temperature inactivates the virus completely. Commercial disinfectants based on invert soap, amphoteric soap, or orthodichlorobenzene are not effective against CIAV.

Treatments with iodine or hypochlorite are effective, but require two hours at 37 °C with final concentrations of 10% rather than the generally recommended concentrations of 2%. Formaldehyde or ethylene oxide fumigation for 24 hours does not inactivate CIAV completely. CIAV is also resistant to acid treatment at pH 3 for three hours. Treatments with disinfectants with pH 2 are widely used by the SPF industry and are apparently effective in inactivating the virus (78, 322).

CIAV is also resistant to treatment with 90% acetone for 24 hours (258). As a consequence, acetone-fixed slides of CIAV material may remain infectious and need to be sterilized prior to final disposal. CIAV is resistant to heating at 56 °C or 70 °C for one hour and at 80 °C for 15 minutes (64, 87, 338). However, it is only partially resistant to heating at 80 °C for 30 minutes, and is completely inactivated within 15 minutes at 100 °C (87). With regard to heat inactivation of CIAV, it is important to distinguish between CIAV in liquid substrates and in a dried state. Lyophilized CIAV is resistant to treatment at 120 °C for 30 minutes (312). Inactivation of CIAV in infected chicken byproducts requires a core temperature of 95 °C for 35 minutes or 100 °C for 10 minutes, whereas fermentation is ineffective (280).

Strain Classification

Antigenicity

No antigenic differences have been recognized among various Japanese, European, and American isolates of CIAV using polyclonal chicken antibodies (64, 164, 295, 297, 330). As a consequence, it is generally accepted that all strains belong to one serotype (160, 191). However, based on differences in reaction patterns with mAbs (166, 231, 243, 278) and DNA sequence differences resulting in changes in the predicted protein folding patterns (218), it is expected that strains may differ in antigenicity. Importantly, one of the mAbs that differentiates among CIAV isolates neutralizes some isolates, but not others, suggesting relevance of this antigenic difference (278).

A second serotype of CIAV, represented by CIAV-7, has been reported (249, 250). CIAV-7 has physical characteristics similar to CIAV (250), and produces similar clinical disease and gross and microscopic lesions (249). However, thymic and bone marrow lesions and anemia produced by CIAV-7 are much milder than those generated by CIAV. Furthermore, the lack of any antigenic cross-reactivity using polyclonal chicken sera and lack of cross hybridization under low-stringency conditions suggest that CIAV-7 is a novel virus rather than a new

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serotype of CIAV. This virus has not been further reported since the original description.

Molecular Differences

Partial or complete genome sequences of numerous strains from different parts of the world have been determined and AA sequences predicted. The genetic variation among isolates is limited and generally less than 5%, with most of the variation in the VP1 coding region. As more CIAV sequences have been determined, slightly more variability has been found. For example, Nogueira et al. (184) found over 10% nucleotide sequence differences between partial sequences of some CIAV samples obtained from Brazilian chickens after 1998 and sequences of older CIA isolates. However, these nucleotide sequence differences led to relatively few AA sequence differences in VP1. Molecular evolution model analyses of 46 complete VP1 sequences identified 6–8 AA positions in VP1 that are subject to positive selection (306). Analysis of a smaller number of partial VP1 sequences found evidence for positive selection in only one of fifteen sequences compared (93). Phylogenetic analysis of 121 complete CIAV genome sequences generated eight lineages, without clear relationships to time or location (141). Although some of the lineages contain only isolates from a specific region such as Eastern Asia, others include viruses from multiple continents, including Asia. Some isolates appear to have arisen by recombination between genomes of different molecular groups (63, 99, 141, 344). In each case where the points of recombination have been mapped, one falls within the VP1 gene and one falls within the non-protein-coding region. Apparent phylogenetic relationships may be influenced by adaptation to culture (36). Phylogenetic groups based on nucleotide and AA sequences often differ from each other (e.g., 60, 130, 194, 285). Furthermore, it is not clear whether these groups differ biologically. For these reasons, the significance of groupings based on phylogenetic relationships of CIAV sequences remains unclear.

Minor differences in predicted AA sequences have been noted, especially for the AAs 139–151 of VP1 (hypervariable region), and also at the carboxy terminus of VP2 and VP3 (218). The predicted protein structure of VP1 is affected by the observed differences in the hypervariable region. Furthermore, recognition by a neutralizing mAb that distinguishes among CIAV isolates depends on the presence of glutamic acid at AA position 144 within the hypervariable region (278). Chimeric constructs in which a fragment encoding the hypervariable region had been exchanged between the highly passaged Cux-1 and low-passage CIA-1 strains demonstrated that differences in the hypervariable region can influence virus replication in MSB-1 cells (218). Noqueira et al. (184) and van Santen et al. (286) reported that the inability to isolate CIAV strains in MSB-1 was not solely linked to changes in the hypervariable region. The importance of the hypervariable region for *in vivo* pathogenicity is not clear. Meehan et al. (168) examined the pathogenicity of a number of chimeric viruses with multiple changes, including those in the hypervariable region, and concluded that changes in the hypervariable region did not contribute disproportionately to pathogenicity. However, studies with changes only in this region have not been reported, and the question of the importance of the hypervariable region for the pathogenicity remains unresolved. Unfortunately, many authors have interpreted the finding that differences within the VP1 hypervariable region affect replication and spread of CIAV in cultured cells (218) to imply that CIAV with certain AA sequences in this region replicate poorly in chickens and have lower transmission rates. This has led to unsubstantiated conclusions on the pathogenicity of CIAV detected by PCR based on its VP1 hypervariable region deduced AA sequence.

Pathogenicity

Although it is generally accepted that strains isolated worldwide do not differ substantially in pathogenicity, very few studies have directly compared different strains under identical experimental conditions. Yuasa and Imai (330) compared 11 isolates, which were each passaged 12 times in MSB-1 cells prior to inoculation. Minor differences in virulence were found when chicks were inoculated at 7 days but not at 1 day of age; inoculation at 14 days of age failed to induce lesions. Rimondi et al. (220) compared two isolates and found that only one of them caused severe depletion of thymocytes when inoculated into two-week-old chickens. However, because the inocula were quantitated only by genome copy number, equivalence of dosages of the two isolates could not be assured. Natesan et al. (181) compared the pathogenicity of four isolates in one-day-old chicks and detected no differences. Toro et al. (274) reported thymus and bone marrow lesions when 10-week-old broiler breeders were infected with strain 10343, but comparative studies with other isolates in these birds were not reported.

Attenuation

Attenuation of CIAV by cell-culture passages has been problematic with conflicting results; details can be found in (225, 228). Attenuation of Cux-1 was reported after 49 passages in cell culture by von Bülow and Fuchs (296), while Todd et al. (263) found that Cux-1 became substantially less pathogenic only after 173 passages in MSB-1 cells. Moreover the attenuation at this level was not stable even when molecular clones were analyzed (264). Additional serial passages were analyzed at p320 and some molecular clones were indeed further attenuated (157), but the stability of the attenuation was not reported. Attenuation of isolates other than Cux-1 was

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also attempted but without success after 40 (88, 328) or 129 (254) passages. Chowdhury et al. (36) reported that two Malaysian CIAV isolates were partly attenuated at p60 and one was further attenuated at p123 in MSB-1 cells. Stability of attenuation and which changes were responsible for attenuation were not examined.

In addition to cell-culture passage, site-directed mutagenesis of VP2 was used to attenuate CIAV (204, 205). Inoculation of these mutant viruses in cell culture suggested that the mutants were attenuated. The results in chickens were less clear (128) and the stability of the one mutant that was apathogenic in chicks was not established. As noted above, mutant CIAV expressing truncated VP3 lacking its C-terminal 24 AA exhibited reduced pathogenicity in chickens (309).

Schat (225) reviewed the molecular changes associated with attenuation resulting from cell-culture passage and concluded that the molecular basis for attenuation is complex and poorly understood. One molecularly-cloned culture-attenuated CIAV has an AA change in VP1 at AA position 394 and thus VP1 AA 394 has been designated a pathogenicity determinant (320). This finding has often been misused to conclude that any CIAV with the wild-type AA at VP1 position 394 is highly virulent or highly pathogenic, although this AA position exhibits no variability in CIAV identified in or isolated from chickens or other sources.

Laboratory Host Systems

CIAV can be propagated and assayed in cell cultures, one-day-old chicks, or in chicken embryos.

Cell Cultures

The use of cell cultures is the preferred method for virus isolation and propagation since Yuasa et al. (335) reported that some chicken lymphoblastoid T cell lines (e.g., MSB-1 and MDCC-JP2) and the B cell line LSCC-1104B1 are suitable for propagation and assay of CIAV. However, many other chicken T cell and B cell lymphoblastoid cell lines, whether producers or nonproducers of the respective transforming viruses, are resistant to CIAV (25, 300, 335). Two mammalian lymphoblastoid cell lines tested are resistant to CIAV (94).

MSB-1 cell cultures have been preferred for *in vitro* cultivation, although sublines of MSB-1 differ in their susceptibility to infection. Some strains of CIAV, e.g., CIA-1 (148), do not replicate in one subline of MSB-1 (MSB-1-L) and poorly in another subline of MSB-1 (MSB-1-S), whereas both sublines are susceptible to infection with Cux-1. Furthermore, the sensitivity of susceptible MSB-1 cells to CIAV is reduced after subculturing the cells, in some cases for as little as eight weeks (25, 286, 297). The MDCC-CU147 (CU147) cell line is superior to MSB-1 for propagation of CIAV, including the CIA-1 strain (25), regeneration of CIAV from CIAV genomes molecularly cloned directly from field specimens (286), and virus titrations (25). Unfortunately, this cell line is difficult to propagate (286).

Virus titrations require subculturing of inoculated cells every two to four days until cells inoculated with the endpoint dilution of CIAV are destroyed (297, 335). Alternatively, endpoints can be determined by PCR (284) or immunofluorescence assay (25).

Primary lymphoid cell cultures such as ConA-stimulated chicken peripheral blood mononuclear cells can also be used for isolation and propagation of CIAV (150), and would provide an alternative where CIAV-susceptible lymphoblastoid cell lines are not available. It is possible that CIAV isolates refractory to isolation in MSB-1 cells might be isolated in primary lymphoid cells, but this has not been tested. Cux-1, which had already been isolated and propagated in MSB-1 cells, could be propagated in primary splenocytes and bone marrow cells, but the titers achieved were at least 30-fold lower than in MSB-1 cells (158).

Chickens

Inoculation of one-day-old chicks free of maternal antibodies can be used to isolate and propagate CIAV in instances where the clinical syndrome suggests that CIAV may be present, but *in vitro* virus isolation and/or PCR assays are negative. Positive chicks develop anemia and gross lesions in lymphoid tissues and bone marrow after 12–16 days (338). Mortality may occur between 12 and 28 days postinoculation (PI) but usually remains low, rarely exceeding 30%. Neonatal (331) or embryonal bursectomy (148) can enhance the sensitivity of isolation, especially if samples with low titers are analyzed. Chicks with maternal anti-CIAV antibody are resistant to CIAV infection and cannot be used for isolation or propagation of CIAV (334).

Chicken Embryos

Propagation of CIAV in chicken embryos following yolk sac inoculation has been reported (303). Moderate virus yields were obtained after 14 days from all parts of the embryo, but not from yolk or chorioallantoic membrane. Depending on the virus isolate, lesions including embryo mortality may be present or absent between 16 and 20 days of incubation (48, 137, 254, 303).

Pathobiology and Epizootiology

Incidence and Distribution

CIAV is ubiquitous in all major chicken-producing countries of the world (reviewed in 302). A recent survey involving seven clinical poultry veterinarians from around the world confirmed that the virus has a worldwide prevalence. The importance of CIAV

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infections is less clear because most breeder flocks are vaccinated, preventing clinical disease. However, in countries with very virulent infectious bursal disease virus (vvIBDV) using IBDV vaccine strains of intermediate pathogenicity, clinical CIA may occur (Schat, personal observations). The impact of subclinical immunosuppression is often difficult to estimate and was not addressed in the responses from the poultry veterinarians.

Natural and Experimental Hosts

The chicken is the only known host for CIAV. CIAV has been detected in or isolated from feces of stray mammals and ferrets fed chicken meat, but no studies have addressed whether the virus replicates in these hosts (70, 141, 208, 343). All ages of chickens are susceptible to infection, but susceptibility to anemia rapidly decreases in immunologically intact chicks during the first one to three weeks of life (87, 221, 330, 337, 338), although some strains have been reported to cause a reduction in hematocrit values after experimental infection of ten-week-old broiler breeders (274). Chickens three weeks of age and older continue to be susceptible to effects of CIAV on immune function (153, 154, 215, 240, 274, 310).

Antibodies to CIAV have been detected in Japanese quail but not in ducks, pigeons, or crows in Japan, but information on the specific species was not provided (69). Fancy chicken breeds in the Netherlands were frequently positive for CIAV antibodies (52). A survey in Ireland found CIAV antibodies in jackdaws, rooks, and rare avian breeds, but not in pigeons, pheasants, or ducks (26). McNulty et al. (162) failed to detect antibodies in turkey and duck sera. Turkey poults inoculated at one day of age with high doses of the virus were resistant to infection and did not develop antibodies to CIAV (160). However, a virus similar to CIAV, but with low pathogenicity in chickens, has been isolated from turkeys (230).

Transmission

CIAV spreads both horizontally and vertically. Although most experimental infections are established by intramuscular injection, natural horizontal transmission most likely occurs through the fecal–oral route based on the presence of high concentrations of virus in the feces of chickens for five to seven weeks after infection (101, 336) and successful experimental inoculation (254, 283). However, infection via the respiratory route, as shown in chicks after intratracheal inoculation (221), also may be possible in the field. In addition to virus shedding through the feces, virus can also be shed through feather follicle epithelium (46). Transmission occurs readily via contaminated litter (114). CIAV spreads easily among chickens in a group only if they are immunosuppressed (337). In naturally exposed flocks, it commonly takes two to four weeks until most birds have seroconverted (162, 248, 295). Isolation may prevent early seroconversion; 70% of grandparent flocks that were imported into Sweden and kept in quarantine remained seronegative until 16 weeks of age (65).

It is important to differentiate vertical transmission in commercial flocks from that in SPF flocks experiencing sporadic breaks. In the former, vertical transmission of virus through hatching eggs is considered to be the most important means of dissemination (35, 65). Vertical transmission occurs when antibody-negative hens become infected by horizontal infection or by semen from infected cocks (102). Egg transmission only occurred from 8 to 14 days after experimental infection of hens (102, 339). After the development of immune responses egg transmission of virus could not be demonstrated, even when birds were stressed by injections with betamethasone or exchanging hens in cages. Field observations indicate that vertical transmission can occur during a period of three to nine weeks after exposure with peak transmission at one to three weeks. The duration of egg transmission in a flock depends on the rate of spread of infection and development of immunity to CIAV (18, 35, 67, 291). In contrast to earlier studies showing the absence of vertical transmission after the development of antibodies, later studies demonstrated CIAV DNA in progeny of hens with high titers of neutralizing antibody. Detection of CIAV relied on highly sensitive nested PCR, and no disease or lesions were found in the progeny (21, 93).

Seroconversion patterns in SPF flocks suggest a more complex situation than described for commercial birds. Seroconversion in SPF chickens has been reported for commercial and noncommercial SPF flocks and often occurs during the first laying cycle (27, 68, 78, 161, 162, 227, 322, 332). When CIAV was accidentally introduced in three genetically different SPF flocks maintained at Cornell University, seroconversion coincided with the development of sexual maturity even while birds were housed in a CIAV-infected environment. Seroconversion was less than 100% in birds of different genetic strains during a 60-week-period while birds were kept in colony cages in an environment contaminated with CIAV. However, CIAV DNA could be detected in both seronegative and seropositive birds by nested PCR assays of gonadal tissues and spleens, even in chickens that had been antibody positive for more than 40 weeks (27, 28).

Horizontal spreading in SPF flocks kept in cages may be less efficient than described for field flocks. Miller et al. (176) followed seroconversion in a flock of 90 chicks hatched from eggs supplied by an SPF producer. All birds were bled monthly and one bird seroconverted at six weeks of age; this bird was euthanized. The remainder of the flock remained seronegative until 16 or 20 weeks of age when two additional birds seroconverted. In a horizontal transmission experiment, a rooster shedding virus

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through semen was placed in a cage flanked by cages with seronegative chickens, which remained virus-negative over at least a two-month-period (29). Significant differences in seroconversion rates were noted among genetic strains (27), but even between different generations of the same genetic strains, seroconversion ranged from close to 0–95% (176). Schat and Schukken (227) followed seroconversion of SPF flocks in a completely closed environment during an eight-year period and found remarkable fluctuations in the percent seroconversions from flock to flock. Examination of tissues from embryos obtained from hens positive for viral DNA in the gonads showed that the embryos can carry the viral DNA without signs of virus replication, thus continuing the transmission cycle (173). These data strongly support the hypothesis, first proposed by McNulty (160), that CIAV can establish a latent infection (177, 225).

Incubation Period

In experimental infections, anemia and distinct histologic lesions can first be detected at eight days after parenteral inoculation of virus. Clinical signs generally develop after 10 to 14 days, and mortality begins at 12 to 14 days after inoculation (89, 255, 338). Clinical signs are delayed and milder after oral inoculation compared to intramuscular inoculation (254, 283).

Under field conditions, congenitally infected chicks show clinical signs and increased mortality beginning at 10 to 12 days of age, with a peak at 17 to 24 days (35, 67, 86, 124). In heavily infected flocks, there can be a second peak of mortality at 30 to 34 days (67, 124), probably due to horizontal infection.

Clinical Signs

The only specific sign of CIAV infection is anemia, with a peak at 14 to 16 days PI. Anemia is characterized by hematocrit values ranging from 6–27%. Affected birds are depressed and become pale. Weight gain is depressed between 10 and 20 days after experimental infection. Affected birds may die between 12 and 28 days PI. If mortality occurs, it generally does not exceed 30%. Surviving chicks completely recover from depression and anemia by 20–28 days PI (87, 221, 256, 300, 338), although retarded recovery and increased mortality may be associated with secondary bacterial or viral infections. Secondary infections, causing more severe clinical signs, are frequently seen in field cases, but they may also occur inadvertently in experimental chicks (66, 86, 291, 300).

Hematology

In general, hematocrit values greater than 27% are considered normal, but values may vary between inbred lines of chickens (116). Normal values are lower in white leghorn chicks than in broilers and decrease in both types of birds with increasing age (79, 80, 83). Blood of severely affected chicks is more or less watery, the clotting time is increased, and the blood plasma is paler than normal. Hematocrit values begin to drop below 27% at eight to 10 days PI, are mostly in the range of 10–20% at 14 to 20 days, and may drop to 6% in moribund birds. In convalescent chicks, hematocrit values increase after 16 to 21 days and return to normal (29–35%) by 28 to 32 days PI (89, 105, 222, 255, 338).

Low hematocrit values in CIAV-infected chickens are due to a pancytopenia as a consequence of infection of hemocytoblasts as early as three to four days PI (3, 241, 255), resulting in markedly decreased numbers of erythrocytes, white blood cells, and thrombocytes. Anisocytosis has been noticed as early as eight days PI. Juvenile forms of erythrocytes, granulocytes, and thrombocytes begin to appear in the peripheral blood by 16 days PI, and the incidence of immature erythrocytes may exceed 30% several days later. The blood picture in convalescent chicks returns to normal by 40 days (255). Decreased clotting is most likely the direct consequence of thrombocytopenia and may lead to the hemorrhages associated with CIA. Coinfection with IBDV may aggravate the thrombocytopenia (212).

Morbidity and Mortality

The outcome of CIAV infection is influenced by a number of viral, host, and environmental factors. Uncomplicated infectious anemia, especially if caused by horizontal infection, may result in nothing more than slightly increased mortality and transient poor performance of affected flocks, and, therefore, it could even go unobserved in commercial settings. However, subclinical infections with CIAV can aggravate other diseases (see Immunosuppression).

Morbidity and mortality are considerably enhanced if chicks are dually infected with CIAV and Marek's disease virus (MDV), reticuloendotheliosis virus (REV), or IBDV, probably due to virus-induced immunosuppression (39, 197, 221, 300, 301, 337). Because lymphocytic depletion of the bursa often precedes lymphocytic depletion of the thymus associated with CIAV infection in commercial chickens (248, 276), immunosuppression by other viral agents, such as IBDV, likely plays an important role in the outcome of CIAV infection in commercial flocks. Certain strains of reovirus also can be immunosuppressive in chickens (66, 234), which may explain the enhanced pathogenicity of CIAV in the presence of reovirus (66). Dual infections between *Cryptosporidium baileyi* and CIAV can enhance CIA as well as cryptosporidiosis under experimental conditions (104). Occasional outbreaks of disease due to concurrent infections have been reported in commercial flocks (53).

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Pathology

Lesions associated with CIA may vary dependent on the route of infection, age of exposure, viral dose, and immune status of the host. Moreover, CIAV infection may often be involved in diseases caused by other pathogens and can be complicated by other pathogens. The pathology will be described for uncomplicated infections mostly based on experimental infections, as part of the hemorrhagic-aplastic anemia syndrome, and as a complicating factor in other diseases.

Gross Lesions

Thymic atrophy (Figure 8.2), sometimes resulting in an almost complete absence of thymic lobes, is the most consistent lesion especially when chicks develop age resistance to anemia (87, 119, 240, 255). The thymic remnants may have a dark reddish color. Bone marrow atrophy is the most characteristic lesion seen and is best evaluated in the femur (89, 255). Affected bone marrow becomes fatty and yellowish or pink (Figure 8.3). In some instances, its color appears dark red, although distinct lesions can be detected by histologic examination. Bursal atrophy is less commonly associated with CIAV infection. In a small proportion of birds, the size of the cloacal bursa (bursa of Fabricius) may be reduced. In many cases, the outer bursal wall appears translucent, so plicae become visible. Hemorrhages in the proventricular mucosa and subcutaneous and muscular hemorrhages are sometimes associated with severe anemia (87, 89, 148, 222, 255, 256, 300). More pronounced hemorrhages or bursal atrophy, and lesions in other tissues, for example, swollen and mottled livers (89, 222) have also been reported but may be caused by secondary infections with other agents.

Hemorrhagic-Aplastic Anemia Syndrome

Outbreaks of infectious anemia in field flocks are mostly associated with the so-called hemorrhagic syndrome, with or without concurrent (gangrenous) dermatitis (Figure 8.4) (e.g., 18, 35, 54, 67, 291, 333). CIAV is also

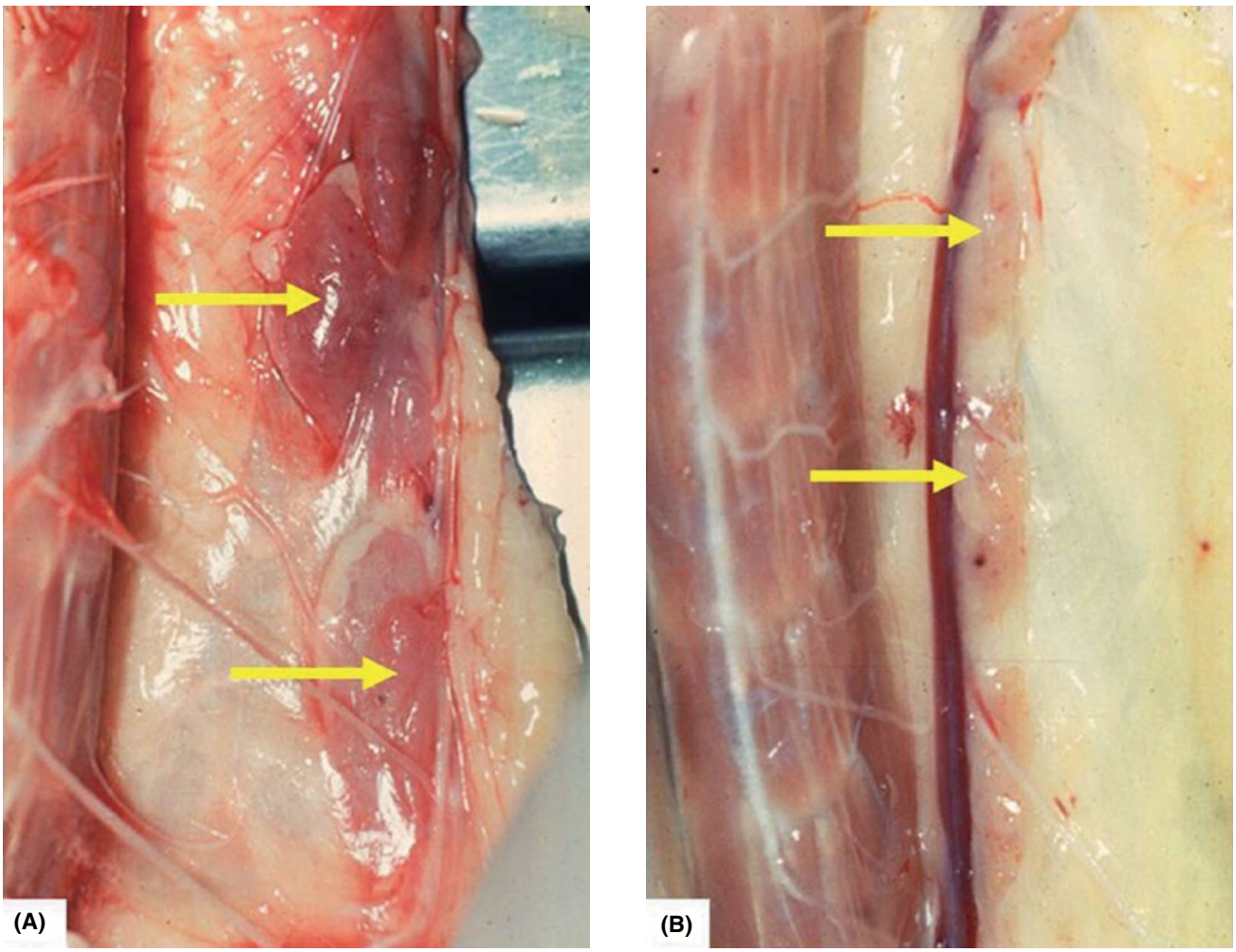


Figure 8.2 Thymus atrophy in chickens infected with CIAV. (A) Control thymus. (B) Thymus with chicken CIAV–induced atrophy, 14 days PI with the CIA-1 strain of CIAV. (B. Lucio and L. Hu)

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Figure 8.3 Femur with normal dark red bone marrow (top) and femur with pale aplastic bone marrow (bottom), 14 days PI with the CIA-1 strain of CIAV. (B. Lucio and H.L. Shivaprasad)

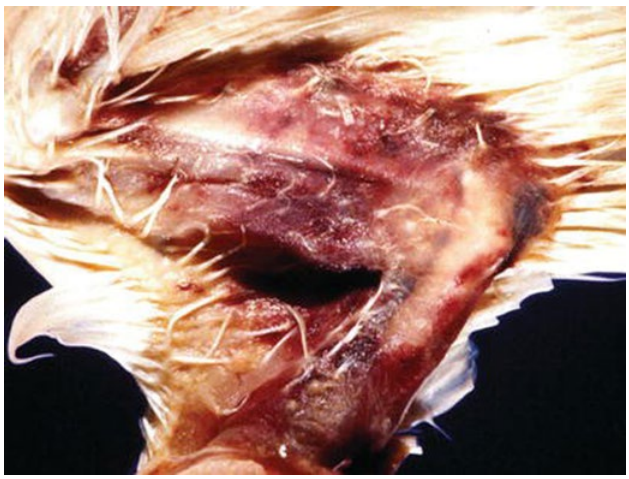


Figure 8.4 Lesions in chickens associated with chicken infectious anemia and hemorrhagic anemia disease. Gangrenous dermatitis (blue wing disease). (Cornell University collection)

involved in the etiology of aplastic anemia associated with inclusion body hepatitis (IBH) (301) and with the IBH/hydropericardium syndrome or infectious bursal disease (IBD) (212). Hemorrhages seen in chickens with IBD may, in most instances, be a sequel of CIAV rather than IBDV infection.

Characteristic lesions of so-called hemorrhagic syndrome are intracutaneous, subcutaneous, and intramuscular hemorrhages (Figures 8.5 and 8.6). Punctuate

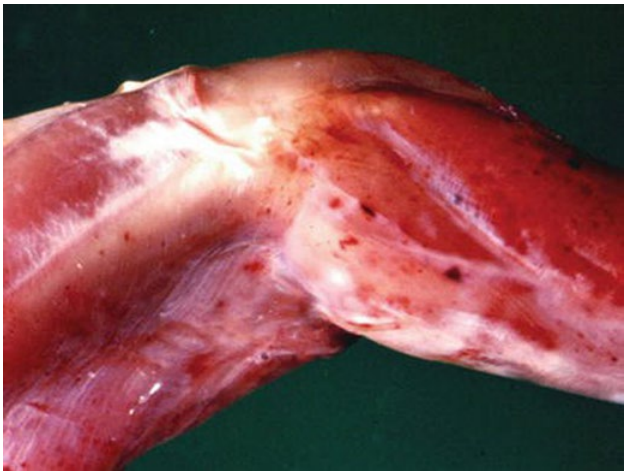


Figure 8.5 Lesions in chickens associated with chicken infectious anemia and hemorrhagic anemia disease. Hemorrhages in thigh and leg muscles. (Cornell University collection)

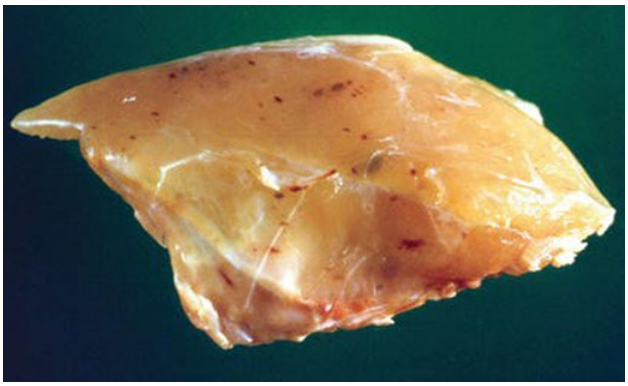


Figure 8.6 Lesions in chickens associated with chicken infectious anemia and hemorrhagic anemia disease. Hemorrhages in breast muscle. (Cornell University collection)

hemorrhages may be present even more frequently in the mucosa of the distal part of the proventriculus (Figure 8.7). Intracutaneous or subcutaneous hemorrhages of the wings are often complicated by severe edema and subsequent dermatitis, which may become gangrenous due to bacterial infection (67). Subcutaneous hemorrhage of shanks and feet may result in formation of ulcers. Affected chicks also sometimes appear to be predisposed to develop pododermatitis.

Hemorrhages are not consistently seen in anemic chicks, although their occurrence is mostly correlated with the severity of anemia. Increased clotting time associated with thrombocytopenia, therefore, does not completely explain hemorrhages. Endothelial lesions and impaired liver functions, partly caused by viral infection and enhanced by secondary bacterial infection, are likely to be more important in the pathogenesis of hemorrhagic diathesis.

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Figure 8.7 Lesions in chickens associated with chicken infectious anemia and hemorrhagic anemia disease. Hemorrhages in proventriculus. (Cornell University collection)

Microscopic Pathology

Histopathologic changes in anemic chicks have been characterized as panmyelophthisis and generalized lymphoid atrophy (89, 120, 148, 255, 256, 298, 300). In the bone marrow, atrophy and aplasia involve all compartments and hematopoietic lineages (Figure 8.8). Necrosis of residual small cell foci may occasionally be seen. Hematopoietic cells are replaced by adipose tissue or proliferating stroma cells. Regenerative areas consisting of proerythroblasts appear 16–18 days after experimental infection, and there is hyperplasia of bone marrow between 24 and 32 days PI in birds that recover.

Severe lymphoid depletion is seen in the thymus, starting with the cortical lymphocytes, but the non-lymphoid leukocytes and stroma cells are not affected. The thymus cortex and medulla become equally atrophic, with hydropic degeneration of residual cells and occasional necrotic foci (Figure 8.9). In chicks that recover, repopulation of the thymus with lymphocytes becomes distinct at 20–24 days, and the morphology returns to normal by 32–36 days PI.

Lesions in the cloacal bursa may be present. These lesions consist of mild to severe atrophy of the lymphoid follicles with occasional small necrotic foci, infolded epithelium, hydropic epithelial degeneration, and proliferation of reticular cells. Repopulation of lymphocytes until complete recovery is similar to that in the thymus.

In the spleen, depletion of T cells with hyperplasia of reticular cells is seen in the lymphoid follicles as well as in the Schweigger-Seidl sheaths. Necrotic foci in follicles or sheaths have been observed rarely.

In the liver, kidneys, lungs, proventriculus, duodenum, and cecal tonsils, lymphoid foci are depleted of cells, making them smaller and less dense than those in unaffected birds. Liver cells are sometimes swollen, and hepatic sinusoids may be dilated.

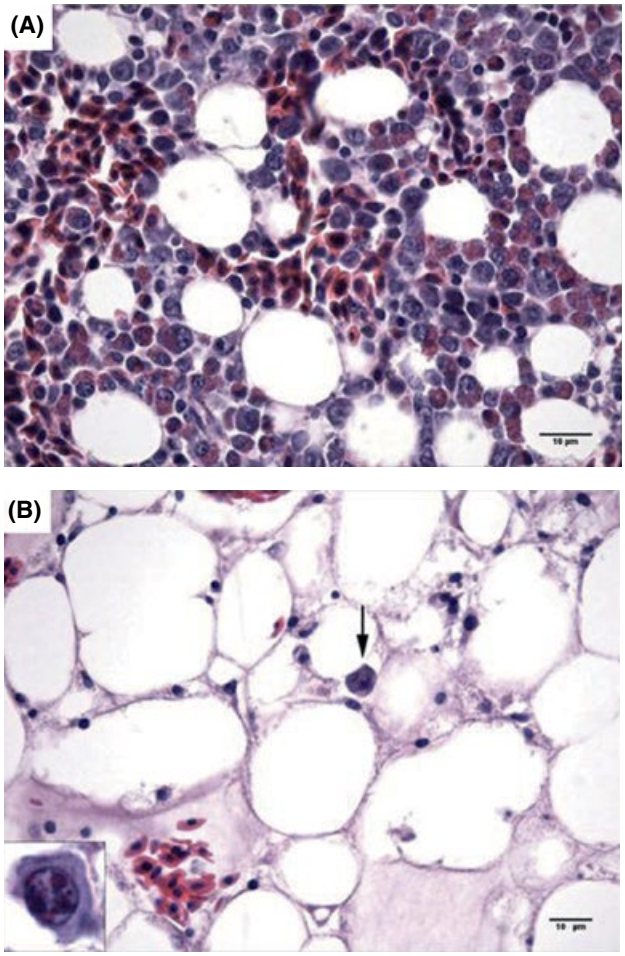


Figure 8.8 (A) Normal femoral bone marrow from a 4-week-old broiler chicken. H & E, bar = 10 microns. (B) Femoral bone marrow from a 4-week-old broiler chicken with CIAV lesions. Note atrophy of hematopoietic and myeloid tissue and presence of fat cells. Few cells have small intranuclear inclusion bodies (arrow) and insert (×1400). H & E, bar = 100 microns. (O. Fletcher)

Small eosinophilic nuclear inclusions have been detected in altered, enlarged cells of affected tissues, predominantly in the thymus and bone marrow, where they are most frequent at five to seven days after experimental infection (89, 241).

Ultrastructural Lesions

Few ultrastructural studies have been described for CIAV-infected chicks (85, 89, 90, 121). Changes in hematopoietic cells and thymocytes were first observed at six days PI and were most advanced at eight days PI. The affected cells had electron-dense regions in the cytoplasm and inclusion bodies consisting of homogeneous or fine granular materials. In addition, irregular plasma membranes, vacuolization, and pseudopod formation were seen. Between days 12 and 16 PI, many degenerative cells were seen, as well

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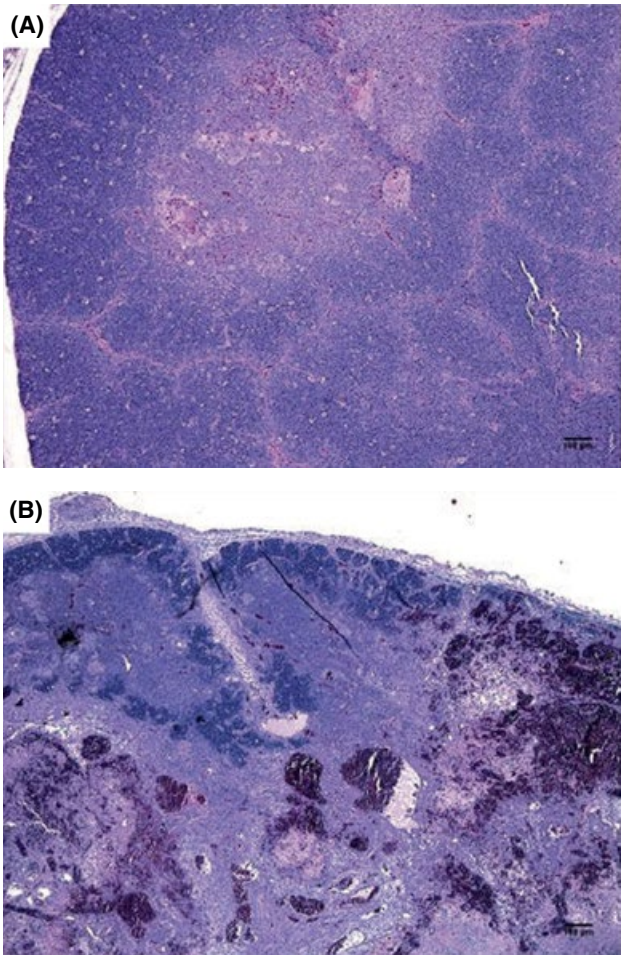


Figure 8.9 (A) Normal thymus from a five-week-old broiler chicken. H & E, bar = 100 microns. (B) Thymus from a five-week-old broiler chicken with CIAV lesions. Note the large region of hemorrhage and necrosis resulting in absence of demarcation between medulla and cortex. H & E, bar = 100 microns. (O. Fletcher)

as actively phagocytizing cells. Apoptotic bodies were present in infected thymocytes. After 20 days, regeneration started to occur.

Pathogenesis

The basic events during the pathogenesis of CIAV infection have been elucidated by sequential histopathologic (89, 240, 241, 255), ultrastructural (85, 90, 121) and immunocytochemical studies (103, 240, 241). Hemocytoblasts in the bone marrow and lymphoblasts in the thymus cortex are primarily involved in early cytolytic infection at six to eight days PI leading to a rapid depletion by apoptosis of these cells. Besides enlarged proerythroblasts and degenerating hematopoietic cells, macrophages with ingested degenerated hematopoietic cells have been observed in the bone marrow. Depletion of lymphoid cells and occasional necrosis in the cloacal bursa, spleen, and lymphoid foci of other tissues have not been detected before 10 to 12 days PI (89, 241, 255, 300). Repopulation of the thymus with lymphocytes, repopulation of the bone marrow with proerythroblasts and promyelocytes, and recovery of hematopoietic activity, beginning 16 days PI all appear to coincide with the beginning of antibody formation (see Immunity). These events result in complete recovery by 32–36 days.

Treatment of formalin-fixed thymus tissues with proteases III or XIV, needed to unmask viral antigens (159), facilitated immunocytochemical studies (4, 103, 106, 241). Large numbers of cortical thymic lymphoblasts become virus positive within four to six days PI. In addition, intrasinusoidal and extrasinusoidal hemocytoblasts, reticular cells in the bone marrow, and mature T cells in the spleen can be virus antigen positive. Infected cells in the thymus and bone marrow are most abundant at six to seven days PI and can be detected until 10 to 12 days or even later. Viral antigen has also been demonstrated in lymphoid tissues in many other organs (241). Infection of proventriculus, ascending part of the duodenum, kidney, and lung could provide an explanation for virus shedding. Infected cells in these tissues usually cannot be detected for more than 22 days after infection at one day of age (241), although virus may persist in tissues until 28 days and in rectal contents until 49 days or later (336). CIAV also can persist in neoplastic lymphocytic infiltrations caused by MDV (8).

Although CIAV has a tropism for lymphoid tissue, particularly for the thymus cortex (120), susceptibility of thymocytes or spleen cells to infection is not dependent on the expression of particular cell markers such as CD4 or CD8 (4, 120). Transient severe depletion of $CD4^+$ and $CD8^+$ lymphocytes, or a selective decrease in cytotoxic T lymphocytes (CTL), may play an important role in the mechanism of CIAV-induced immunosuppression (4, 39, 106, 120).

Age Resistance

Under experimental conditions, age resistance to anemia develops rapidly during the first week of life and becomes complete by three weeks or even earlier in immunologically competent chickens. The degree of resistance may vary based on the virulence of the virus, dose, and route of infection (87, 222, 274, 330, 337). Development of age resistance is closely associated with the ability of the chicken to produce antibodies against the virus (330, 337). Chickens infected at six weeks of age with high doses of CIAV rapidly develop neutralizing antibodies and do not shed virus, whereas chickens infected with a lower dose require more time to develop detectable antibody and do shed virus (58). Antibody development is considerably delayed by immunosuppression, for example, by simultaneous infection with IBDV (111, 221, 337) or by bursectomy (105, 331). Dual infection with IBDV increases the persistence of CIAV in blood cells of

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chickens infected at six weeks of age and prolongs viral shedding (111).

The finding that embryonally bursectomized chickens develop severe anemia when challenged at five weeks of age (105) emphasizes the ability to produce antibodies as an important mechanism of age resistance. In contrast to age resistance to anemia, numerous studies have demonstrated a lack of age resistance to the subclinical disease characterized by lymphocytic depletion of the thymus and spleen, and reduced cellular immune responses (96, 153, 154, 215, 220, 240, 274, 310). Inoculation of six-week-old chickens with relatively low doses of CIAV (10^4 tissue culture infective doses [$TCID_{50}$]) via the ocular route resulted in virus replication in thymus, spleen, and liver, and subclinical disease resulting in reduced weight gain (126).

Although most CIAV experiments have been conducted in leghorn chickens, Joiner et al. (122) showed that broilers also exhibit age resistance to clinical disease and have low viral loads under experimental conditions. However, under commercial conditions, anemia in association with CIAV infection and accompanied by bacterial and parasitic diseases indicative of immunosuppression is observed in chickens up to 130 days of age (47, 277, 308). This suggests that the concept of age resistance may not always be valid in commercial operations, possibly due to environmental factors or other pathogens that affect the immune function of the flocks.

Route of Infection and Virus Dose

Virus dosage affects the severity of anemia or the proportion of affected chicks, but infection can be established by intramuscular injection of doses as low as $10^{0.75}$ $TCID_{50}$ (163, 221, 338). The route of inoculation also plays a role in experimental infection because infection by contact usually does not cause anemia in immunologically intact chicks, in contrast to immunologically compromised birds (221, 337). Oral, nasal, or ocular infection routes are much less effective than parenteral inoculation in inducing disease (221, 254, 283, 325).

Genetic Resistance

There is little information on genetic resistance to infection and disease. Hu (107) suggested that S13 (MHC: $B^{13}B^{13}$) chicks seemed to be more susceptible to disease than N2a (MHC: $B^{21}B^{21}$), and P2a (MHC: $B^{19}B^{19}$) chicks. This observation is compatible with the finding that S13 chickens have a poor seroconversion rate after natural exposure and after vaccination with a commercial vaccine using an adjuvant. S13 chickens had only a 73% seroconversion seven weeks post vaccination, while the N2a and P2a strains were 100% and 85% seropositive, respectively (27). In an experiment designed to detect MHC influences on CIAV susceptibility in four-week-old broiler chickens, Joiner et al. (122) found no statistically significant differences among MHC types in seroconversion rates and viral loads two weeks PI.

Immunity

Active Immunity

Antibody responses are the major arm of protective immunity to CIAV, but neutralizing antibodies cannot be detected until three weeks PI of susceptible one-day-old chicks. Titers are low (1:80) and show little increase (1:320) until four weeks. Chickens inoculated intramuscularly at two to six weeks of age have a faster response with neutralizing antibody detectable as early as four to seven days and with maximum titers (1:1280–1:5120) at 12 to 14 days PI (58, 332, 336). Humoral antibody formation is delayed if chickens are infected orally rather than intramuscularly (254, 283). Yuasa et al. (336) reported that increasing antibody production coincides with decreasing virus concentrations in chicken tissues. However, comparing the levels of antibody detectable by ELISA at 14 days PI among individual chickens inoculated at four weeks of age, Joiner et al. (122) found that higher virus levels corresponded to higher antibody levels, suggesting that higher antibody levels were a result of greater stimulation by virus.

Seroconversion in horizontally infected breeder flocks may be detected as early as eight to nine weeks of age, and most flocks have antibodies to CIAV at 18–24 weeks (112). High titers of neutralizing antibody persist in all birds of a flock for at least 52 weeks. The prevalence of antibodies detected by indirect immunofluorescence assays, however, may decrease with increasing age (112, 162) and is frequently less than 100% in a flock (81, 162). Antibodies detected by a commercial ELISA kit will remain present until 60 to 80 weeks of age in CIAV-infected SPF flocks (27, 227). There is no information on the importance of cell-mediated and non-specific immunity, although Hu et al. (105) noted that some embryonally bursectomized birds recovered from anemia in the absence of antibodies.

Passive Immunity

Maternal antibodies provide complete protection of young chicks against CIAV-induced anemia (334). This protection can be abrogated if chicks are immunosuppressed by other factors, including viral infections, especially infections such as IBDV that affect humoral immune responses (221, 298). Maternally derived immunity, including protection against experimental challenge, persists for about three weeks (162, 199). Furthermore, vertical transmission of the virus is unlikely to occur from antibody-positive hens, but viral DNA can still be transmitted (21, 28, 93, 173). Outbreaks of infectious anemia in the field are often correlated with the absence of anti-CIAV antibody in the respective parent flocks (35, 65, 291, 333).

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Immunosuppression

Impairment of the immune response by CIAV infection may result directly from damage to hematopoietic and lymphopoietic tissues and subsequent generalized lymphoid depletion and perhaps also from cytokine imbalances. Splenocytes from experimentally infected one- to seven-day-old chicks had depressed responses to mitogen stimulation between 7 to 15 days but not at 18–21 days PI (5, 19, 195, 200). Depressed mitogen responses were also noted between 14 and 21 days after oral infection of three-week-old chickens (154). Decreases in macrophage functions such as Fc receptor expression, interleukin (IL)-1 production, phagocytosis, and bactericidal activity were noted after infection of one-day-old and three-week-old chicks (154, 155). Although transient, effects on macrophage function persisted longer than effects on T cell mitogen responses, up to six weeks PI (154, 155). Interferon (IFN) production by mitogen-stimulated splenic lymphocytes *in vitro* was increased at eight days and decreased between 15 and 29 days PI (5, 6, 154). IFN-γ mRNA levels were also increased in the spleens and peripheral blood of CIAV-infected chickens five, seven, or ten days PI in some (153, 310, 311), but not all (77), studies. Although IFN-γ mRNA levels in the spleen subsequently decreased, at 14 days PI they were not reduced compared to uninfected control chickens (153). T cell growth factor production (presumed to be IL-2) after *in vitro* stimulation was also decreased between 14 and 21 days PI (154). Similarly, a decrease in levels of IL-2 mRNA in spleens and peripheral blood of infected chickens was detected in some (310, 311), but not all (153), studies. CIAV infection interfered with the increase in both IFN-α and IFN-γ mRNA levels in blood cells induced within four hours in response to vaccination with trivalent inactivated IBDV/NDV/IBV vaccine (215). This marked effect on early innate responses was found one, two, and three weeks PI in chickens infected at four weeks of age. However, chickens exhibit an innate immune response to CIAV infection; four days PI of one-day-old chicks, IFN-α and IFN-β mRNA expression was increased in the thymus (77). Markowski-Grimsrud and Schat (153) found that CIAV infection significantly reduced the development of antigen-specific CTL for MDV and REV in chickens infected with CIAV after three weeks of age, suggesting that CIAV can impact vaccinal immunity and recovery from infections when CMI responses are important.

CIAV as a Cofactor in Other Diseases

Based on the impact of clinical or subclinical CIAV infection on specific and non-specific immune responses, it is not surprising that infection has been linked to increased susceptibility to other pathogens. Immunosuppression in anemic CIAV-infected birds has been linked to increased bacterial and fungal infections (86, 216, 256, 300, 304) and to enhanced pathogenicity of adenovirus (273, 301), reovirus (64), and infectious bronchitis virus (IBV) (276, 287). Experimental infection with CIAV reduced CD4+/CD8+ T cell ratios and suppressed generation of IBV-specific IgA-secreting cells in Harderian glands, delayed development of IBV-specific IgA in tears, prolonged respiratory signs, and delayed IBV clearance in IBV-infected chickens (282, 287). Genetic characterization of IBV during serial passages of IBV vaccine in CIAV-infected chickens provided experimental evidence supporting the assumption that CIAV-induced immunosuppression allows persistence of the vaccine virus in flocks, and can lead to establishment of a presumably more fit IBV vaccine subpopulation in vaccinated flocks (74). Experimental infection with CIAV and *Salmonella enterica* subspecies enteric Serovar Enteritidis resulted in a decrease in the number of gut-associated T cells, IgA^+ cells and the level of intestinal *Salmonella*-specific IgA compared to chickens infected with *Salmonella* alone. However, there was no significant increase of *Salmonella* positive cells in dually infected birds (235). Experimental CIAV infection increased mortality and severity of tracheal and air sac lesions in *Mycoplasma gallisepticum*-infected SPF broiler chickens (214). Experimental infection with CIAV seven days prior to inoculation of *Plasmodium juxtanucleare* resulted in increased levels of parasitemia and higher mortality rates in young chicks (237). Hagood et al. (92) found a significant association between both presence of CIAV DNA and thymic atrophy and coccidiosis, gangrenous dermatitis, or respiratory disease in commercial broiler flocks. De Boer et al. (49) used live attenuated ND LaSota-type vaccine in one- and ten-day-old chicks, which were infected at one day of age with CIAV. Severe respiratory distress was noted in the dually infected chicks without affecting the HI titers against ND virus. Impaired humoral immune response to inactivated ND vaccine may occur (20, 40) but is not a usual phenomenon in commercial flocks (82). Toro et al. (275) demonstrated an exacerbating effect of CIAV infection on bursal atrophy resulting from IBDV infection in commercial chickens under experimental conditions. Commercial broiler chickens with maternal antibodies to both CIAV and IBDV, and vaccinated against IBDV *in ovo*, when exposed to CIAV and IBDV in drinking water under experimental conditions, exhibited bursal atrophy at an earlier age (20 days of age) and higher IBDV levels in cloacal bursae at 20 days of age compared to chickens exposed to IBDV alone.

Dual infections with CIAV and MDV have led to increased early mortality and increased incidence of MD (71, 196, 299, 340). A high proportion of MDV isolates from 14- to 24-week-old layers exhibiting acute MDV infection also contain CIAV, and inoculation of CIAV into commercial chickens reactivates latent MDV infections

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(71). Two factors may influence the degree of interactions. Infection with a CIAV enhanced the incidence of lymphoproliferative MD lesions when low doses of MDV were given, while CIAV decreased the incidence of MD lesions induced by high doses of MDV (118). The virulence of the MDV strain may also influence the outcome of dual infections. Miles et al. (172) found that coinfection with CIAV and very virulent (vv)MDV strains exacerbated the mortality and thymus atrophy, but that this was less evident with vv+MDV strains. Haridy et al. (95, 97) examined interactions between virulent (v)MDV and CIAV and vvMDV and CIAV. SPF chickens were inoculated with MDV at one day of age and CIAV at four weeks of age, to mimic effects of CIAV infection in commercial flocks occurring after maternal antibody has decayed. The CIAV challenge of MDV-infected chickens increased mortality compared to infection with MDV alone, although these differences did not reach statistical significance. CIAV infection increased the severity of lymphoproliferative lesions in sciatic nerves of chickens infected with vMDV, but not vvMDV, and also did not increase severity of lymphoproliferative lesions in most other tissues. Regeneration of thymus and spleen was negatively impacted by the combination of vMDV and CIAV. This was not observed in birds challenged with vvMDV and CIAV, probably because vvMDV alone already causes severe depletion of lymphocytes in these organs. Depletion of CD4+ and CD8+ cells was also observed in the spleen and thymus after dual infection with MDV and CIAV, supporting the finding that CIAV can deplete CTL after dual infections (153). MDV vaccinal immunity is depressed by CIAV infection even if infection occurs at 14 days of age (195, 200, 329) and, based on the ablation of MDV-specific CTL responses (153) probably at later times as well.

Diagnosis

Isolation and Identification of CIAV

Detailed procedures for the isolation and identification of CIAV have been published (242). Virus can be isolated from most tissues, buffy coat cells, and rectal contents from diseased chickens with maximum virus titers detected at seven days PI (242). Virus titers will decrease after antibodies develop, but whole blood, buffy coat cells, and thymic homogenates were found to be infectious for at least 14 days PI, even from birds with neutralizing antibodies (283, 299, 327).

Lymphocytes from the spleen or buffy coat are preferred sources for virus isolation. Clarified homogenates can be heated for five minutes at 70°C (87) or treated with chloroform to eliminate or inactivate possible contaminants before inoculating cell cultures.

MDCC-CU147 or MSB-1 cell cultures are preferred for virus isolation and titrations (25, 328, 335). Some CIAV strains do not readily replicate in MSB-1 cells and differences in susceptibility of MSB-1 sublines have been reported (218). Freshly prepared cultures containing 2×10^5 cells/mL and seeded at 10^5 cells/cm^2 should be used. Cells are inoculated with 0.1 mL/1 mL of culture with 1 : 20 or greater dilutions (or serial 10-fold dilutions) of appropriately prepared tissue homogenates. Cultures are split every two to four days for 10 passages or until cell death is observed. Microscopic examination of cultures between 36 and 48 hours after passage is recommended to distinguish between virus-induced cytopathic effects (Figure 8.10) and nonspecific cell degeneration. Isolation of CIAV should be verified by CIAV-specific antibody or PCR analysis and sequencing.

Bioassay by intramuscular or intraabdominal inoculation of susceptible one-day-old chicks is the most sensitive method available for primary isolation of CIAV. This approach can be used if CIAV is suspected, but virus was not isolated in cell culture. The bioassay is as much as 100-fold more sensitive than cell culture and the sensitivity can be further increased by bursectomy (105, 331). Between 14 and 21 days PI hematocrit values are examined; values below 27% are considered indicative of the presence of CIAV (148, 222). Postmortem examination for bone marrow atrophy can be used in the case of non-anemic birds. Confirmation that CIAV is present in the lesions by PCR or immunohistochemistry is important.

DNA-Based Detection of CIAV

Polymerase Chain Reaction-Based Techniques

PCR assays have become the assay of choice for the detection of CIAV DNA in infected cell cultures, chicken tissues, archived formalin-fixed paraffin-embedded tissues, or vaccines (59, 192, 247, 259, 260, 270). PCR assays have proven to be specific and much more sensitive than cell-culture isolation of the virus and facilitate sequence analysis. The potential presence of CIAV in vaccines is an important concern for vaccine manufacturers. The European Pharmacopoeia requires avian viral vaccines to be free of adventitious agents based on testing in embryonating hen eggs, cell cultures, and two-week-old SPF chicks. The move away from *in vivo* tests has led to the developments of standardized PCR assays (11, 134, 201). The USDA Center for Veterinary Biologics has published official guidelines for the use of a CAV-specific PCR test (12), which are expected to be updated in the near future.

Very high sensitivity is achieved with a nested PCR, which, however, is also most sensitive to cross-contamination (28, 247). Loop-mediated isothermal amplification assays have been developed for a number of pathogens including CIAV (108). This technique is

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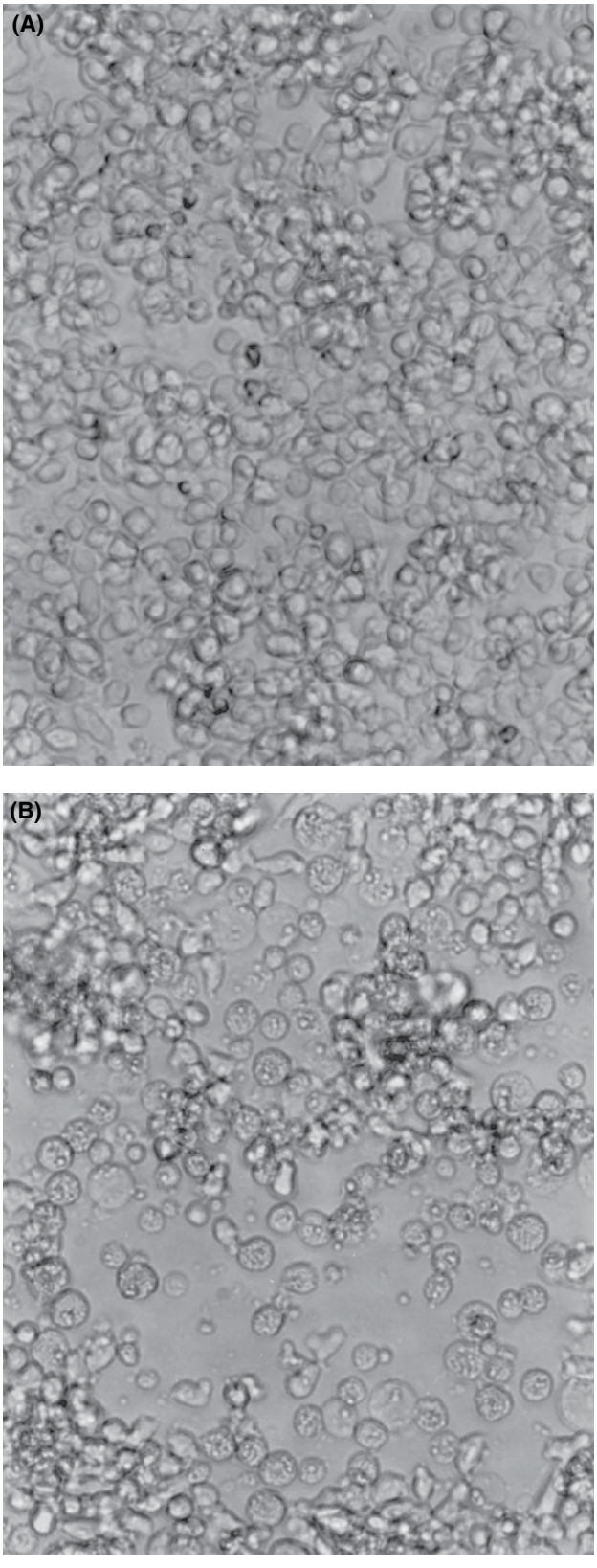


Figure 8.10 Cytopathic effects in cultured MSB-1 cells 2 days PI with CIAV. (A) Uninfected cells. (B) Cells infected with a high dose of virus. Unstained. ×230. (V. von Bülow)

highly sensitive, does not require a thermocycler, and positive samples can be detected by observing color change during the reaction. Real-time PCR assays for the quantitation of viral DNA and RNA have also been developed (e.g., 152, 284) and have replaced the conventional PCR in many laboratories. For routine assays primers are best selected from the conserved ORF regions. DNA can be extracted from the same tissues as used for virus isolation. Miller et al. (173) used nested PCR to screen embryonal tissues and egg membranes obtained after hatching to analyze the presence of viral DNA in offspring of SPF hens. *In situ* PCR assays have been used to detect CIAV-infected cells in the absence of detectable levels of VP3 (28).

DNA Probes

DNA probes for the detection of CIAV DNA in formalin-fixed, paraffin-embedded tissues have been described (10, 58, 183) and, although seldom used for diagnosis, can be of interest if historical samples are available. The use of DNA probes for the detection of CIAV by dot-blot hybridization, has been reviewed (228), but such probes are no longer used.

Detection of CIAV by Antibodies

Detection of CIAV using indirect immunohistochemistry or immunofluorescence is a standard approach in pathogenesis studies and has been reviewed in the 12th edition of *Diseases of Poultry* (228).

Electron Microscopy

Electron microscopy for routine diagnostic examination for CIAV is not recommended due to its lack of sensitivity.

Serology

Three serological assays are routinely used: ELISA-based assays, indirect immunofluorescence assays, and virus neutralization (VN) tests. Selection of a given test depends on the purpose of the serological examination and costs associated with each assay.

Enzyme Immunoassays

Various commercially available enzyme-linked immunosorbent assay (ELISA) techniques for the detection and measurement of CIAV antibodies in chicken sera have been developed and are routinely used to screen breeder flocks in countries where vaccines are available (65), but false-positive responses have been reported (171). Antigens are generally prepared from partially purified virus preparations grown in MSB-1 cells, which may include MDV antigens. Recombinant technology has been

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used to produce VP3 as a fusion protein in bacterial systems (203) or VP1, VP2, and VP3 in baculovirus systems (115). VP3 and VP2, but not VP1, proteins could be used as ELISA antigens. Unfortunately, these antigens will not detect VN antibodies. However, Todd et al. (269) developed a blocking ELISA using a mAb, 2A9, that reacts with field isolates from different parts of the world and recognizes a VN epitope (166, 268). A similar assay, using the same neutralizing mAb, but an Australian CIAV isolate as antigen, has also been developed (257). The blocking assays have advantages in terms of costs (269) compared to the indirect assay described earlier (268) and yield fewer false positive results than commercial indirect ELISA kits that do not use a blocking format (257).

Indirect Fluorescent Antibody (IFA) Tests

The IFA test (162, 297, 332) for the detection of antibodies is a standard IFA test. Although it has been largely replaced by ELISA tests, it is still a useful assay especially as a follow-up test to confirm ELISA-positive results for SPF chickens. CIAV-infected MSB-1 or CU147 cells are used as the source of antigen. Cells are collected just before the beginning of cell lysis, usually 36–42 hours after inoculation, placed on glass slides, and acetone fixed. Fluorescent staining of rather small, irregularly shaped granules in the nucleus of enlarged cells (Figure 8.11) is considered evidence for antibody in the test serum. The concurrent appearance of fluorescent, somewhat irregular circular structures is also specific, but less frequent. This pattern of immunofluorescence is considered typical of tests with neutralizing CIAV antibody (22, 166, 268). Positive and negative reference sera should always be included in FA tests. Uninfected cells need to be used as a control, because sera may have antibodies against MDV and CU147 and MSB-1 cells can express MDV antigens in the nucleus and cytoplasm. Nonspecific staining and background staining masking specific reactions can be largely reduced by using sufficiently diluted test sera, that is, 1:40 to 1:100 or even more (162). Non-specific staining due to direct binding of anti-IgG conjugates (149) may be prevented by selection of pretested conjugates.

Virus-Neutralization Tests

The VN test (297, 336) is considered the gold standard for the detection of serum and yolk antibodies. Micro-well plates are recommended if large numbers of sera have to be examined (113, 123). It may take up to five weeks, requiring eight to nine subcultures, before the assay is completed; however, results can be obtained much earlier, and subcultures can be omitted, if the virus concentration in the mixture is increased to $10^{5.0}$ to $10^{5.5}$ $TCID_{50}$/0.1 mL (198, 295, 297). In this instance, inoculated cultures should be examined microscopically for CIAV-specific CPE after two and three days. One subculture may be required if complete destruction of virus-control cultures

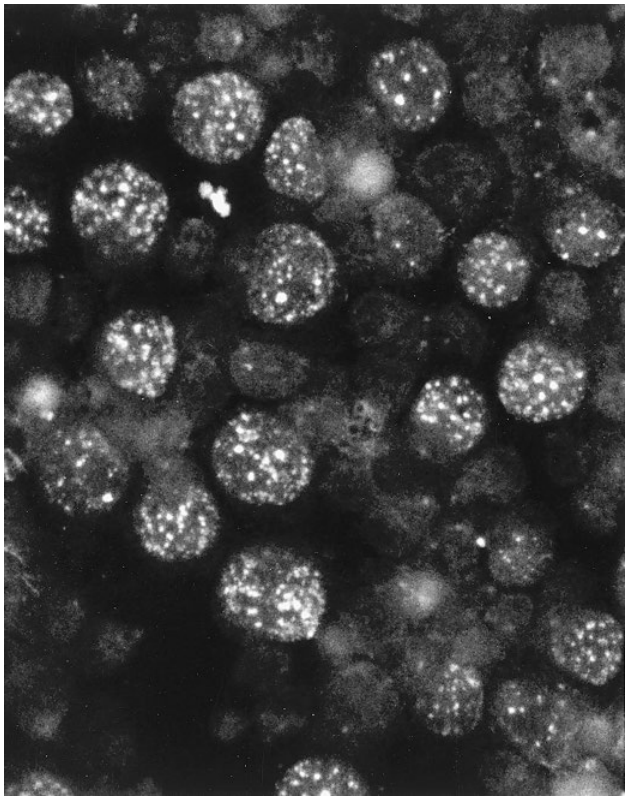


Figure 8.11 CIAV antigens detected by immunofluorescent staining in cytospin preparations of MSB-1 cells harvested at 40 hours PI. Antigens are seen in enlarged cells with characteristic intranuclear granular fluorescence. ×400. (V. von Bülow)

is desired to establish the endpoint. Subcultures can also be omitted if virus replication is assessed by PCR three to four days PI (284).

Qualitative VN tests for flock screening can be made with a constant serum dilution of 1:80–1:100 and a high dose of test virus as described in the previous paragraph. Lower serum dilutions are not recommended because they can occasionally be cytotoxic or cause nonspecific inhibition of the virus. This type of test can be rendered semi-quantitative by making a series of subcultures; the relative antibody level is indicated by the number of subcultures in which the inoculated cells stay alive (295, 297).

Direct comparisons between an ELISA test, IFA assay, and VN test were made by Otaki et al. (198). The VN test was more sensitive than the other two assays and the IFA assay frequently gave false positive results, especially when sera were tested at dilutions of less than 1:50. Unfortunately, no reports compare commercial ELISA kits with VN assays.

Differential Diagnosis

Infection criteria have only limited value in diagnosis of CIAV-induced disease, because CIAV is virtually ubiquitous among chickens. Demonstration of the virus, viral

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antigens, or viral DNA may be considered etiologically significant if detected at sufficiently high levels in a high proportion of affected birds. In chickens under six weeks of age, a typical combination of signs, hematologic changes, gross and microscopic lesions, and flock history are suggestive of CIA. However, no particular lesions can be considered pathognomonic.

Aplastic anemia, but not a pancytopenia, with a concurrent atrophy of thymus and cloacal bursa, and depressed immune response also can be caused by osteopetrosis virus. Anemia induced by erythroblastosis virus can be distinguished from CIAV-induced anemia by microscopic examination of blood smears. MDV can cause severe atrophy of the thymus and cloacal bursa, especially after infection with very virulent viruses (24, 172). IBDV induces atrophy of lymphoid tissues with typical histologic lesions but normally does not affect the thymus. MDV and IBDV normally do not cause anemia, although anemia has been described with some strains of MDV (76). Aplastic anemia that may be associated with acute IBDV occurs and disappears much earlier than CIAV-induced anemia (193). Adenovirus is a major cause of an inclusion body hepatitis-aplastic anemia syndrome that occurs most frequently between 5 and 10 weeks of age (41). It does not, however, induce aplastic anemia after a single infection of experimental chickens.

Intoxication with high doses of sulfonamides, or mycotoxins such as aflatoxin, can result in aplastic anemia and "hemorrhagic syndrome." Aflatoxin also may impair the immune system. In the field, however, chickens are rarely exposed to doses of aflatoxin or sulfonamides that are sufficient to cause acute disease. On the other hand, subclinical intoxication of chickens might add to the pathogenicity of CIAV or *vice versa*.

Intervention Strategies

Management Procedures

Attention should be paid to management and hygiene procedures to prevent immunosuppression by environmental factors or other infectious diseases and to prevent early exposure to CIAV. Improved hygiene has reduced seroconversion rates, but this may cause problems when flocks are exposed later in life (65, 125, 156). Eradication of CIAV is virtually impossible under field conditions and may be difficult on infected SPF premises. In the latter case, this is not only because of the high resistance of CIAV to disinfection, but also because viral DNA can be transmitted vertically, which may be reactivated during the laying cycle (28, 173). Monitoring of breeder flocks for the presence of CIAV antibody should be done to avoid vertically transmitted disease outbreaks or to test the efficacy of vaccinations.

The survey of poultry veterinarians indicated that breeder flocks are frequently vaccinated following the recommendations of the vaccine manufacturer. In some instances, the producers rely on natural exposure. In addition management procedures are recommended. The effectiveness of strict hygiene and biosecurity may be beneficial but is probably not very effective at the production level in many parts of the world (Schat, personal observations).

The potential to develop transgenic chickens expressing short-hairpin (sh) RNAs targeting CIAV VP transcripts may provide a method to develop SPF flocks resistant to CIAV. *In vitro* assays have shown that these shRNAs can decrease CIAV replication in MSB-1 cells (100).

Vaccination

Current vaccination strategies are based on the prevention of vertical and horizontal transmission of virus to very young chicks by immunization of breeder flocks and have been successful in reducing the incidence of anemia in young chicks (65). Artificial exposure of young breeder flocks was originally achieved by transfer of litter from CIAV-infected flocks or by providing drinking water containing CIAV-positive tissue homogenate. This method is still used in countries where vaccines are not available or where vaccines are not applied for economic reasons. However, these procedures are very risky with regard to hygiene and level of exposure and should be discouraged (291). Commercial live vaccines for pullets are available in several countries (290, 292, 293). Vaccination should be performed at about 9 to 15 weeks of age, but never later than three to four weeks before the first collection of hatching eggs to avoid the hazard of vaccine virus spread through the egg. Vaccines can be applied in the drinking water or by injection. Based on the negative effect of CIAV on the generation of CTL when infection occurs after maternal antibodies have disappeared (153) vaccination for broilers may also be necessary.

Several strategies utilizing various combinations of natural exposure, monitoring for seroconversion, and vaccination of breeders are actually used in the broiler industry. Smith (238) surveyed 68 complexes of eight, large, vertically-integrated, broiler production companies across the United States to determine which strategies are most commonly used for broiler breeders. He found that the majority of operations relied on natural exposure. Half of the operations did not routinely test for seroconversion, but some of those operations vaccinated breeders in new or cleaned houses where natural exposure might not occur. Approximately one-third of the operations relying on natural exposure did routinely test for seroconversion and subsequently applied commercial vaccines to flocks that did not exhibit adequate

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seroconversion. Smith noted the relatively high cost of commercial live CIAV vaccines as the reason for reliance on natural exposure. However, approximately one-third of the complexes surveyed did routinely vaccinate all breeder pullets between 10 and 12 weeks of age. Males are not usually vaccinated.

Although the live vaccines are considered safe and efficacious, further studies are needed to examine potential deregulation of immune responses as a consequence of vaccination. Vaziry et al. (289) showed that inoculation of one-day-old SPF chickens with a vaccine licensed for pullets resulted in persistence of the vaccine virus in the thymus and spleen in some birds, altering the thymopoiesis and inducing a low antibody response to CIAV. Currently, all live CIAV vaccines are intended for use in parent and grandparent flocks for vaccination of chickens older than eight weeks of age. Thus biosecurity measures must be employed to prevent infection before vaccination.

The paucity of information on the stability of current vaccines and the effects of vaccination on immune responses have led to several approaches to develop safer vaccines. Inactivated vaccines have been tested in SPF breeder hens. Vaccinated hens showed seroconversion and their offspring were protected against challenge (202, 345). Unfortunately, viral titers in MSB-1 cells and embryos are generally low (160) and therefore inactivated vaccines may not be cost-effective. Kaffashi et al. (126) evaluated CIAV with specific mutations in VP2. One of the mutants (E186G) caused minimal lesions and protected against challenge. However, the stability of this VP2 mutant needs to be established, especially in light of spontaneous mutations found in cell cultures infected with another VP2 mutant (R129G). Schat et al. (226) inoculated one-day-old SPF chickens with antigen–antibody complexes and showed that specific combinations of antigen and antibody did induce a protective response against challenge with a different strain. However, additional studies need to be conducted in maternal antibody-positive chicks to determine if this approach can be used. Based on the work by Koch et al. (131) and Noteborn et al. (188) indicating that co-expression of VP1 and VP2 is required for induction of neutralizing antibodies, recombinant vaccines expressing VP1 and VP2 are possible. Moeini et al. (178, 179) inoculated two-week-old SPF chickens with plasmids co-expressing VP1 and VP2 or VP1 alone. Inoculation with plasmids expressing VP1 + VP2, but not VP1 alone resulted in the induction of neutralizing antibodies and a Th1 response. Linkage of the MDV VP22 coding sequence to the CIAV VP1 gene significantly increased the titers of neutralizing antibodies. Sawant et al. (224) also examined the immune response of SPF chickens inoculated with plasmids expressing VP1 + VP2 and found both antibody and CMI responses were increased when recombinant high mobility group box 1 protein produced in bacteria was included as an immunomodulating adjuvant. However, protection of chickens against challenge with CIAV was not evaluated in any of the plasmid vaccination studies. Shen et al. (236) demonstrated antibody and Th1 responses as well as protection against reduction in hematocrit following CIAV challenge when SPF chickens were vaccinated with recombinant VP1 lacking its amino-terminal portion along with recombinant pigeon gamma interferon, both produced in *Escherichia coli*. In this case it was not necessary to synthesize VP1 in the presence of VP2 in order to elicit a protective immune response. Moeini et al. (180) also showed that *Lactobacillus acidophilus* displaying purified VP1, which was synthesized in *E. coli* as a fusion protein with a cell wall binding domain in the presence of VP2, can be potentially used as an oral vaccine although the antibody titers were rather low (180). Finally, a plant-based vaccine may be possible. *Nicotiana benthamiana* transfected with VP1 and VP2 coding sequences did express these proteins, although experimental inoculation of chickens was not reported (135).

Treatment

No specific treatment for chickens affected by CIAV infection is available. Treatment with broad-spectrum antibiotics to control bacterial infections usually associated with CIA might be indicated. The potential for herbal immunomodulatory and hematinic supplements to ameliorate the immunosuppressive, anemia, and growth suppressive effects of CIAV infection has been investigated (16, 132, 138).

Circovirus Infections in Commercial Flocks

Karel A. Schat and Vicky L. van Santen

Circovirus infections in ducks (DuCV), geese (GoCV), and pigeons (PiCV), are briefly described in the following sections because these viruses affect commercially important species. Detailed information on the economic significance, history, etiology, morphology, and virus replication of *Circovirus* infections in free-living and domesticated avian species can be found in the section Circovirus Infections of Avian Species by Woods and Latimer in Chapter 8 of *Diseases of Poultry*, 13th edition. (315).

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Circoviruses have an ambisense, single-stranded, covalently-closed, circular DNA genome. The genomes of circoviruses code for three major proteins. The three major open reading frames (ORFs) are referred to as V1/ORF1/*rep*, C1/ORF2/*cap*, and C3/*ORF3*. ORF1 is located on the viral strand, while the other two are located on the complimentary strand. ORF3 is located within ORF1, but on the opposite strand. ORF1 codes for the replication initiator protein (REP) and ORF2 codes for the capsid (CAP) protein (reviewed in 315). DuCV ORF3 codes for VP3, an apoptosis-inducing protein similar to VP3 of porcine circovirus 2 (317). PiCV, DuCV, and GoCV have a worldwide distribution causing economic losses mostly associated with immunosuppression (reviewed in 315).

Duck and Goose Circovirus

Etiology

DuCV was identified in Germany by PCR from a mulard duck with a feathering problem and poor body condition. The sequence of this isolate resembled the typical sequence of circoviruses and numerous typical circovirus virions were present in the cloacal bursa (98). Shortly afterwards, DuCV was detected in Muscovy, mulard, and Pekin ducks in Hungary (72) and Taiwan (34), and in commercial Pekin ducks in the United States (13). Since then, many DuCV sequences have been added to the databases. Sequence analysis indicates the existence of two distinct lineages: DuCV-1 and DuCV-2 (31, 73, 314, 319, 347). Recently a recombinant DuCV was isolated with sequences from both DuCV-1 and -2 (253).

A closely related virus (GoCV) has been isolated and sequenced from a commercial flock of geese with a runting syndrome (246, 271). Additional sequences were reported from China and Taiwan (33, 323).

Epizootiology and Transmission

Infections with DuCV and GoCV are presumed to be worldwide based on the molecular isolation of DuCV and GoCV from different continents with a duck and/or goose industry. Limited serological data showed that the majority of duck and geese flocks are positive for antibodies against DuCV (146, 147) and GoCV (232). Transmission is likely to occur by the fecal/oral route. However, DuCV has been detected in low percentages in one-day-old ducklings and embryos (143) suggesting the possibility of vertical transmission.

Pathology and Pathogenesis

Infections with DuCV are often associated with feathering problems, low weight gain, and secondary infections with *Riemerella anatipestifer* and *Salmonella enteritidis* (13, 31, 244). Hattermann et al. (98) and Soike et al. (244) reported feather dystrophy especially in the dorsal region with hemorrhagic feather shafts. Histopathological examination showed heterophilic inflammatory infiltration of the follicular and perifollicular tissues of the skin and lymphoid depletion, necrosis, and histiocytosis of the cloacal bursa. Electron microscopic examination of bursal tissue showed the presence of circovirus particles. Immunosuppression as a consequence of DuCV replication in lymphoid cells is the likely cause of the secondary bacterial infections. Subclinical infections without reported secondary infections have also been reported (30). The lack of virus propagation in cell culture has prevented more detailed studies on the pathology and pathogenesis of infection in the absence of secondary infections. Recently, Li et al. (140) injected 10-day-old ducklings with a plasmid containing two copies of DuCV DNA resulting in infection (group 1) or with wild-type virus (group 2). Sera from the two infected groups were positive for DuCV by PCR at 10 and 15 days PI and for antibodies at 15 and 21 days PI for group 2 and 1, respectively. Daily weight gains for groups 1 and 2 were significantly lower than for the uninfected controls. In addition feathering disorders and depression were observed in the two infected groups.

Guo et al. (91) infected 21-day-old geese with a bursa homogenate containing GoCV. Infected geese developed diarrhea between 17 to 30 days PI and a few birds developed feather disorders, while the controls remained healthy. There was also a significantly slower growth rate in the infected geese. Hemorrhages were observed in the liver, lung, heart, and thymus. Histopathological examination revealed severe depletion of lymphocytes and histiocytosis in the cloacal bursa. Apoptotic cells were present in the medulla and cortex of the bursa.

Diagnosis and Intervention Strategies

Isolation of DuCV or GoCV in cell culture has not been reported. Mészáros et al. (170) mentioned isolation in transformed Muscovy duck cell lines, but the qPCR analysis showed the recovery of less virus than was inoculated onto the cultures. The authors did not have positive sera for DuCV and were therefore unable to prove viral replication.

Several molecular techniques including conventional PCR and qPCR (34, 72, 305) and LAMP (318, 340) assays have been reported for DuCV and GoCV. Several serological assays are available for the detection of antibodies against GoCV and DuCV. Scott et al. (232) developed an indirect immunofluorescence assay using baby hamster kidney cells expressing GoCV capsid protein. An indirect enzyme-linked immunosorbent assay (ELISA) test was developed for DuCV by expressing capsid protein in *Escherichia coli* (146). However the yield of protein was

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rather low due to the nuclear localization signal (NLS). Using a truncated CAP gene lacking the NLS improved the capsid protein yield resulting in the development of a reliable ELISA test (147).

There is no treatment or vaccine available to control DuCV or GoCV.

Pigeon Circovirus

Etiology

During the 1980s and 1990s circovirus-like particles were detected in the cloacal bursa of pigeons submitted to diagnostic laboratories in Australia, the United States, and Europe. Sequence analysis confirmed the presence of circovirus with the typical genome structure consisting of the V1/*rep*, C1/*cap*, and C3/*ORF3* genes and stem-loop sequence (reviewed in 315). Currently five clades are recognized based on sequence analysis of a large number of isolates. Frequent mutations and deletions especially in the *cap* gene, as well as recombination are driving the continuous evolution of PiCV (43, 109, 144, 251, 307).

Epizootiology and Transmission

PiCV has a worldwide distribution, which is likely facilitated by the racing industry. Transmission occurs most likely in the rearing loft via the fecal/oral route (reviewed in 315). Air samples obtained from 15 pigeon lofts with PCR-positive pigeons were positive for PiCV providing evidence that aerosol transmission is highly likely (57). The presence of PiCV in embryos, gonads, and semen indicates that vertical transmission is also possible (61, 62, 245). Based on the presence of circovirus inclusion bodies in the third eyelid, transmission through lacrimal fluids may also be possible (109).

Pathology

Cloacal bursal atrophy is the only gross lesion associated with PiCV infection, which may lead to immunosuppression and secondary infections. Lymphoid cell depletion in the cloacal bursa and the presence of botryoid inclusion bodies in bursal macrophages are frequently associated with PiCV infections (reviewed in 315). Apoptosis has been associated with lymphoid cell depletion (1).

The young pigeon disease syndrome (YPDS) occurs mostly in racing pigeons between 4 and 12 weeks of age. YPDS frequently results in high morbidity and mortality. Clinical signs include reluctance to fly, apathy, fluffed feathers, fluid-filled crop, diarrhea, vomiting, and anorexia (217, 233). Although PiCV is most likely a major factor in this multifactorial disease syndrome, experimental infection with PiCV did not result in YPDS or in decreased antibody responses to pigeon paramyxovirus-1 vaccine (229).

Diagnosis and Intervention Strategies

The presence of botryoid inclusion bodies is a strong indication that PiCV is involved in any pigeon disease. Confirmation of the presence of PiCV can be done using PCR, real-time PCR (62), or LAMP assays (279). However, there is no clear correlation between copy numbers and PiCV-induced disease. ELISA assays using *E. coli*-produced capsid antigens are available (136, 252). There are currently no vaccines available. Strict hygiene in the lofts is recommended but unlikely to eliminate the risk of infection.

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9

Adenovirus Infections

Introduction

Scott D. Fitzgerald

Summary

Agents, Infections, and Diseases. Avian adenoviruses fall into three genera, *Aviadenovirus*, *Siadenovirus*, and *Atadenovirus*. The aviadenoviruses affecting fowl are further subdivided into 5 species (A–E) and 11 serotypes; additional species are known in turkeys, geese, ducks, and wild birds. Many of the Aviadenoviruses are subclinical and may only produce disease when birds have other concurrent infections, with the notable exceptions of strains of fowl adenovirus-1 producing gizzard erosions, strains of fowl adenovirus-4 producing hydropericardium syndrome, certain strains of fowl adenovirus species D and E producing inclusion body disease, and strains of fowl adenovirus-1 producing quail bronchitis virus. However, members of the *Siadenovirus* genus (turkey hemorrhagic enteritis virus, marble spleen disease virus), and *Atadenovirus* genus (egg drop syndrome virus) serve as primary pathogens causing disease, mortality, or significantly decreased egg production.

Diagnosis. The current primary method of detection is PCR for molecular detection of hexon genes, but older methodologies including virus isolation on cell cultures, serotyping by virus neutralization, and immunohistochemical staining or *in situ* hybridization are utilized in many laboratories.

Intervention. Vaccines have been developed against the more pathogenic species and strains. Use of antibiotics to reduce secondary bacterial infections in the face of an outbreak is commonly utilized to reduce mortality. Control of other immunosuppressive diseases within flocks is an important management tool as well. Some of the diseases such as egg drop syndrome are primarily spread by vertical transmission, so careful selection of replacement and breeding birds to avoid introducing infection can be a valuable control method.

Adenoviruses are common infectious agents in poultry and wild birds worldwide. Many of the viruses replicate in healthy birds with little or no apparent signs of infection, although they can quickly take on the role of opportunistic pathogens when additional factors, particularly concurrent infections, adversely affect the health of the avian host. Some adenoviruses however (e.g., turkey hemorrhagic enteritis virus, quail bronchitis virus, and egg drop syndrome virus) are primary pathogens in their own right, and others continually turn up in specific disease situations, indicating a degree of guilt by association, although the results of experimental infections to elucidate pathogenic intent have not always been successful.

The first avian adenovirus was isolated in 1949 when material from a case of lumpy skin disease in cattle was inoculated into embryonated chicken eggs (12). Other early unintentional isolates of fowl adenoviruses were the chicken embryo lethal orphan (CELO) isolates made in embryonated eggs (13) and the Gallus adeno-like (GAL) viruses from chicken cell cultures (4). The first isolate of an avian adenovirus from diseased birds was from an outbreak of respiratory disease in bobwhite quail (*Colinus virginianus*) by Olson (11). Human adenoviruses were isolated in 1954 during investigations of respiratory disease (8) and initially were called adenoidal-pharyngeal-conjunctival agents, but the name adenoviruses subsequently was adopted (7).

Most of the viruses replicate readily in avian cell cultures derived from tissues, such as liver or kidney. Replication takes place in the nucleus and is accompanied by the development of intranuclear inclusions, which may aid histopathological diagnosis (9). The general properties required for classifying an isolate as an adenovirus have been defined by the International Committee on Taxonomy of Viruses (ICTV) (3). This report recognized two genera, *Mastadenovirus* and *Aviadenovirus*, within the adenovirus family, with human

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Table 9.1 Classification of adenoviruses.

Genus	Old classification	Species of host affected	Disease examples from this chapter
Mastadenovirus	*Mastadenovirus*	Human, simian, bovine, equine, murine, porcine, ovine, caprine, etc.	• None
Aviadenovirus	Group I avian adenoviruses	Chickens, turkeys, ducks, geese, pigeons, various wild bird species	• Inclusion body hepatitis • Quail bronchitis virus • Hydropericardium syndrome • Gizzard erosions
Siadenovirus	Group II avian adenoviruses	Chickens, turkeys, pheasants, frogs, raptors	• Hemorrhagic enteritis (turkeys) • Marble spleen disease (pheasants) • Splenomegaly (chickens)
Atadenovirus	Group III avian adenoviruses	Ducks, bovine, ovine, deer, possums, snakes	• Egg drop syndrome
Ichtdenovirus		White sturgeon (fish)	• None

adenovirus type 2 and CELO virus as the respective type species (Table 9.1). The aviadenoviruses are serologically distinct from mastadenoviruses (10) and differ also in their genome organization (3). The genus contains most of the characterized adenoviruses isolated from chickens, turkeys, and geese (see Aviadenovirus Infections). These viruses often were previously referred to as group 1 avian adenoviruses in the literature (9).

However, two of the most important adenoviruses causing significant disease in avian species, namely hemorrhagic enteritis (see section on Hemorrhagic Enteritis and Related Infections) and egg drop syndrome virus (see subchapter on Atadenovirus [Egg Drop Syndrome and Related Infections), show substantial differences at the molecular level. Currently, hemorrhagic enteritis (HE) virus, along with the related viruses of marble spleen disease (MSD) virus of pheasants and splenomegaly virus of chickens, and a recently isolated virus from a frog form the genus *Siadenovirus* named to reflect one of their unique genome characteristics, namely the presence of a gene coding for sialidase (5, 6). Egg drop syndrome (EDS) virus, along with certain related adenoviruses isolated from ruminants, marsupials, and reptiles, are now classified as members of the genus *Atadenovirus*, reflecting their high adenine-thymidine (AT) content (1, 2, 3, 6) (Table 9.2). In addition, a recently characterized adenovirus from a fish appears unrelated to currently recognized genera and represents a fifth adenovirus genus *Ichtadenovirus* (1, 3).

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Aviadenovirus Infections

Michael Hess

Summary

Agent, Infection, and Disease. Fowl aviadenoviruses (FAdV) can be separated in 5 different species (A–E) with various genotypes and 12 serotypes. Some geno- or serotypes induce hepatitis–hydropericardium syndrome (HHS), inclusion body hepatitis (IBH), and adenoviral gizzard erosion (AGE). FAdVs are mostly egg-transmitted or spread fecal-orally.

Diagnosis. The preferred diagnostic tests for FAdV-induced diseases include: virus isolation, demonstration of FAdV genome by polymerase chain reaction (PCR), specific gross lesions, and histopathological changes in target tissues (liver [HHS and IBH] or gizzard [AGE]) of clinically-affected birds. Anti-FAdV antibodies can be measured by ELISA (broad cross-detection) or neutralization assay (serotype specific).

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Table 9.2 Members of Adenoviridae affecting birds.[a]

Genus *Aviadenovirus* (abbreviation) [strains/isolates]
Fowl adenovirus *A*
Serotypes: Fowl adenovirus 1 (FAdV-1) [112, CELO, Phelps, QBV, OTE, H1, Fontes, PLA2]
Fowl adenovirus *B*
Serotypes: Fowl adenovirus 5 (FAdV-5) [340, M2, Tipton, TR22]
Fowl adenovirus *C*
Serotypes: Fowl adenovirus 4 (FAdV-4) [341, 506, Da60, H2, J2, K31, K1013, KR5]
Fowl adenovirus 10 (FAdV-10) [C-2B, CFA20, M11, SA2]
Fowl adenovirus *D*
Serotypes: Fowl adenovirus 2 (FAdV-2) [685, GAL-1, H3, IDA1, P7, SR48]
Fowl adenovirus 3 (FAdV-3) [75, H5, SR49]
Fowl adenovirus 9 (FAdV-9) [93, A2]
Fowl adenovirus 11 (FAdV-11) [161, 380, UF71]
Fowl adenovirus *E*
Serotypes: Fowl adenovirus 6 (FAdV-6) [168, CR-119, WDA6]
Fowl adenovirus 7 (FAdV-7) [122, X11, YR36]
Fowl adenovirus 8a (FAdV-8a) [58, T8, TR59]
Fowl adenovirus 8b (FAdV-8b) [764, B3, CFA3]
Duck adenovirus B (DAdV-2) [GR]
Falcon adenovirus A (FaAdV-1)
Goose adenovirus A (GoAdV-1) [P29, N1, 569]
Pigeon adenovirus (PiAdV-1) [IDA4, IDA5]
Turkey adenovirus B (TAdV-1, TAdV-2) [D90/2, T2]
Genus Atadenovirus
Duck adenovirus A (DAdV-1, egg drop syndrome) [127, AAV-2]
Genus Siadenovirus
Raptor adenovirus A (RAdV-1)
Turkey adenovirus A (TAdV-3) [marble spleen disease virus, avian adenovirus splenomegaly virus, turkey hemorrhagic enteritis virus]

[a] Adapted from https://link.springer.com/referenceworkentry/10.1007/978-0-387-95919-1_2

Intervention. Vaccination of breeder hens with inactivated or live vaccines is used in certain areas with occurrence of IBH and HHS.

Introduction

Definition and Synonyms

The genus *Aviadenovirus* within the Adenoviridae family includes the important fowl aviadenoviruses (FAdV), separated into five species (A–E) (39). Aviadenoviruses from geese (GoAdV-A), turkeys (TAdV-B-D), pigeons (PiAdV-A), and ducks (DAdV-B) are compiled in other species. Related viruses, but not yet approved as species, are some viruses from psittacines and parrots. Different to the past recent data strengthen the role of some FAdV strains as primary pathogens, mainly in chickens. This could be demonstrated for some FAdV-1 strains (species A) causing adenoviral gizzard erosion (AGE and some FAdV-4 strains (species C) which play a major role in the etiology of HHS. In addition, other strains, mainly those belonging to species D and E, can cause severe liver damage leading to the condition known as Inclusion Body Hepatitis (IBH). Finally, quail bronchitis (see the subchapter on Quail Bronchitis) is caused by FAdV-1. In recent years different non-virulent FAdVs were modified to be used as viral vectors. Several reviews on aviadenoviruses are available highlighting different subjects, bearing important information on aviadenoviruses together with this chapter in previous editions of *Diseases of Poultry* (9, 10, 18, 40, 51, 71, 74, 114, 121).

Economic Significance

With such variability in disease association, it is very difficult at present to assess the overall economic importance of aviadenoviruses. However, the high mortality noticed in flocks suffering from HHS or IBH and the growth retardation in connection with IBH and AGE, result in very substantial economic losses (114).

Public Health Significance

There is no evidence of productive infection of human cells by aviadenoviruses, and therefore any public health implications are likely to be minimal. However, there is growing interest in the use of FAdV as a gene transfer vehicle for use in humans and possibly other species. Two different viruses belonging to two different serotypes (FAdV-1 and FAdV-9) have been shown to be able to transduce human cell lines without productive replication (17, 73).

History

In the decades following the first report of the isolation of an avian adenovirus in 1949, adenoviruses were isolated from healthy and diseased birds in geographically different regions and typed by cross neutralization tests (40, 71, 74). In the period afterwards it was demonstrated that infectious bursal disease virus (IBDV) and chicken infectious anemia virus (CIAV) were capable of enhancing the virulence of FAdV (27, 137). Later on, field and experimental data demonstrated the primary role of FAdV in certain diseases, arguing to reassess the virulence of FAdV (26, 30, 70, 81, 93, 128).

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Etiology

Classification

Within the Adenoviridae, species designation depends on at least two of a number of key criteria that include calculated phylogenetic distance, restriction enzyme fragmentation, host range, pathogenicity, cross neutralization, and possibility of recombination (39). Within the genus *Aviadenovirus* five species of fowl aviadenoviruses, designated with the letters A–E, are recognized, based largely on molecular criteria in particular sequencing data (Table 9.2). In recent years the complete nucleotide sequences of numerous representatives of all FAdV species and serotypes have been determined, enabling more comprehensive phylogenetic studies (3, 14, 34, 37, 61, 64, 85). Furthermore, the complete sequences of duck, turkey, and pigeon aviadenoviruses were established supporting the classification in different species (45, 62, 63, 132, 144). Nucleotide identity values between *Aviadenovirus* species might be as low as 32% whereas between FAdV species it increases to 75%, with the smaller left and larger right end of the genome being the most variable regions. Finally, FAdVs from diseased birds have been sequenced but no distinctive virulence marker has been determined so far (50, 55, 67, 126, 135, 145). Deletions at the right end of genome were noticed in virulent FAdV-4 from China but the function needs still to be elucidated (50, 98, 143).

Viruses within each species can also be further subdivided into serotypes based largely on the results of cross neutralization tests or into genotypes.

Morphology

Ultrastructure and Symmetry

The virion is composed of 252 capsomeres, surrounding a core 60–65 nm in diameter (107). Capsomeres are arranged in triangular faces with six capsomeres along each edge. There are 240 nonvertex capsomeres (hexons) of 8–9.5 nm diameter and 12 vertex capsomeres (penton bases). Vertex capsomeres carry projections known as fibers. Mammalian adenoviruses have one fiber on each penton base, and FAdV have two. In most cases, both fibers are of similar length as they are transcribed from a single gene. Different to this, members of species FAdV-A and FAdV-C have two fiber genes (61). FAdV-1 possesses two fibers of different lengths (i.e., 46 and 10 nm). How the vertex capsomere is assembled to accommodate two fibers is not fully understood. The fiber proteins are of high importance for virus–cell interaction as demonstrated for FAdV-1 (130). This might also explain the importance of the fiber protein as virulence factor with varying results based upon phylogenetic variations (36, 59, 65, 90, 96). Variations in the number of fiber genes, either one or two, were also reported for aviadenoviruses from turkeys and ducks (62, 63, 144).

Ultrastructural studies demonstrate the accumulation of virus particles in the nucleus of infected cells, and these often form crystalline arrays which differ in morphology and density depending on the content of viral protein and DNA (Figure 9.1). Large intranuclear inclusions are also clearly visible in tissues from infected birds or in infected cell cultures by cytochemical or immunostaining methods (Figure 9.2), and these may be useful in diagnosis of adenovirus infections.

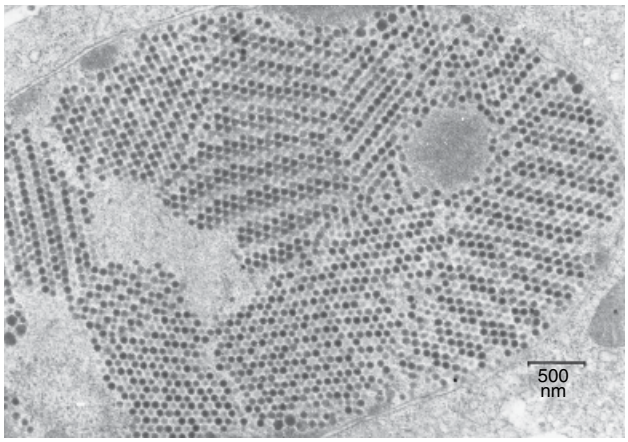


Figure 9.1 Adenovirus-infected chick liver cell culture (48 hours postinfection). Adenovirus particles almost fill the nucleus.

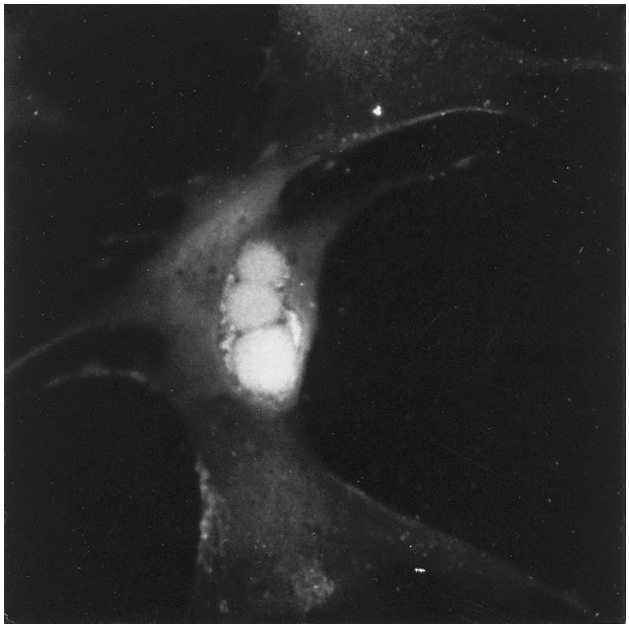


Figure 9.2 Growth of FAdV-8 (764) in chick kidney cell cultures. Intranuclear inclusions stained by direct immunofluorescence.

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Size and Density

The adenovirus virion is a non-enveloped, icosahedral structure 70–90 nm in diameter (107). Densities between 1.32 and 1.37 g/mL in cesium chloride (CsCl) have been estimated for aviadenoviruses. Similar differences in density, which have been attributed to differences in DNA content and base composition in different isolates, have also been found in human adenoviruses.

Chemical Composition

The nucleic acid is double-stranded DNA, which accounts for 17.3% of the FAdV-1 virion, with the remainder being protein (48). The guanine–cytosine (G–C) content of fully sequenced *Aviadenovirus* genomes varies between 45% (GoAdV-4) and 68% (TAdV-1). Between 11 and 14 structural polypeptides have been described for FAdV-1, with hexon as the most abundant one.

Virus Replication

Adenovirus replication is divided into two well-defined phases. The early phase involves the entry of virus into the host cell and the transfer of the virus DNA to the nucleus, which is followed by the transcription and translation of the so-called early (E) genes (39). Afterwards, proteins coded by the early genes redirect cellular functions, in order to facilitate replication of the virus DNA and the consequent transcription and translation of the late (L) genes, coding for the virus structural proteins. Assembly of the viral proteins into complete virions is completed in the nucleus followed by disruption of the nuclear membrane and release of virus by destruction of the cell. Few studies have investigated genes specific for aviadenoviruses, mainly in the interest of developing FAdVs as vectors (18, 22, 49, 139).

Susceptibility to Chemical and Physical Agents

All avian adenoviruses tested so far have shown typical adenovirus properties (74). They are resistant to lipid solvents such as ether and chloroform, sodium deoxycholate, trypsin, 2% phenol, and 50% alcohol. They are resistant to variations in pH between 3 and 9 but are inactivated by a 1:1000 concentration of formaldehyde. They are inhibited by the DNA inhibitors IuDR and BuDR.

Adenoviruses in general are inactivated in aqueous solution after exposure to 56 °C for 30 minutes and heat stability is reduced by divalent ions. FAdVs show more variability and are apparently more heat resistant. Some strains survive 60 °C and even 70 °C for 30 minutes. Although most research has found that divalent cations destabilize adenoviruses, some have found no effect, emphasizing the need to standardize procedures.

Strain Classification

Antigenicity

The adenovirus hexon is the major capsid protein and contains type-, group-, and subgroup-specific antigenic determinants against which antibodies are produced (39, 71). The group-specific determinants are shared by all aviadenoviruses, but are not present in the siadenoviruses or atadenoviruses.

Type-specific determinants give rise to antibodies that neutralize viral infectivity, and, therefore, the neutralization test has been widely used to separate isolates into serotypes (40, 71). The current classification of aviadenoviruses is shown in Table 9.2.

Immunogenicity and Protective Characteristics

Neutralizing antibodies produced against the type-specific epitopes should provide protection, but there are only very limited data available about the contribution of individual epitopes and the duration of protection. Recombinant proteins underline the protective capability of certain epitopes on structural proteins (112, 118).

Molecular Classification

Based upon genomic differences FAdVs are separated into 5 genotypes designated A–E including all 12 serotypes (Table 9.2) (39). Polymerase chain reaction (PCR) has been applied for detection of FAdV for nearly two decades, with primer sequences mainly based on the hexon gene (40, 83, 102, 138, 142). Sequencing of variable regions of the hexon gene can be used to allocate viruses to species A–E and to establish genotypes within species which is now frequently carried out (60, 72, 113). Variation within loop one of the hexon gene can also be used for differentiation of viruses by high resolution melting curve analysis or pyrosequencing (100, 127). However, applying sequences of hexon and fiber gene for typing reveals certain inconsistencies for a limited number of strains (113). The highly conserved 52K gene can be used as target to quantify FAdV by real-time PCR (38).

Laboratory Host Systems

Most chicken isolates have been obtained from chick kidney (CK) or chicken embryo liver (CEL) cells. A chicken Leghorn male hepatoma (LMH) cell line can also be used for isolation and propagation of FAdV (7, 87). Although chicken embryo fibroblasts (CEF) appear to be less sensitive than other cells, adaptation and attenuation of virulent FAdV-4 to a quail fibroblast cell line (QT35) was reported (71, 116).

Great variation exists between different strains of FAdV following embryo propagation and some of them might not induce lesions (19). Yolk sac, in comparison to the allantoic cavity, was found most susceptible reaching

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the highest virus titers. For egg-adapted viruses the chorioallantoic membrane (CAM) might be used. Signs and lesions produced in the embryo were death, stunting, curling, hepatitis, splenomegaly, congestion, and hemorrhage of body parts, with urate accumulations in the kidneys. Hepatocytes usually contained basophilic or eosinophilic intranuclear inclusion bodies (6). No correlation can be found between high embryo mortality and clinical signs in experimentally infected day-old specific pathogen free (SPF) birds.

Aviadenoviruses have been isolated from a variety of bird species. Furthermore, numerous reports describe the presence of FAdV in different species of birds, including psittacines, raptors, and various wild birds. Overall, homologous cell systems are advantageous for isolation (40).

Pathogenicity

Different serotypes, and even strains of the same serotype, can vary in their ability to produce illness and death (113). With some isolates, a relationship has been found between genotype and virulence but not between serotype and virulence. In comparison to other strains FAdV-1 produces a variety of tumors when inoculated into hamsters and transforms human and hamster cells (74).

In many studies, the route of inoculation, the age and type of birds, the virus dose, and the type of virus has been extremely important in producing disease (114). Natural routes of infection induce mortality only in younger birds, whereas parenteral inoculation has to be applied in birds from three weeks of life onwards. Inducing high mortality via oral or eye/nostril infection is a distinctive feature of very virulent FAdV. Coinfection with IBDV or CIAV enhanced the pathogenicity of some FAdV (27, 137). In contrast, presence of an adenovirus-associated parvovirus may reduce the growth of FAdV in cell cultures (74).

Pathobiology and Epidemiology

Incidence and Distribution

FAdV are distributed widely throughout the world, and domestic avian species of all ages are susceptible. Other avian species appear to be susceptible to infection with FAdV as well as their own adenoviruses.

In the last two decades reported viruses inducing IBH in geographically different regions belong mainly to species D or E, predominantly serotypes 2, 8a, 8b, and 11 (114). However, considering the differences in nomenclature, the technology and use of reference viruses in laboratory diagnosis and assignment of certain serotypes could be misleading limiting the comparison between individual studies.

Hepatitis hydropericardium syndrome (HHS) first appeared in 1987 in Pakistan and spread later on not only to numerous Asian countries but also to the Middle East, and Central and South America (9, 51, 114, 120). The disease is similar to IBH and it appears that the only difference between HHS and IBH is that the mortality rate and incidence of HHS is higher. In nearly all studies in which the virus was further characterized it turned out to be a FAdV-4 strain.

Adenoviral gizzard erosion due to FAdV have been reported in the last two decades from Europe, Japan, and Korea (114). In the majority of field outbreaks FAdV-1 was identified as an etiological agent of AGE; exceptionally FAdV-8a and -8b were noticed (66, 89).

Natural and Experimental Hosts

FAdVs are widespread in chicken flocks independent of the vaccination status of breeders (25). The different influences on pathogenicity limit the link between the presence of a certain virus and pathogenicity (41). In addition to infecting chickens, FAdV have been recovered from turkeys, pigeons, psittacines, ostriches, guinea fowl, and a mallard duck.

Particles that resemble adenoviruses have been observed by electron microscopy in thin sections of tissue taken from wild birds or those kept in captivity. Without further characterization of the viruses they can't be attributed to the genus *Aviadenovirus*.

In addition to FAdV, turkeys are also infected with TAdV-1–5, which grow in cells of turkey origin but either do not grow or grow poorly in cells of fowl origin.

Another separate species are goose adenoviruses (GoAdV-1–5) detected and isolated from geese (39). These viruses grow in cells of both goose and fowl origin. Duck aviadenoviruses have been isolated from Muscovy ducks in France and China (11, 144). DAdV were propagated in chicken as well as duck cells and are so far allocated in DAdV-B (Table 9.2). Viruses genetically different to FAdV were isolated in tissue culture or identified by deep sequencing from pigeons (40, 132).

Age of Host Commonly Affected

In case of IBH and HHS usually young chicks are affected, especially broilers up to five weeks of age (114). AGE seems less restricted to appearing at a specific age, although economic consequences seem more substantial in younger birds. Disease in mature birds is rarely reported but some viruses can influence hatchability.

Transmission, Carriers, and Vectors

Vertical transmission is important in the spread of FAdV and, consequently, in induction of disease (133). In the embryonating egg, viral antigens can be detected in egg

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yolk and the albumen and virus is often reactivated in cell cultures prepared from embryos and young chicks taken from infected flocks (71, 109). This has been one of the strongest motivations for establishing SPF flocks as FAdV might remain latent and undetected. In the field it could be demonstrated that infection during rearing with virulent FAdV and high levels of antibodies prevented subsequent vertical transmission (99). Reduced hatchability in breeders following natural or experimental infection affected hatchability for five weeks (31, 133). Cross infections resulting in the mixing of several serotypes can take place when birds from different breeder flocks are brought together.

Horizontal transmission is another route for FAdV to spread, mainly via feces where it survives for weeks. Virus excretion depends on the FAdV strain and the age of birds but intermittent excretion needs to be considered in addition to the applied test system (38). Older birds showed lower peak titer of adenovirus present in the feces with an earlier decline in virus titer, and excretion persisted for a shorter time (91).

Aerial spread between farms does not appear to be important, except when cleaning of depopulated houses takes place and dust is transmitted between farms. Spread by fomites (e.g., egg trays and egg trolleys) and transport can also be important. For transmission of FAdV-4, personnel were identified as an important vector (5). Spread of FAdV-4 as a result of a contaminated vaccine poses an important risk (4, 52).

It is not uncommon to isolate two or even three strains from one bird, suggesting that there is little cross-protection, despite the presence of high levels of neutralizing antibody to a single serotype. There appears to be a second period, around peak egg production, when FAdV are often detected. Presumably, the stress associated with egg production or the increased levels of sex hormones at this time causes reactivation of the virus, ensuring maximum egg transmission to the next generation. Virus is present in feces, the tracheal and nasal mucosa, kidneys, intestine, and cecal tonsils. Therefore, virus could be transmitted in all excretions, but the highest titers are found in feces. Virus may also be present in the semen, presenting a potential risk where artificial insemination is used.

Incubation Period

The incubation period depends on the age of birds and route of infection but can be very short (24–48 hours).

Clinical Signs

In a previous edition of this textbook the isolation of FAdV from birds with varying diseases was described. However, this chapter focuses only on such disease conditions for which an etiological link can be established based upon experimental data (41). Despite this, FAdV should also be considered in multifactorial diseases (21).

IBH is characterized by sudden onset of mortality peaking after 3–4 days and usually stopping on day 5 but occasionally continuing for 2–3 weeks. Morbidity is low; sick birds adopt a crouching position with ruffled feathers and die within 48 hours or recover. Mortality might be only slightly elevated but occasionally it might reach values as high as 30% (114). Higher mortality appears in younger birds less than three weeks of age. IBH is predominantly seen in meat-producing birds and it may start in the first week of life (15, 53, 56, 81). Disease in young birds is most likely caused by vertical transmission, although no clear differentiation between vertical and horizontal introduction can be made.

Experimental studies indicate that immunosuppression induced by IBDV infection appears to facilitate adenoviruses in producing IBH (27, 137). In Korea, FAdV infections with high mortality often coincided with the presence of CAV (15). On the other hand, numerous outbreaks in different parts of the world confirmed the primary etiology of FAdV in causing IBH (8, 16, 30, 82, 128). In India IBH was often associated with the presence of aflatoxins in the feed (125).

Clinical signs of HHS are very much similar to IBH, with the overall disease being much more severe with mortality up to 80% (9, 51, 114, 120). Due to the severity of the disease, some elevated mortality might be noticed in mature birds.

Growth retardation, reduced uniformity, and a higher selection rate are the reported clinical signs in broilers suffering from AGE (23, 31). However, it might also be that the infection is only noticed at slaughter without clinical signs being seen on the farm (92). Increased weekly mortality of 0.2% and reduced laying performance were reported in layers (54, 67). Due to vertical transmission of FAdV-1 hatchability can be reduced (31).

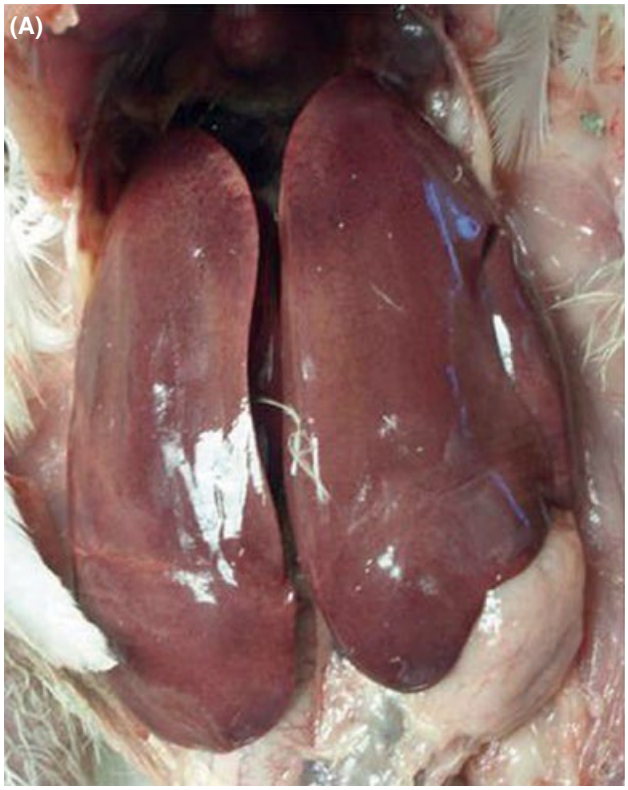
In some experiments, in addition to gizzard erosions, investigators also found pancreatitis, hepatitis, cholecystitis, and cholangitis, indicating infection spread through a number of digestive organs beyond the gizzard (95). High mortality was reported in one experimental study which is contradictory to other reports (23).

Pathology

Gross Lesions

The main lesions of IBH are pale, friable, swollen livers. Small white foci can be seen on the liver and petechial or ecchymotic hemorrhages may be present (Figure 9.3). Swollen kidneys frequently coincide with glomerulonephritis (140). Lesions including atrophy of the bursa and thymus, aplastic bone marrow, and hepatitis have been described in natural outbreaks and experimental

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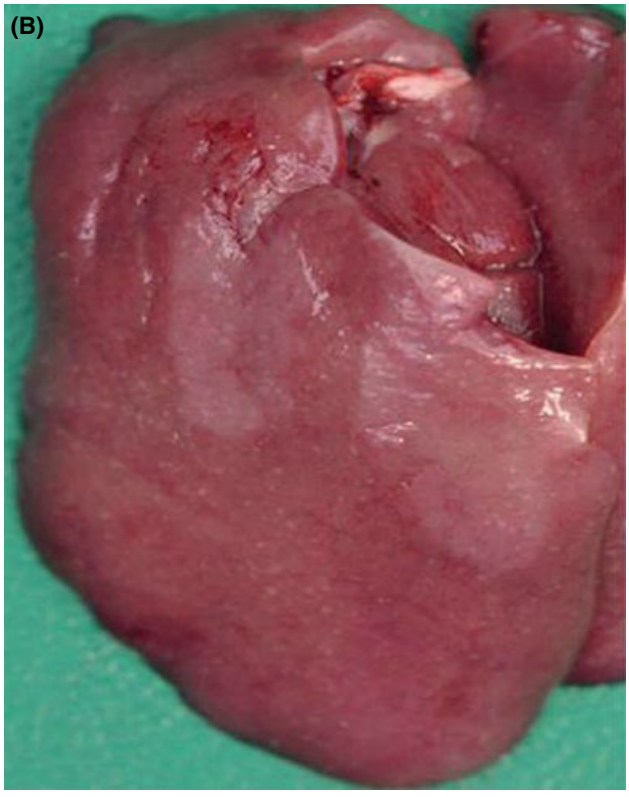


Figure 9.3 Liver from birds showing inclusion body hepatitis, (A) from outbreak in broilers, (B) from experimentally infected specific pathogen free (SPF) birds.

studies (114). Following ocular or oral infection of day-old SPF birds gross lesions peaked between 4–10 days postinfection (69, 129). Broilers are more susceptible with higher mortality due to the severe metabolic imbalance and the heavy destruction of the pancreas in addition to the liver (68).

For HHS, gross lesions in the liver and kidneys are similar to IBH except that they are more severe. In addition, there is an accumulation of clear, straw-colored fluid in the pericardial sac, pulmonary edema, swollen and discolored liver, and enlarged kidneys with distended tubules showing degenerative changes (Figure 9.4) (1, 46, 145). Petechial hemorrhages and focal necrosis are present in the heart and liver (9, 42, 79, 120). Multifocal necrosis in the pancreas is reported in severe outbreaks of HHS (84). Ascites might be noticed in severely infected birds. Effects on lymphatic organs noticed during field outbreaks should consider possible coinfections, although various experimental studies indicate atrophy of thymus and bursa in the case of HHS and IBH (9, 120, 129).

AGE is characterized by distended gizzards with hemorrhagic fluid and multiple black patchy erosions within the koilin layer (Figure 9.5) (2, 23, 31, 115). A number of experimental studies using oral or nasal/ocular inoculation in birds from 1–53 days of age



Figure 9.4 Hepatitis hydropericardium syndrome (HHS) in a specific pathogen free (SPF) chicken three days postinfection following intramuscular infection with virulent FAdV-4 at 21 days.

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Figure 9.5 Experimentally induced gizzard erosion in specific pathogen free (SPF) broilers (M. Hess).

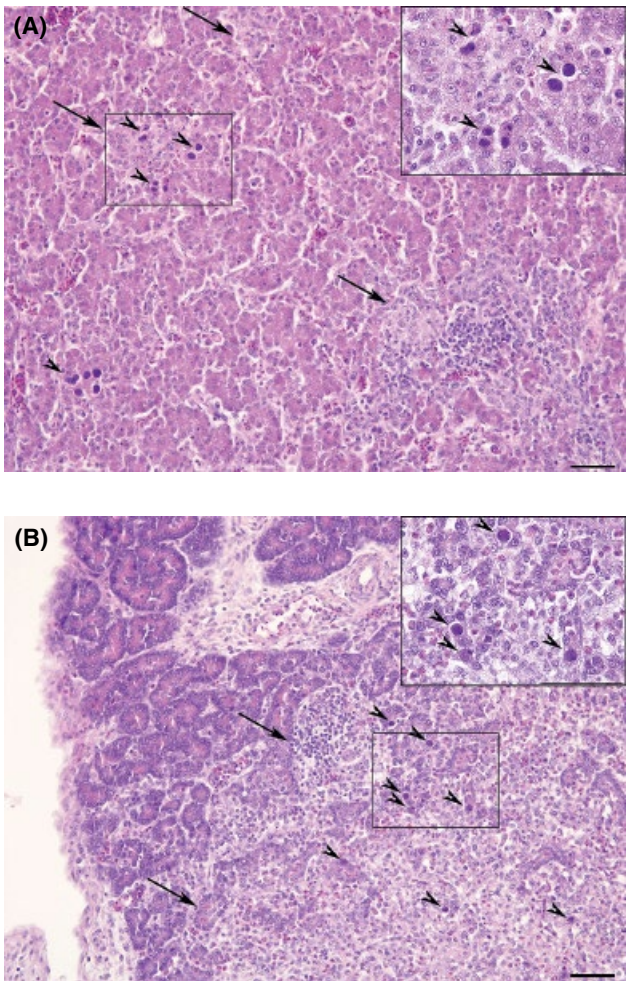


Figure 9.6 Liver (A) and pancreas (B) of a chicken experimentally infected with a fowl aviadenovirus-D strain at seven days postinfection. Arrows indicate areas of necrosis and inflammation with infiltrations of mononuclear cells together with granulocytes. Basophilic intranuclear inclusion bodies are present in hepatocytes of the liver, respectively acinar cells of the pancreas. The inserts represent higher magnifications of the defined selections. Scale bars = 50 µm.

reproduced gizzard erosions from 3–18 days post-inoculation (114). Focal pancreatitis and gizzard erosions have been described in broiler chickens in both natural outbreaks and experimental studies in Japan (89, 95). Virulent FAdV-1 has an affinity for the gizzard which is different to FAdV inducing IBH and HHS and the high load of FAdV-1 in the gizzard coincidences with macroscopic lesions (32, 88). High mortality was reported in one experimental study which is contradictory to other reports (23).

Microscopic Lesions

Intranuclear inclusion bodies are seen in organs with lesions; in cases of IBH and HHS in the liver, pancreas, kidneys, gizzard, and intestine. They are mainly basophilic, large, round, or irregularly shaped with a clear, pale halo, or, occasionally, eosinophilic without virus particles in intranuclear bodies developing at a later stage (Figure 9.6). Glomerulonephritis characterized by an increase in the glomerular area and the average glomerular cell count was noticed during a severe outbreak of IBH (140). Pancreatic necrosis might also be seen and in some cases gizzard erosions are reported (114). Myocarditis and hemorrhages in the heart can be noticed in chickens and ducks who died due to HHS (9, 13, 120). Atrophy of follicles of the bursa of Fabricius and a loss of lymphocytes with hemorrhages can be noticed. Following experimental infection of SPF birds with virulent FAdV-4 severe immunosuppression was induced, with depletion of B and T cells in lymphoid organs (77, 116).

Adenovirus-induced gizzard erosions coincide with intranuclear inclusion bodies containing adenovirus antigen in glandular epithelial cells which are associated with necrosis of the koilin layer, and infiltration of the lamina propria, submucosa, and muscle layers by macrophages and lymphocytes (2, 23, 31, 32, 57, 80, 92, 93, 94, 95). Intranuclear inclusions have also been demonstrated in necrotic pancreatic acinar cells (80, 95, 131). Pancreatitis, hepatitis, cholecystitis, and cholangitis, indicating infection spread through a number of digestive organs beyond the gizzard can be seen (88, 89).

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Aviadenovirus Infection in other Birds

FAdVs were isolated from various bird species, mainly in cases of IBH, indicating a less strict host affiliation. However, clinical relevance is often limited to single case reports similar to adenoviruses which were detected in tissues of birds without being further characterized.

Aviadenovirus Infection in Turkeys

FAdV, TAdV, and less well-defined adenovirus particles are reported in turkeys. However, no evidence has been found in studies investigating the influence of aviadenoviruses on growth performance or any other adverse disease condition, with rare appearances of IBH (124).

Aviadenovirus Infection in Geese and Ducks

GoAdV was isolated during an outbreak of HHS in 8-day-old goslings in two different farms in Hungary (42). The disease lasted for about three weeks and the cumulative mortality reached 6%. DAdV-2 was isolated from 5-week-old Muscovy ducks with increased mortality over a 10-day period (11). DAdV-3 was isolated from diseased ducks and seen to induce lesions in duck embryos as well as hepatitis, myocarditis, and nephritis in experimentally-infected day-old commercial and SPF ducks but only limited changes in SPF chickens were seen (144). The appearance of virulent FAdV-4 in Peking ducks in China led to mortality of up to 20% but virus replication in ducks is less efficient (13, 97).

Aviadenovirus Infection in Pigeons

Except some single cases of IBH earlier reports on specific diseases in pigeons due to an aviadenovirus infection, including PiAdV-1 and -2, were not confirmed by recent studies (40, 132, 136).

HHS has also been seen in pigeons and the virus was typed as FAdV-4 (76). Using liver suspension the disease was reproduced in broilers and Controlled using the poultry vaccine.

Aviadenovirus Infection in Guinea Fowl and Quail

Less well characterized aviadenoviruses were isolated from Guinea fowl with gizzard erosions, ventriculitis, and pancreatitis. FAdV-4 was isolated from quails showing HHS during postmortem and the disease was reproduced in 4-week-old broiler chickens (106).

Aviadenovirus Infection in Ostriches

Adenoviruses have been associated with illness, death, and poor hatchability on ostrich farms. In a study in which 3-day-old ostrich chicks were inoculated with an ostrich-derived FAdV-A isolate, all died (24). Mortality of up to 30% in young ostriches was noticed in China due to HHS (12).

Immunity

Aviadenoviruses have a common group-specific antigen that is distinct from that of siadenoviruses and atadenoviruses (39). Following infection, birds rapidly develop neutralizing (type-specific) antibodies that are detectable after one week and reach peak titers after three weeks.

It has been found that birds were resistant to reinfection with the same serotype 45 days after primary infection, which was also possible after 8 weeks, eliciting a secondary response of both neutralizing and precipitating antibodies. Virus excretion can occur, despite the presence of humoral antibodies, and maternal antibodies do not protect against natural routes of infection. It seems that short-lived, transient, local immunity prevents reinfection while circulating antibodies protect mainly against invasion of the internal organs, with consequences on viral load in tissues (110). With regard to protection, neutralizing antibodies are obviously not solely responsible for protection (112, 117).

Diagnosis

Isolation and Identification of Aviadenoviruses

Specimens of choice for virus isolation are feces, cecal tonsils, pharynx, kidney, and affected organs (e.g., liver, in IBH). A 10% suspension of tissue in medium is prepared and, in the case of FAdV, inoculated onto either chick embryo liver cells (CEL), chick kidney cells (CK), or on LMH cells. If attempting to isolate aviadenoviruses from other avian species, it is preferable to use cells from the species being investigated, although chicken cells can be used (40). Embryonated eggs are insensitive for primary isolation of most aviadenoviruses, although yolk sac or CAM infection was demonstrated for some FAdV isolates, leaving it as an alternative in laboratories lacking cell culture technology.

Confirmation of a virus isolate as an adenovirus can be carried out by electron microscopy. Immunocytochemistry can be used to detect adenoviruses in infected cells by staining with an avian adenovirus antiserum labeled with a fluorescent dye (Figure 9.2). To identify the serotype of a virus that has been isolated, virus neutralization tests with the isolate against standard reference antiserums to all known serotypes has to be applied (40). For FAdV this laborious procedure is now mostly substituted by PCR combined with other procedures using nucleotide differences within the loop one region of the hexon gene to allocate isolates to individual genotypes, which correlate mostly with serotypes (29, 60, 72, 100, 127). Developing primers for other, more conserved, regions allows extension of the PCR beyond aviadenoviruses but prevents further subtyping of FAdV genotypes. Primers that detect individual species or serotypes can also be developed

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(103, 138) and FTA™ papers were reported as an appropriate source for viral DNA (75). Nested and real-time PCRs were found beneficial to increase sensitivity in comparison to virus isolation and can be used for quantification (38, 105). In birds without lesions cecal tonsils should be used to isolate and detect FAdV and liver and pancreas are targeted internal organs (35, 110).

Detection of adenovirus DNA in tissue samples by *in situ* hybridization (ISH) has been reported (40). The widespread occurrence of FAdV highlights the importance of ISH and immunohistochemistry to clarify etiologies, especially for AGE (31, 57, 94). If these techniques are not available, then H&E staining of infected cell monolayers, or tissue sections, and demonstration of intranuclear basophilic inclusions provides a nonspecific indication of the presence of DNA-containing virus (Figure 9.6).

Serology

Hemagglutination is a unique feature of FAdV-1 strains, some of which hemagglutinate rat and/or sheep erythrocytes, therefore hemagglutination inhibition is not applicable (74).

The ELISA or indirect immunofluorescence assay detect group-specific antibodies, considering that results depend on the antigen used (40). Type-specific antibodies normally have been detected using the serum-neutralization test, but ELISA can also be used for this purpose even though presence of multiple serotypes complicate interpretation. Recombinant nonstructural proteins 33K and 100K can be used to differentiate between infected and vaccinated animals (141). Partial hexon protein can be applied to detect homologous antibodies with good sensitivity (44, 101). The presence of humoral antibody gives no indication of the state of local immunity at mucosal surfaces.

Intervention Strategies

Management Procedures

Aviadenoviruses are resistant to inactivation but it is possible to eliminate them from most environmentally-controlled houses with impervious floors and walls that can be made airtight. Because FAdV are effectively transmitted vertically through the embryonated egg, effective control would have to start at primary breeder level. However, experience with SPF flocks has indicated that horizontal spread is also a major problem, and it takes some efforts to keep a commercial flock free from FAdV infection. As both IBDV and CIAV can potentiate the pathogenicity of FAdV the first step must be to control or eliminate these two viruses.

Vaccination

As evidence mounts that certain serotypes/genotypes may be primary pathogens, the possibility of vaccination is more appealing (114). Autogenous vaccines are used in areas without licensed products or to improve coverage of certain strains present in the field. Considering vertical transmission breeders are within the focus of vaccination and a certain antibody level is requested for complete protection of progenies (108). This is further supported by IBH outbreaks in broilers in Australia which occurred despite vaccination of breeders 1–3 times between 9–18 weeks of age, with a live vaccine consisting of FAdV-8b and adequate protection against CAV and IBDV (128). In addition to a FAdV homologous to the vaccine, a heterologous strain was sometimes isolated from diseased birds, indicating a lack of cross-protection. Contrary to this, an inactivated oil-emulsion FAdV-4 vaccine induced a high level of protection against various serotypes, not only in vaccinated/challenged SPF birds but also in broilers originated from vaccinated breeders (47).

Complete protection against IBH could be induced in progenies obtained up to 50 weeks of life of a grandparent stock vaccinating at 10 and 17 weeks of life with an inactivated vaccine containing isolates of species FAdV-D and FAdV-E (8). Using a polyphosphazene in combination with avian beta defensin 2 as adjuvant elicited an antibody response and upregulation of selected cytokines in the spleen even after *in ovo* vaccination of a killed FAdV-8b vaccine (20, 111).

Soon after the first reports about HHS appeared a vaccine prepared by inactivating homogenates of liver from infected birds has been used extensively with apparent success in Pakistan to control the disease (114). Subsequently, vaccines prepared on tissue culture or in embryonated eggs were developed (58, 78). Adaptation of a virulent FAdV-4 isolate to QT35 cells resulted in attenuation and protection of subsequently vaccinated birds (117). The lack of neutralizing antibodies prior to challenge underlines the importance of cellular immunity for protection. One study has shown enhanced protection of progenies against HHS by vaccinating broiler breeders against both CIAV and FAdV-4, compared to lesser protection achieved by vaccinating against either disease alone (134). Structural proteins fiber 2 or penton base of FAdV-4 expressed as recombinant proteins induced a high level of protection against HHS (112, 118). On the other hand, only 40% of protection was achieved with the 100K nonstructural protein (119).

Contrary to IBH and HHS, maternal antibodies seem to be less protective in preventing AGE, as commercial broilers with antibodies, infected within the first week of life, displayed severe lesions (32, 94). A nonvirulent FAdV-1 strain administered as live vaccine orally to

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day-old birds induced complete protection following challenge three weeks later with a virulent field isolate (33).

The genomes of FAdV-1 (CELO), and strains belonging to species FAdV-C and FAdV-E have been shown to have substantial capacity for insertion of foreign DNA sequences, and consequently there is substantial interest in the development of such viruses as a vaccine vector for use in mammalian and avian species (17, 18, 73, 86). Anticancer activity in human cells was demonstrated by incorporating the herpes simplex virus type 1 thymidine kinase (TK) gene into the FAdV-1 genome (122). Experiments in which the VP2 gene of IBDV was incorporated into FAdV genomes demonstrated efficient expression and protection of challenged birds (28, 123). Furthermore, the S1 gene of IBV and chicken interferon gamma were successfully expressed in such vectors (43, 104).

Altogether, these developments reflect the broad range of FAdV, from severe primary pathogens to nonvirulent vectors.

Atadenovirus (Egg Drop Syndrome of Chickens and Related Infections)

Joan A. Smyth

Summary

Agent, Infection, and Disease. Egg drop syndrome of chickens (EDS'76) is an atadenoviral disease affecting the oviduct of laying chickens. The virus, which originated in ducks, is transmitted vertically and laterally and causes production of pale, thin-shelled, and shell-less eggs. Disease has occurred in many countries worldwide, but not in the United States to date.

Diagnosis. Due to the transient presence of diagnostic atadenoviral inclusions and difficulty in identifying acutely affected birds, pathologic evaluation rarely provides definitive diagnosis. Development of virus specific hemagglutination inhibiting antibodies coincident with drop in egg production or shell abnormalities is highly indicative.

Intervention. Vaccination of hens during rear with inactivated vaccine prevents disease.

Introduction

Definition and Synonyms

Egg drop syndrome-76 (EDS) virus was originally designated as the sole member of the subgroup III avian adenoviruses. In 2005 it was reclassified as a member of the genus *Atadenovirus* of the Adenoviridae family (11) (Table 9.1). Its species name is duck atadenovirus A (5), and its strain name is duck adenovirus 1 (DAdV-1) or egg drop syndrome virus (EDSV) (11). The disease should not be confused with a more recently reported condition known as egg drop syndrome of ducks or duck egg drop syndrome, a disease caused by a flavivirus. There are rare reports of DAdV-1 causing respiratory disease in geese (34) and ducks (16, 19).

Economic Significance

While cost analysis figures are not available, outbreaks of EDS in laying hens will cause significant loss of both hatching eggs and eggs produced for human consumption. The disease has been well controlled in many countries for many years, either by vaccination and/or eradication programs. It is critically important that live virus is not introduced into breeding hens of any type, because the virus can be vertically transmitted. To this end, the European Pharmacopoeia, for example, requires that all avian viral vaccines are tested to ensure freedom from EDSV.

Public Health Significance

The virus affects only avian species and, therefore, has no public health significance.

History

Egg drop syndrome of chickens was first described in 1976 (62), and became commonly referred to as EDS'76. Hemagglutinating adenoviruses were isolated (44), and later shown to be the cause of the disease. No antibody to these viruses was found in chickens before 1974 (44), but antibody was common in ducks (9). Egg drop syndrome virus was isolated from normal ducks (18) and showed optimal growth in duck cells, so it was hypothesized in 1977 that this was a duck adenovirus, probably introduced into chickens through a contaminated vaccine (44).

Etiology

Classification

Egg drop syndrome virus is classified as an adenovirus based on its morphology, replication, and chemical composition. The virus is not related to 11 fowl

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adenoviruses, or to duck adenovirus-2 (DAdV-2), using serum neutralization (SN) or hemagglutination inhibition (HI) tests (3, 15). Although the fowl adenovirus group specific antigen is not detected by immunodiffusion or immunofluorescence tests in EDSV preparations, common antigenic determinants were demonstrated in experiments wherein aviadenovirus group-specific antibodies, which had developed in chickens after inoculation with an aviadenovirus and regressed, reappeared following inoculation with EDSV (42).

Sequencing of the EDSV genome revealed significant differences from the subgroup I avian adenoviruses (32), for example, smaller genome size (33.2 kb compared to 43.8 kb for FAdV-1), absence of some early genes that are present in aviadenoviruses, and presence of other genes that have no obvious homology with known aviadenoviruses (32). In turn, the EDSV genome was substantially larger than turkey hemorrhagic enteritis and marble spleen disease viruses (subgroup II avian adenoviruses), Genomically, EDSV is very similar to ovine adenovirus strain 287, goat adenovirus 1, bovine adenoviruses 4, 5, and 8, and a possum adenovirus. These viruses, and the recently discovered Psittacine atadenovirus A, are sufficiently different from other adenoviruses to warrant classification as a separate adenovirus genus. This genus is called *Atadenovirus*, reflecting the high AT content of the viral DNA (11, 12). Although initial isolates of EDSV were from chickens (44), the virus is recognized as having originated from ducks, hence its species name is Duck atadenovirus type A (5), and its strain name is duck adenovirus type 1 (DAdV-1), or EDSV (11). Only one serotype is recognized (see Strain Classification).

Morphology

Ultrastructure

Purified virus particles had typical adenovirus morphology with triangular faces having six capsomeres on the edge and a single 25 nm fiber projecting from each vertex (37) (note, aviadenoviruses have two fibers). Surface structure detail is not obvious in non-purified preparations (3, 43, 65). It is possible to distinguish EDSV particles from the fowl adenoviruses in some electron microscope preparations (Figure 9.7). In ultrathin sections, intranuclear virus particles measured 70–75 nm in chick embryo liver cells (CEL) (3), and 68–80 nm in oviduct (58).

Size and Density

Egg drop syndrome virus particles in negatively stained preparations measured 76–80 nm (43).

Most researchers found multiple bands upon centrifugation of EDSV in cesium chloride, variously between 1.28 and 1.33 g/mL. Infectivity, hemagglutinating ability and particle morphology differed between the bands (37, 57, 59, 65). The discrepancies between studies were explained, at least partially, by Zsak and Kisary (68), who reported that density and hemagglutinating ability of EDSV particles depended upon whether the virus was grown in cell cultures or embryonated eggs and upon the purification method.

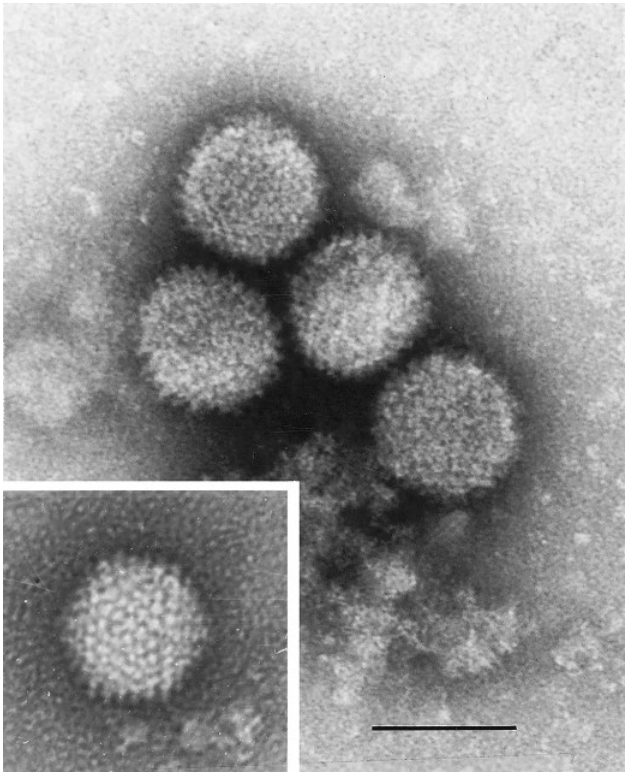


Figure 9.7 Four particles of egg drop syndrome virus (EDSV). Although individual capsomeres are well resolved, typical adenovirus morphology is not apparent. Inset: Fowl adenovirus type 8 (FAdV-8) particle showing well-defined, triangular faces. Scale bar = 80 nm.

Chemical Composition

Labeling with H3-thymidine and inhibition with iododeoxyuridine showed that EDSV contained DNA (3, 37, 59, 65). The molecular weight of the DNA was estimated at 22.6×10^6 d compared with 28.9×10^6 d for FAdV-1 (Phelps) (68). EDSV has 13 structural polypeptides, at least 7 of which correspond with polypeptides of FAdV-1 (59, 67). Egg drop virus syndrome, like other atadenoviruses, has a gene for a unique structural protein (p32k) (11) and an F-box motif which is very rare among viral proteins (14)

Hemagglutination

Egg drop syndrome virus agglutinates erythrocytes of chickens, ducks, turkeys, geese, pigeons, and peacocks but does not agglutinate rat, rabbit, horse, sheep, cattle, goat, or pig erythrocytes (3, 39).

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An initial fourfold fall in hemagglutinin (HA) titer was reported after 16 hours at 56°C, and thereafter the titer remained stable for 4 days; no HA activity was detected after 8 days at 56°C. The HA was destroyed by heating to 70°C for 30 minutes. Hemagglutinin activity was stable for long periods at 4°C (3, 45) and was resistant to treatment with trypsin, 2-mercaptoethanol, ethylenediaminetetraacetic acid, papain, ficin, and 0.5% formaldehyde at 37°C for 1 hour, but the titer was greatly reduced by treatment with potassium periodate and 0.5% glutaraldehyde (57).

Virus Replication

In cell culture, EDSV replicates in the nucleus, producing intranuclear inclusions in a similar fashion to aviadenoviruses (1–3). *In vivo*, EDSV replicates in epithelial cells of the infundibulum, tubular shell gland, pouch shell gland, isthmus, and in nasal mucosa and spleen of experimentally infected hens (55, 58).

Susceptibility to Chemical and Physical Agents

Egg drop syndrome virus was resistant to chloroform and variations in pH between 3 and 10. The virus was inactivated by heating for 30 minutes at 60°C, survived for 3 hours at 56°C, and was stable in the presence of monovalent but not divalent cations (3, 65). Infectivity was not demonstrated after treating with 0.5% formaldehyde or 0.5% glutaraldehyde (57).

Strain Classification

Only one serotype of EDSV has been recognized (25, 65). By restriction endonuclease analysis, four genotypes were recognized (50, 60). One genotype included isolates made over an 11-year period from European chickens, and a second included isolates from ducks in the United Kingdom. A virus isolated from chickens in Australia formed the third genotype and one isolated from a duck in India represented the fourth. A 2004 study of Japanese isolates from chickens showed no evidence of change of the virus after two decades of the virus circulating in chickens (61).

Although all isolates of EDSV from chickens and from ducks in Europe appeared to be of similar virulence (7), isolates from ducks in the United States produced no effect on egg production in chickens (63) or affected only egg size (17).

Laboratory Host Systems

Egg drop syndrome virus replicated to high titers in duck kidney, duck embryo liver, and duck embryo fibroblast cell cultures and grew well in chick embryo liver cells (CEL), less well in chick kidney cells (CK), and poorly in chicken embryo fibroblast cells (CEF) and turkey cells. No replication was detected in a range of mammalian cell cultures (3). The virus grew to high titers in goose cell cultures (68) and also grew very well when inoculated into the allantoic sac of embryonated duck or goose eggs producing HA titers of 1/16,000–1/32,000. No growth was detected in embryonated chicken eggs (3, 67).

Pathobiology and Epizootiology

Incidence and Distribution

Egg drop syndrome virus has been isolated from chickens (and ducks) in many countries worldwide (see earlier editions of *Diseases of Poultry*) but to date however, EDS has not been reported in chickens in the United States or Canada.

Natural and Experimental Hosts

Although disease outbreaks have been recorded mostly in laying hens, it is thought that the natural hosts for EDSV are ducks and geese. Hemagglutinin inhibition (HI) antibody to EDSV is widespread in domesticated ducks and geese, as well as in wild ducks and waterfowl (see earlier editions of *Diseases of Poultry*).

The virus has been isolated from healthy domestic ducks (9, 63), from diseased ducks (6, 16, 27, 29, 51), and it has been suggested that EDSV may cause rough, thin shells and decreased egg production in ducks (27, 38). With the exception of Canadian ducklings with proliferative tracheitis in which intralesional adenoviral inclusions were confirmed (16), it remains possible that the viruses recovered from diseased ducks were incidental, since the virus can be found commonly in healthy ducks.

Infection also was shown to be common in geese (35, 40, 69), but goslings and geese experimentally infected with the virus showed neither illness nor change in egg production (69). However, a severe respiratory disease outbreak was reported in young goslings, in which adenoviral inclusion bodies were found in the trachea and bronchial epithelium of affected birds. Egg drop syndrome virus was recovered from affected geese and the disease was reproduced experimentally (34).

Quail (*Coturnix japonica*) are susceptible to infection and develop classic signs of EDS (26). There is no evidence of naturally occurring infection of pheasants (8, 35). There is one report of naturally occurring infection in turkeys associated with egg production problems (13), but disease was not produced in turkeys experimentally (46). Guinea fowl may be infected naturally or experimentally. Soft-shelled eggs were produced in one experimental study (30), but there was no disease in another study (64).

A wide range of chicken breeds have been shown to be equally susceptible to experimental infection, and

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chickens of all ages are susceptible to EDSV infection. Introduction of EDSV into a laying site affected egg production in all ages of laying hens. The appearance of disease at around peak egg production (45) may be due to reactivation of latent virus.

Antibody to EDSV has been detected in some species of free living birds, for example, owls and house sparrows (see earlier editions of *Diseases of Poultry*).

Transmission, Carriers, and Vectors

Egg drop syndrome outbreaks could be divided into three types; that is, classical, enzootic (or endemic), and sporadic. In the initially observed classic form of the disease, primary breeders were infected, and the main method of spread was vertically through the embryonating egg (43). Although the number of infected embryos was probably low (10), later lateral spread of virus was efficient. In many cases, chicks infected *in ovo* did not excrete virus or develop HI antibody until the flock had achieved greater than 50% egg production. At this stage, the virus was reactivated and excreted, resulting in rapid lateral spread due to multiple foci of infection.

The virus became established in commercial egg-laying flocks in some areas. This enzootic form of disease was often associated with a common egg-packing station, probably due to contamination of egg trays. Both normal- and abnormal-shelled eggs produced during the period of virus growth in the shell gland contained virus on their exterior and interior (53). Droppings also contained virus, but such excretion was intermittent, and the virus was often present only at low titer (23). In the adult bird, presence of virus in the feces probably arises from contamination by oviduct exudate/secretions (55). Spread can occur when birds are transported in inadequately cleaned trucks or when unused food has been moved between sites. Needles or blades used for vaccination or bleeding of viremic birds, if not properly sterilized, can transmit infection. Lateral spread of virus is slow and intermittent, taking up to 11 weeks to spread through a cage house. Spread of virus between birds on litter is usually faster (23, 62), but in one case, spread to an adjoining pen was prevented by a wire fence.

The sporadic form of disease appears to result from spread of EDSV from domestic or wild ducks, geese, and possibly other wild birds, to hens through drinking water contaminated by droppings. While such cases tend to be sporadic, they are important because such an infected flock could become the focus for establishing endemic infection.

Clinical Signs

Following experimental infection, most workers detected the first signs of disease after 7–9 days for example, (22, 41, 66), but in some experiments, signs did not appear until 17 days postinoculation (PI) (45).

The first sign was loss of eggshell pigment. This was quickly followed by production of thin-shelled, soft-shelled, or shell-less eggs (Figure 9.8). Thin-shelled eggs were often rough, with a sandpaper-like texture, or had a granular roughening of the shell at one end of the egg. If the abnormal eggs were discarded, there was no effect on fertility or hatchability. The fall in egg production was very rapid or extended over several weeks. Outbreaks usually lasted 4–10 weeks, and egg production was reduced by up to 40%; however, there was often compensation later in lay, so that the total number of eggs lost was typically 10–16/bird. If disease was due to reactivation of latent virus, the fall usually occurred when production was between 50% and peak. Small eggs have been described in naturally occurring outbreaks (43), but no effect on egg size was found in experimental infections (41). Watery albumen has been described (41, 45, 62), although no effect on albumen was reported by other workers (25, 41, 65). Age at infection may be important. Birds infected at one day of age produced apparently normal eggs except for impaired albumen quality and smaller size (22). If birds were infected in the late stages of egg production and force molted, egg production was normal on resumption of laying.

An apparently different clinical syndrome is seen if some birds have acquired antibody before latent virus is reactivated. There may be failure to achieve predicted egg production, and onset of lay may be delayed. If a careful examination is made, it can often be established that there has been a series of small clinical episodes of classic

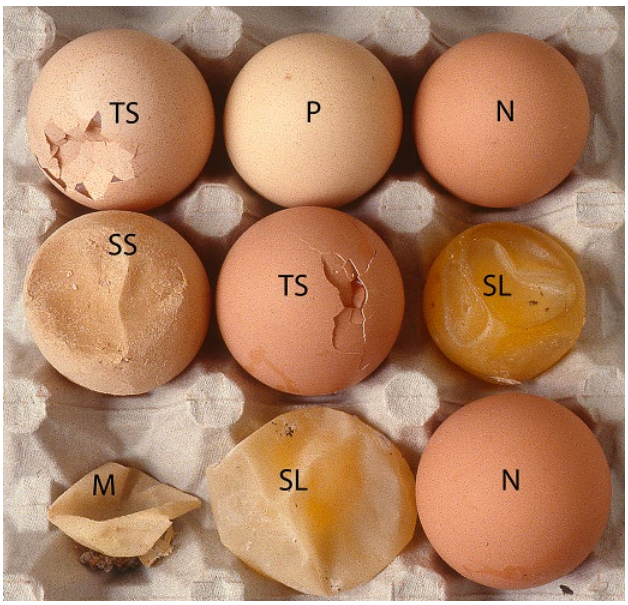


Figure 9.8 Eggs from hens infected with egg drop syndrome virus (EDSV). P: pale shelled egg, TS: thin shelled egg, SS: soft shelled egg, SL: shell-less egg, M: shell membrane remnant, N: normal brown eggs.

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EDS. Presumably, presence of EDSV-specific antibodies slows down the spread of the virus between the birds. A similar picture often has been observed in birds housed in caged units, where spread of the virus can be slow.

Inappetence and dullness have been described in some affected flocks, but these are not consistent findings. The transient diarrhea described by some authors is probably due to secretions from the oviduct mixing with the droppings (55). Egg drop syndrome virus does not cause clinical disease in growing chickens in the field. Oral infection of susceptible day-old chicks resulted in increased mortality in the first week of life (22), but there was no increase in mortality in many flocks of chickens produced by virus-infected parent flocks.

Pathology

Gross Lesions

Following experimental infection with EDSV, mucosal edema and exudate within the pouch shell gland (PSG) commonly occurred within 9–14 days PI (55, 58). Mild splenomegaly, flaccid ovules, and eggs in various stages of formation in the abdominal cavity also were reported (58). In naturally occurring outbreaks of EDS, inactive ovaries and atrophied oviducts were sometimes present. In one outbreak, uterine edema was observed (39).

Microscopic Lesions

The major pathologic changes occurred in the PSG. Virus replication occurs in the nuclei of surface epithelial cells, and intranuclear inclusion bodies were detectable from seven days PI onward (Figure 9.9) (55, 58). Many affected cells were sloughed into the lumen, and there was a rapid and severe inflammatory response with mucosal edema and heterophilic infiltration of the epithelium and lamina propria, together with macrophages, plasma cells, and lymphocytes, in the lamina propria. Inclusion bodies were not seen after the third day of abnormal egg production, but viral antigen persisted for up to one week (52). As lesions progressed, heterophils were less common and the mononuclear cells dominated. The sloughed surface epithelium was replaced

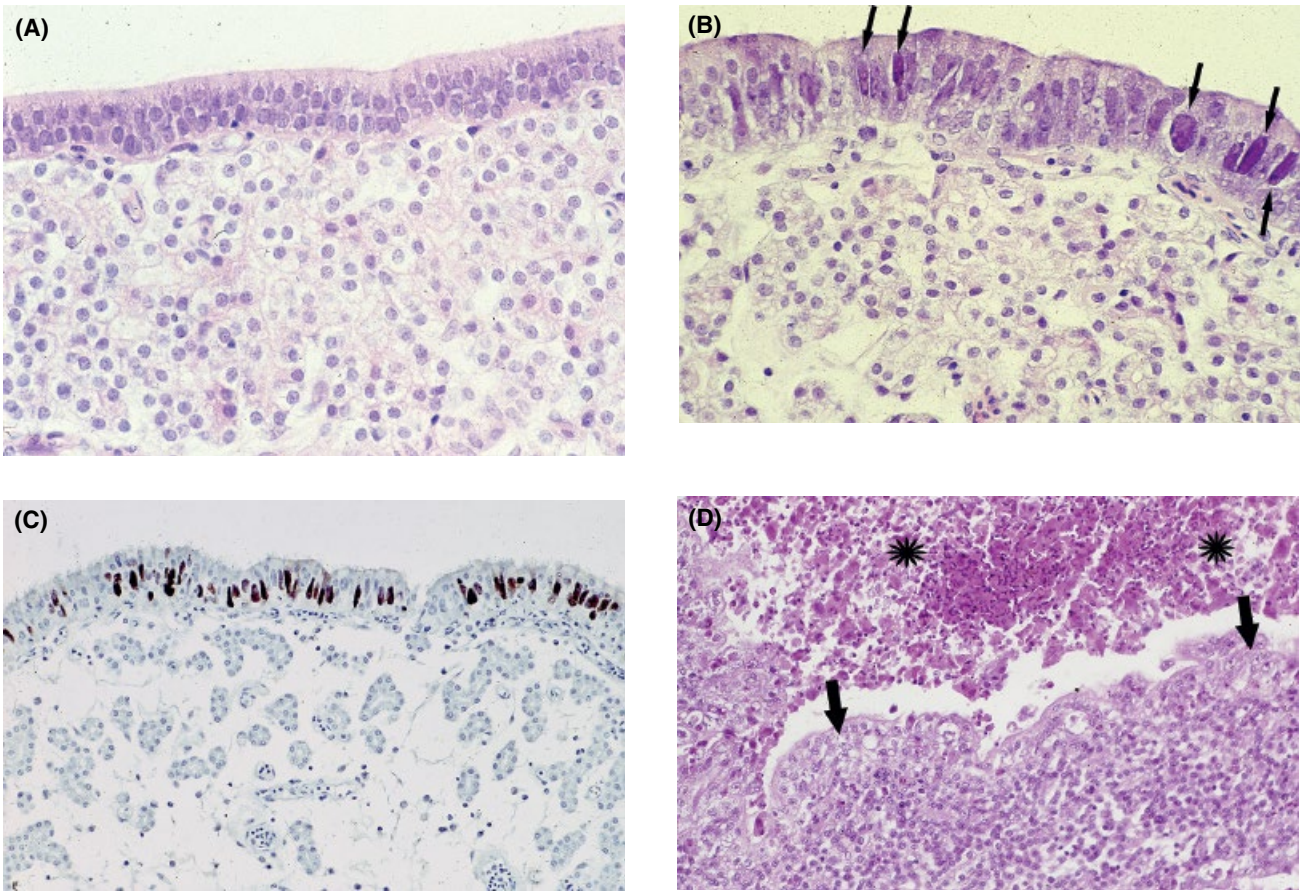


Figure 9.9 (A) Normal mucosa, pouch shell gland (PSG). Surface epithelium consists of a single layer of columnar cells, many of which are ciliated; underlying these are tubular glands. (B) Numerous enlarged nuclei (arrows) with margined chromatin and eosinophilic to amphophilic inclusions, PSG, day 9 postinfection. (C) Immunolabeled egg drop syndrome virus (EDSV) antigen (brown) in surface epithelium. PSG, day 9 postinfection. (D) Epithelial hyperplasia (arrows) and heterophilic exudate in lumen (asterisks), PSG, day 11 postinfection.

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initially by squamous to cuboidal epithelium (54), with rapid return to the normal pseudostratified, ciliated, columnar epithelium. In some recovering and recovered birds which were producing normal eggs, a few areas of surface cuboidal epithelium and a few lymphoid aggregates or minimal loose infiltrates of lymphocytes and plasma cells persisted (52, 54).

Most descriptions of the pathology of birds from naturally occurring disease outbreaks do not include the finding of inclusion bodies or the acute inflammatory and necrotizing phase of the disease. This is due to the transient nature of these lesions and the difficulty in finding acutely affected birds among the thousands of birds which may be present in an affected flock, where not all birds will have been infected simultaneously (54).

Ultrastructural

Lesions at the ultrastructural level have not been reported in detail. Adenovirus particles were found in PSG surface epithelial cells, macrophages in the lamina propria, and desquamated epithelial cells (54, 58).

Pathogenesis of the Infectious Process

Oral inoculation of adult hens with EDSV resulted in limited virus replication in the nasal mucosa and viremia (55). At 3–4 days PI, virus replication occurred in lymphoid tissue throughout the body. At 7–20 days PI, evidence of substantial virus replication was detected in the PSG (Figure 9.9) with lower levels of replication in other parts of the oviduct. Replication in the shell gland was associated with a pronounced inflammatory response and production of eggs with abnormal shells (55, 58, 66). Studies in duck embryo fibroblasts suggest that EDSV enters cells by clathrin-mediated endocytosis (33).

Unlike the aviadenoviruses and siadenoviruses of birds, EDSV does not appear to replicate in intestinal epithelium, and presence of virus in the feces of laying birds is probably due to contamination with infected oviduct secretion/exudate (55).

Immunity

Following experimental infection with EDSV, antibody is detectable by indirect fluorescent antibody test (IFA), ELISA, and SN and HI tests five days after infection and by double immunodiffusion (DID) test after seven days (4). Antibodies reached a peak after 4–5 weeks; immunoprecipitating antibodies were more transient.

Birds excrete EDSV in the presence of high levels of HI antibody, but some birds that excreted the virus failed to develop antibody (22).

Antibody is transferred to the embryos through the yolk sac, and young chicks had high EDS HI titers (geometric mean titers, 6–9 $\log_2$). This antibody had a half-life of three days (25). Active antibody production was not stimulated until the chicks reached 4–5 weeks of age, when maternal antibodies were almost undetectable (25). Some chicks which were vertically infected developed a latent infection, and failed to develop antibody. With the onset of egg production, the virus was apparently reactivated and virus was excreted. It is not known if all of these chicks developed antibody at this point.

If a flock as a whole develops antibody to EDSV before coming into lay, they will not develop EDS (10).

Diagnosis

Selection of Specimens

A major problem for diagnosis of EDS is the selection of suitable birds for examination. The absence of obvious clinical signs in affected birds, the often slow spread of infection, and the transient nature of virus replication and associated lesions makes it difficult to select birds suitable for diagnostic testing, from among the thousands that may be in the flock. The finding that abnormal eggs contain virus and that the abnormal eggs were produced after the bird had developed EDSV antibodies has allowed a rational approach to diagnosis (53, 54). For serological diagnosis, all birds in a few cages where abnormal eggs are being produced should be blood sampled. If the birds are housed on litter, then birds throughout the house should be bled, because it is usually not possible to determine which birds are producing abnormal eggs in these circumstances. To isolate the virus, abnormal eggs may be fed to antibody-negative laying hens. At the first appearance of abnormal eggs, the hens may be euthanized and virus isolation attempted from the pouch shell gland. To note, it might be possible to detect EDSV in such eggs by sensitive modern methods such as quantitative PCR, but there are no reports of such testing.

Identification of EDSV in Tissue Samples

The successful use of antigen capture ELISA and PCR-based tests has been described for use on field specimens (24, 28, 47–49), but these do not appear to be in widespread use. This may reflect a lack of cases submitted for laboratory diagnosis.

Virus Isolation

The most sensitive medium for EDSV isolation is either allantoic inoculation of embryonating duck or goose eggs derived from a flock free of EDSV infection, or duck or goose cell cultures. These systems have the added

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advantage that they do not support the growth of many chicken viruses. If they are unavailable, chicken cells should be used. CEL were more sensitive than CK; CEF were insensitive (3). Embryonating chicken eggs are not suitable.

In addition to observing for embryo death or cytopathic effect, allantoic fluid from inoculated goose or duck eggs or supernatant from infected cell cultures should be checked after each passage for presence of EDSV HA, using avian erythrocytes (a 0.8% chick erythrocyte suspension is suitable). Alternatively, immunofluorescence, using a labeled EDSV antiserum, can be used to detect the presence of the virus in the cells. Antiserum conjugated to aviadenoviruses will not detect EDSV. Real time PCR can be used to detect increasing amounts of virus (33).

If duck cells are used for isolation, a minimum of two passes are required, and with chick cells, two to five passes are necessary before declaring a specimen negative. The need for extensive passage is partly due to poor growth of the virus on primary isolation in chick cells, and partly because virus titers in the tissues can vary, particularly if the bird being investigated is not at the stage of the disease process when virus titers are maximal.

Serology

Presence of antibodies in nonvaccinated laying flocks indicates infection, but obviously does not indicate when infection occurred. The HI, ELISA, SN, DID, and IFA tests may be used to detect antibody, and are of similar sensitivity (4). While ELISA tests have the advantage of potential automation, nonspecific reactions can occur with antibody ELISA tests for EDSV. When birds were infected with various aviadenovirus serotypes, there were positive reactions in the ELISA, IFA, or DID tests for EDSV, but not in the HI or SN tests (4). Therefore, HI is still considered the best test for serological diagnosis. Antigen for HI test can be prepared in either embryonating duck eggs or in cell culture. Higher HA titers are obtained if duck eggs are used. High HA titers also can be obtained using CEL. A suitable HI test uses 4 HA units of antigen, an initial 1:4 serum dilution, and 0.8% chicken erythrocytes. If nonspecific hemagglutinins are present in the serum, they can be removed by preadsorption of the serum with a 10% erythrocyte suspension. The SN test, using 100 $TCID_{50}$ (tissue culture infective doses) of EDSV, 1-hour reaction time at 37 °C, and duck or chick cell cultures as the indicator system, is sensitive and specific. When using chick cell cultures, it often helps if the endpoints are read by presence of hemagglutinins in the supernatant fluid rather than by cytopathology. The SN test is only really required in diagnostic situations to confirm an unusual HI test result, for example, in an eradication program or to confirm a positive HI result in species in which EDS has not previously been recognized.

Many flocks containing birds that had been vertically infected with EDSV did not develop antibodies during the growing period; antibody only became apparent immediately following the development of clinical signs of the disease. Therefore, even a negative serological test of all birds in a flock, at around 20 weeks of age, gives no guarantee of freedom from infection.

Differential Diagnosis

Egg drop syndrome should be suspected whenever there is failure to achieve predicted egg production levels or if there are falls in egg production, especially if birds are healthy and eggshell changes precede or coincide with the decline. Shell-less eggs are usually a feature of EDS but are often missed because they may be trampled into the litter, be consumed by the birds, or fall through the wire mesh of cage floors. Therefore, flock inspection is best immediately after the lights come on, before the birds become active or belts are run. If the birds are housed on litter, a careful search will reveal shell membranes. Shell-less, soft-shelled, and thin-shelled eggs are characteristic of EDS; misshapen and ridged eggs are not a feature. In an infected flock in which vertical transmission of EDSV has occurred, most if not all cases occur around peak egg production, but any age of flock can be infected by lateral spread.

Although signs of EDS are quite characteristic, diagnosis cannot be made on the clinical picture alone; confirmatory testing is necessary.

Intervention Strategies

Management Procedures

Since classical EDS spreads primarily by vertical transmission through the egg, replacement birds should be derived from uninfected parent flocks. Endemic EDS is often associated with a common egg-packing station; contaminated egg trays have been a major factor in the spread of the disease. Lateral spread is possible because virus is present in droppings, and the virus is resistant to inactivation. Circumstantial evidence exists for spread by personnel and transport, therefore hygienic precautions are required.

Infected birds develop a viremia, thus it is important that vaccination and bleeding needles, or other equipment, should be sterilized between birds.

If infected and uninfected breeding flocks exist within the same organization, separate hatcheries, staff, and transport should be used. If this is not possible, separate setters and hatchers should be used, and hatches should

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be scheduled to take place on different days of the week. Although not recommended, minimum possible precautions are to use separate hatchers and to sex, vaccinate, and dispatch the clean stock before handling potentially infected chicks. It is particularly important to keep infected breeding stock and progeny separate from non-infected birds.

In some areas of the world, particularly where drinking water for birds is derived from dams, lakes, or rivers, EDSV infection has been common. These outbreaks have been controlled either by using water from wells or by chlorination of the water. On mixed species farms, ducks and geese should be carefully segregated from chickens. If possible, all housing should be made wild bird-proof, because it is well documented that wild ducks and geese are often infected with EDSV, and other avian species may also be infected.

It was possible to eradicate the virus from infected breeding stock before vaccines were developed (see earlier editions of *Diseases of Poultry*).

Vaccination

Types of Vaccine

Oil-adjuvant inactivated vaccines are widely used and give good protection against EDS. Birds are usually vaccinated between 14 and 16 weeks of age. If uninfected birds are vaccinated, HI titers of 8–9 $\log_2$ can be expected. If the flock has been exposed previously to EDSV, HI titers of 12–14 $\log_2$ may be found. A HI antibody response can be detected by the seventh day after vaccination, with peak titers achieved between the second and fifth weeks. Vaccinal immunity lasts at least one year (10, 21, 36, 56). Although properly vaccinated birds are protected against disease and do not appear to excrete EDSV, improperly vaccinated birds with low EDS HI titers excreted the virus following challenge (20). An experimental, recombinant subunit vaccine induced good antibody responses and reportedly protected against disease challenge (31).

Field Vaccination

When vertical or lateral transmission of EDSV is a possibility, at-risk flocks can be protected by vaccination in the growing period. If one or more houses on a multi-age laying site become infected due to lateral spread of the virus, the cost of vaccination, combined with costs and effects of handling laying birds in order to administer the vaccine, must be carefully weighed against the economic returns achieved from the protection. It is possible to limit the spread of virus on a site by good hygiene. It is important to remember that the infected egg is potentially the major source of virus.

Treatment

Various treatments have been tried (e.g., vitamins and increasing calcium or protein in the ration), but in controlled trials, no effect could be demonstrated. Therefore, no successful treatment is available.

Hemorrhagic Enteritis and Related Infections

Silke Rautenschlein, Hassan M. Mahsoub, Scott D. Fitzgerald, and F. William Pierson

Summary

Agent, Infection, and Disease. Hemorrhagic enteritis (HE) of turkeys and related infections in other gallinaceous fowl are caused by a group of related Siadenoviruses. These differ from the Aviadenoviruses genomically and ultrastructurally. The HE virus (officially named turkey adenovirus 3, TAdV-3) shows a high prevalence in turkey dense areas worldwide.

Diagnosis. The primary target cells, B-lymphocytes and macrophages, release a variety of cytokines upon infection which incite the development of characteristic lesions. The disease appears as gastrointestinal hemorrhage with splenomegaly in turkeys while pheasants exhibit pulmonary edema and splenomegaly. TAdV-3-infected birds often succumb to secondary infection with opportunists like *Escherichia coli* due to transient immunosuppression.

Intervention. Vaccination has greatly reduced mortality, and contributes to the reduction of antibiotic use because of secondary infections.

Introduction

Definitions and Synonyms

Hemorrhagic enteritis (HE) is an acute viral disease of turkeys four weeks of age and older, characterized by depression, bloody droppings, and death. Clinical disease usually persists in affected flocks for 7–10 days. Due to the immunosuppressive nature of the virus, secondary bacterial infections may extend losses for an additional 2–3 weeks.

Marble spleen disease (MSD) is a condition affecting confinement-reared pheasants 3–8 months of age. The causative virus (MSDV) is serologically indistinguishable from the HE virus (HEV) and has only minor differences

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at the genomic level. The clinical presentation is predominantly respiratory, that is, lung edema, congestion, dyspnea, and death. A similar, albeit milder disease referred to as avian adenovirus splenomegaly (AAS), has been described in broiler breeders.

Economic Significance

Financial losses due to HE and associated secondary infections were high prior to vaccine development (reviewed in [50]). Today, due to the use of vaccines, clinical HE outbreaks are rare and typically associated with a poor vaccination response. The economic impacts of MSD in pheasants and AAS in chickens have not been formally assessed.

Public Health Significance

The viruses responsible for HE, MSD, and AAS are not known to cause illness or seroconversion in humans (31).

History

Hemorrhagic enteritis was first observed in 1936 in Minnesota and described by Pomeroy and Fenstermacher (53). In 1966 and 1979, MSD and AAS were described in ring-necked pheasants and broiler breeders, respectively. Later on, the causative agents were identified as adenoviruses. Additional information about the history of HE can be found in the thirteenth edition of *Diseases of Poultry* (50).

Etiology

Classification

Hemorrhagic enteritis virus, MSDV, and AAS virus (AASV) are very closely related, serologically indistinguishable viruses originally assigned to the genus *Aviadenovirus* and designated as group 2 avian adenoviruses to distinguish them from the other members. The group was shown to be serologically distinct from chicken embryo lethal orphan (CELO) virus and other turkey adenovirus isolates (reviewed in [50]). DNA sequence homologies indicated that HEV and MSDV were different enough from other aviadenoviruses (7, 12, 52) to warrant reclassification. They have since been reassigned to a new genus *Siadenovirus* (Table 9.2) and been given one species name, Turkey adenovirus A (turkey adenovirus 3, TAdV-3) (26). Other members of the genus were described in frog (13), great tit (35), skua (47), and raptors (33). Additional candidate species include psittacine adenovirus 2, Gouldian finch adenovirus 1 (29), Chinstrap penguin adenovirus (37), and Sulawesi tortoise adenovirus 1 (34). Recently two novel types of siadenoviruses (PiAdV-4 and -5) were described in pigeons (5). The genus name is derived from an open reading frame (ORF) in the early transcription region that has a high sequence homology with bacterial genes coding for sialidase. There are also several other putative genus-specific genes that share no sequence similarity with other adenoviruses (12), which include hyd (hydrophobic), E3, ORF7, and ORF8 (47). The origins of the genus, the functional significance of the sialidase homolog, and other genus-specific genes are yet to be determined.

Morphology

Morphologic, histologic, immunologic, and chloroform-resistance studies have been reviewed previously (50). These indicate that HEV, MSDV, and AASV are members of the family Adenoviridae.

Ultrastructure

TAdV-3 viral particles are non-enveloped icosahedrons with a total capsomere count of 252. They occur in empty and dense forms and are arranged intranuclearly in loosely packed aggregates or crystalline arrays (reviewed in [50]). There are 13 structural proteins integrated in the virions (36). The capsid comprises of the trimeric hexon, pentameric penton base, and trimeric fiber, which protrudes from the penton base. Unlike the aviadenoviruses, only one penton fiber is present at each vertex (12). Crystals of the C-terminal head domain of TAdV-3-fiber protein demonstrate the structural characteristics of the fiber head (59). A beta-hairpin insertion in the C-strand of each trimer subunit embraces the neighboring monomer (60). The hexon protein has two functional components: a pedestal region, which is rather conserved across all adenoviruses, and variable loops (L1–L4), of which some may interact with the host immune response (30).

Size and Density

Electron microscopy of intranuclear inclusion bodies and cesium chloride-purified preparations reveal virions to be 60–90 nm in diameter. Buoyant densities are reported to be between 1.32 and 1.34 g/mL (reviewed in [50]).

Chemical Composition

TAdV-3 are linear, double-stranded DNA viruses (12, 28). Complete sequences and maps of the TAdV-3 genome have been published for virulent and avirulent strains (8, 52). The genome length is approximately 26.3 kb, which is one of the smallest adenovirus genomes (11). Guanine and cytosine constitute 34.9% of the

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nucleotides, which is low compared with other adenoviruses (7, 52). The genome consists of eight ORFs in two clusters; one includes ORFs 1, 2, 3, and 4, and the other ORFs 7 and 8; and at least 13 genes (52K, IIIa, penton base, pVI, hexon, EP, 100K, pVIII, fiber, Iva2, POL, pTP, and DBP [52]). Transcriptional mapping identified 5' and 3' untranslated regions (UTR) with a tripartite leader sequence at the 5' UTR comparable in structure to that of mastadenoviruses. Additionally, transcription start sites, splice acceptor and donor-splice site, polyadenylation sites, and leader sequences were determined (1).

It was suggested that TAdV-3 has at least 11 distinct structural polypeptides with molecular weights ranging between 14 and 97 kDa and 9.5 and 96 kDa. Six of these proteins have been further characterized: a 96 kDa polypeptide believed to be the hexon, 51–52 kDa and 29 kDa polypeptides believed to be the vertex penton base and fiber, a 57 kDa homologue of human adenovirus group 2 IIIa protein, and two core nucleoproteins of 12.5 kDa and 9.5 kDa (reviewed in [50]). Recently, two additional structural proteins were identified, that is, sialidase and TaV3gp04 (36).

Virus Replication

Macrophages and B lymphocytes are believed to be the primary target cells (61, 62). The spleen appears to be the major site of viral replication (16, 57); however, infected cells were also detected in a variety of other tissues including intestine, cloacal bursa of Fabricius, cecal tonsils, thymus, liver, kidney, peripheral blood leukocytes, and lung (reviewed in [50]).

At least 14 viral and 18 host proteins were suggested to be involved in virus replication, cell-to-cell spread, and cytoskeleton dynamics (36). The replication strategy at the cellular level is presumed to be similar to that of other adenoviruses. Infection begins by viral attachment facilitated by the penton fiber head to sialic acids (most likely α2,3- and α2,6-linked) on host cell surface (40, 60). The penton base is involved in receptor-mediated endocytosis, most likely via cell surface integrins. A carbohydrate and a protein moiety may be key components of the virus receptor. Preliminary results from virus overlay protein blot assays demonstrated that TAdV-3 may specifically bind to two cell surface proteins on B cells (40). DNA transcription utilizing host RNA polymerase II takes place in the nucleus. Genome replication also occurs in the nucleus and involves virus-encoded DNA-dependent DNA polymerase and the formation of a pan-handle intermediate with base pairing occurring at inverted terminal repeats. The existence of a total of 23 ORFs was suggested for TAdV-3; those ORFs were recently confirmed to be actively transcribed during *in vitro* replication (1, 9). A tripartite leader sequence was suggested to be involved in the transcription of structural (late) protein genes of the virus (1). Virions are assembled in the nucleus and released upon cell disintegration (12).

Susceptibility to Chemical and Physical Agents

TAdV-3 remains stable under a variety of harsh conditions: heating at 65 °C (149 °F) for 1 hour, liquid at 37 °C (99 °F) for 4 weeks or 4 °C (40 °F) for 6 months, freezing at −20 °C (−4 °F) for 4 years, and at low pH. Infectivity can be destroyed by heating at 70 °C (158 °F) for 1 hour and drying at 37 °C (99 °F) or 25 °C (77 °F) for 1 week. Chlorine- and iodine-based disinfectants are known to be effective against TAdV-3 and quaternary ammonium compounds should also be useful due to their efficacy against non-enveloped viruses (reviewed in [50]).

Strain Classification

Traditionally HEV, MSDV, and AASV strains have been classified according to their host source, that is, turkeys, pheasants, or chickens.

Antigenicity

Antigenic differences have been reported based on monoclonal antibody affinity, but strains are considered to be serologically indistinct and provide cross-protection. The outer coat proteins, that is, hexon, penton fiber, and penton base, appear to be the most antigenic, in part because they are produced in such large amounts. Neutralizing epitopes were identified within the hexon protein (reviewed in [50]).

Immunogenicity or Protective Characteristics

Apart from host virulence characteristics, HEV and MSDV strains are cross-protective. In fact this forms the basis for the current vaccination strategies, that is, strains of MSDV that are virulent in pheasants are used to vaccinate turkeys and strains of HEV that are virulent in turkeys may be used to vaccinate pheasants (reviewed in [50]).

Genetic or Molecular

A comparison between the complete genomes for virulent and avirulent strains of HEV indicates that they are 99.9% identical. However, the occurrence of at least eight mutations in the penton fiber, ORF1, and/or E3 genes may account for variations in virulence; only four relate to amino acid changes (8). Amino acid differences in the C-terminal region of the fiber protein head may allow the differentiation between virulent (Ile354, Thr376) and avirulent strains (Met354, Met376) (60). Also the analysis of the ORF1 sequences at its 5' and 3' ends may help to differentiate strains in the field (3).

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Pathogenicity

It is not uncommon for TAdV-3 isolates to be referred to as avirulent or virulent based on the severity of lesions they produce, that is, splenomegaly in the former and splenomegaly, gastrointestinal hemorrhage, and death in the latter. ORF1, E3, and fib genes were suggested to be key factors affecting the virulence of the TAdV-3 strains (52).

Laboratory Host Systems

TAdV-3 can be propagated in six-week-old turkeys via inoculation with splenic tissue from infected birds. Spleens from these birds can then be harvested three or five days postinoculation (DPI) respectively, to yield significant amounts of virus (16). Inoculation of specific-pathogen-free (SPF) turkey eggs with MSDV on day 24 of incubation also results in infection with peak viral loads in the spleen, intestine, and liver occurring 6 DPI (2). *In vitro* propagation of TAdV-3 in splenocyte culture has been unsuccessful (reviewed in [50]). However, serial passages of TAdV-3 in an immortalized lymphoblastoid cell line derived from a Marek's disease virus-induced tumor of turkey origin (MDTC-RP19) were demonstrated. MDTC-RP19 cells have become the standard system for *in vitro* HE vaccine production. An *in vitro* method using purified peripheral blood leukocytes from turkeys also has been described (64).

Pathogenicity

In the field, mortality due to virulent HE is reported to vary from more than 60% to less than 0.1%. Mortality rates in pheasants naturally infected with MSDV are reported to range between 5% and 20% (reviewed in [50]). Birds experimentally infected with cell culture propagated MSDV or virulent and avirulent strains of HEV failed to develop lung lesions or mortality despite the appearance of typical splenic lesions (19). Therefore, other factors may be necessary to replicate the disease observed in the field (19) or the strains used may have become cell culture adapted.

Pathobiology and Epidemiology

Incidence and Distribution

Despite the high serological incidence of TAdV-3 infection in turkey dense areas, the incidence of HE-related clinical signs became low due to the circulation of avirulent, low virulent, or TAdV-3 vaccine strains (25). MSDV has been documented in confinement pheasant operations on different continents (reviewed in [50]). Seroprevalence data indicated a wide distribution of AASV in mature chickens (50). Reports from China suggest the emergence of HE and splenomegaly associated with TAdV-3 in yellow chickens (38).

Natural and Experimental Hosts

For a complete list of studies that have investigated the natural and experimental hosts of TAdV-3 infections, the reader is referred to the thirteenth edition of this subchapter (50). Natural infections have been documented in turkeys, pheasants, chickens, guinea fowl, and chukar partridges. Serologic survey of 42 different species of wild birds revealed no evidence of infection outside the order Galliformes. Even wild populations of turkeys appear to be at little risk, probably due to their elusive nature. Closely related viruses have been isolated from raptors, psittacines, and a songbird.

Experimentally, HEV will infect ring-necked pheasants, and MSDV and AASV will infect turkeys. However, host genetics appear to influence the severity of clinical disease and lesion formation in both. HEV also produces lesions in golden pheasants and peafowl, but without mortality.

Age of Host Most Commonly Affected

Primarily due to the protection afforded by maternal antibodies, HE is not typically seen in turkeys until about 6 weeks of age, with most field cases occurring between 6 and 11 weeks of age. However, a single case in 2.5-week-old turkeys was reported with the predisposing factor thought to be a lack of maternal antibody (reviewed in [50]). Newly hatched, seronegative turkeys are susceptible to infection, but refractory to intestinal lesion formation (20, 43), suggesting that target cell maturation may be necessary for the development of fulminate disease (21).

As reviewed previously (50), MSD occurs naturally in pheasants 3–8 months of age. Those younger than 4 weeks of age appear to be less susceptible to infection either due to maternal antibodies or insufficient numbers of mature target cells.

Field infection with AASV has been observed in broiler breeders 20–45 weeks of age (50).

Transmission, Carriers, and Vectors

TAdV-3 can be transmitted by oral or cloacal inoculation of susceptible turkeys with infectious feces (reviewed in [50]). Virus can remain viable for several weeks in carcasses protected from drying or in wet fecal material and litter, with the disease commonly reoccurring on infected premises (16). Persistent infection may occur in recovered birds (9), possibly resulting in latent shedding. There is no epidemiologic evidence of egg transmission or other biological vectors (16). Therefore, the most

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Figure 9.10 Three spleens from pheasants experimentally infected with marble spleen disease virus. These spleens are enlarged two to four times the normal size, and diffusely mottled or marbled.

probable route of natural transmission for TAdV-3 is fecal–oral/cloacal ("cloacal drinking") (49). MSDV and AASV are likely transmitted in the same manner, although due to the presence of respiratory lesions, aerosols cannot be ruled out.

Incubation Period

In turkeys, clinical signs and mortality begin 5–6 days after oral or cloacal infection and 3–4 days after intravenous inoculation with infectious splenic homogenate. The incubation period following oral inoculation with MSDV in pheasants and AASV in chickens appears to be similar (reviewed in [50]).

Clinical Signs

HE is characterized by a rapid progression of clinical signs over a 24-hour period. These include depression, bloody droppings, and death. Fecal material containing dark blood is often present around the vent. It also may be expressed from the vent if pressure is applied to the abdomen (16). In naturally infected flocks, signs of disease tend to subside 6–10 days after the appearance of bloody droppings. In pheasants with MSD, death is often peracute. Depression, weakness, dyspnea, and nasal discharge are noted in acute cases (23). The presentation for AASV in chickens is similar to that of pheasants, but generally less severe (50).

Morbidity and Mortality

In field outbreaks of HE, morbidity approaches 100%. Turkeys exhibiting clinical signs usually die within 24 hours or recover completely. Mortality ranges from less than 1% to greater than 60% and averages 10–15% (reviewed in [50])). Up to 80% mortality has been observed in experimental infections (16). If birds survive the acute phase of TAdV-3 infection, immunosuppression still makes them vulnerable to secondary infections leading to a second peak of mortality (25). Morbidity associated with MSD and AAS is similar to that of HE. Mortality associated with MSD in pen-reared ring-necked pheasants averages 2–3%, but may reach 5–20% over a 2- to 3-week period. In mature chickens with AAS, mortality as high as 8.9% has been reported (reviewed in [50]).

Pathology

Gross

Following virulent TAdV-3 infection, dead turkeys routinely appear pale due to blood loss, but are typically in good flesh and have feed in their crops. The small intestine is commonly distended, grossly discolored, and filled with bloody contents (see Figure 9.13 F in the Quail Bronchitis section). The intestinal mucosa is congested and, in some cases, covered with a yellow fibrinonecrotic membrane. Lesions are usually more pronounced in the duodenum, but can extend distally. Spleens are characteristically enlarged, friable, and mottled in appearance; however, those of dead birds tend to be smaller due to contraction of the spleen in response to blood loss. Lungs may be congested, but other organs are generally pale. Hepatomegaly and petechiae in various tissues also have been reported (reviewed in [50]). Lesion formation with virulent strains appears to be dose-dependent (44, 46).

Lesions associated with MSDV infection in pheasants include enlarged, mottled (marbled) spleens (Figure 9.10; also see 9.13G) and edematous congested lungs. Intestinal hemorrhage has not been noted. In broiler breeders infected with AASV, lesions resemble those of MSD (reviewed in [50]).

Microscopic

Splenic lesions present at death include hyperplasia of white pulp and lymphoid necrosis. Basophilic Cowdry type B intranuclear inclusions (INI) can be found within mononuclear cells, that is, macrophages and

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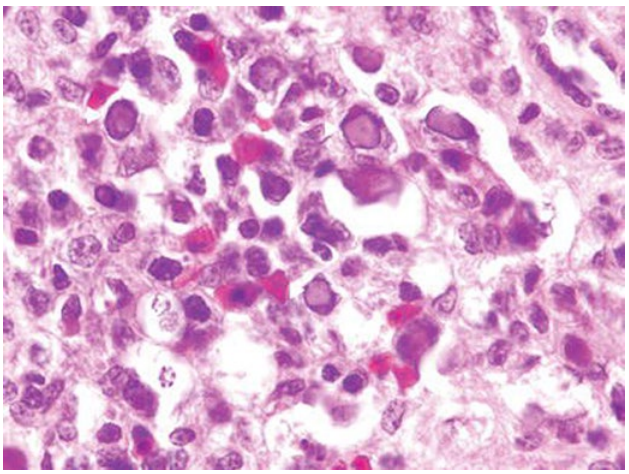


Figure 9.11 Section of spleen from a turkey affected with hemorrhagic enteritis. Nuclei of infected macrophages contain characteristic pale eosinophilic inclusions, with marginated chromatin and eccentric nucleoli. H&E, ×550.

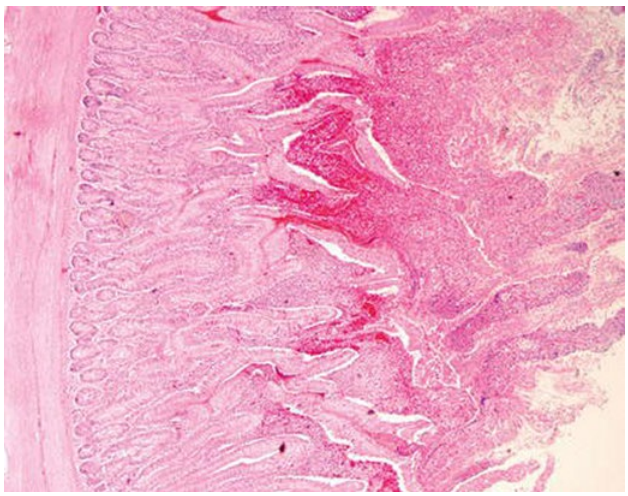


Figure 9.12 Section of small intestine from a turkey affected with hemorrhagic enteritis. Lesions include severe mucosal congestion, sloughing of epithelial cells at tip of villi, and hemorrhage into the intestinal lumen. H&E, ×550.

lymphocytes (Figure 9.11) (57, 62). Proliferation of white pulp surrounding splenic ellipsoids is evident as early as 3 DPI. This leads to large, irregular, confluent islands of white pulp which are grossly visible 4–5 DPI (57). Hematoxylin and eosin (H&E) staining reveals numerous INI in these splenic zones between 3 and 5 DPI but also in other organs (reviewed in [50]). By 4–5 DPI, the white pulp begins to undergo necrosis and by 6–7 DPI it has completely involuted with only occasional plasma cells appearing in the red pulp. In addition to splenic changes, lymphoid depletion is also noted 3–9 DPI in both the cortical and medullary areas of the thymus (27) and bursa of Fabricius (27, 57).

Typical intestinal lesions include mucosal congestion, hemorrhage in the villus tips, and epithelial necrosis (Figure 9.12). Hemorrhage and necrosis are thought to be the result of endothelial disruption rather than destruction because blood vessels in the lamina propria appear intact and diapedesis of red cells is observed. Increased numbers of mononuclear cells with INI are observed in the lamina propria in addition to mast cells, plasma cells, and heterophils. These changes are most pronounced in the duodenum just posterior to the pancreatic ducts, but similar, less severe lesions also may occur in the proventriculus, gizzard, distal small intestine, ceca, cecal tonsils, and bursa of Fabricius (reviewed in [50]).

MSDV and AASV produce splenic lesions similar to those of HEV, but intestinal lesions are absent. In naturally occurring cases of MSD and AAS, vascular congestion of the lung as well as flooding of the pulmonary atria and tertiary bronchi with fibrin and red cells are observed. Large numbers of mononuclear cells with typical INI can be found in the atria and to a lesser extent in other tissues (reviewed in [50]).

Pathogenesis of the Infectious Process

TAdV-3 strains are considered to be lymphotropic and lymphocytopathic (27, 50, 57, 64) with IgM bearing B lymphocytes being the primary target (56, 62). This is consistent with the fact that bursectomy impairs viral replication and lesion formation (20, 50, 61). Macrophages also appear to support viral replication (61, 64). A marked depletion of IgM bearing cells in the spleen and peripheral blood may be observed during the acute phase of TAdV-3 infection (56, 61), leading to a transient inhibition of antibody responses to various antigens as well as suppression of *in vitro* mitogenic responses in B and T cells harvested from infected birds (reviewed in [50]).

Multiple hypotheses regarding TAdV-3 immunopathogenesis have been proposed and reviewed by Pierson and Fitzgerald (50). It was suggested that after oral exposure, TAdV-3 either undergoes an initial round of replication in B lymphocytes located in the intestine and cloacal bursa or it travels directly to the spleen via the peripheral blood. There it replicates to high numbers. This results in an influx of CD4+ T cells and macrophages into the white pulp, presumably in an attempt to clear the virus, and accounts for the hyperplasia observed during the acute phase of infection. Once activated, macrophages produce a variety of cytokines. Type II IFN activates macrophages to produce nitric oxide, which has antiviral, immunosuppressive properties. Type I IFN is also produced in an attempt to limit viral replication. TAdV-3 replication leads to cell death, thus depleting the B cell population. Cytokine-mediated apoptosis of bystander cells also occurs. The cumulative effect is a transient period of immunosuppression.

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The presence of increased numbers of mast cells in the intestine associated with TAdV-3 infection suggests that histamine and prostaglandin may play a role in intestinal lesion formation. Other findings, for example, the presence of few infected cells in the intestine (62) and the ability of cyclosporin A, a T cell inhibitor, to abrogate intestinal hemorrhage, also strongly suggests an immune-mediated pathogenic mechanism (61). Conceivably, the lung lesions associated with MSD and AAS may also be immune-mediated. Epithelial necrosis likely occurs due to a lack in blood supply rather than direct viral damage (49). It was suggested that intestinal hemorrhages in HEV-infected turkeys and also the pulmonary edema in MSDV-affected birds may be the result of an anaphylactoid reaction (49).

It is important to note that transient immunosuppression occurs with virulent as well as avirulent TAdV-3 strains, the effect being more pronounced with the former (50). Independent of virulence, TAdV-3 alone (49) or in conjunction with other infectious agents appears to predispose turkeys to secondary infection especially with *E. coli* in the field, and may allow the development of necrotic enteritis (14). Similar results have been observed experimentally (reviewed in [50]). Combined infections with avian paramyxovirus 2 and *Chlamydia psittaci* have also been reported (4).

Surprisingly, improved weight gains and reduced oocyst shedding have been found in turkeys co-infected with TAdV-3 and *Eimeria meleagrimitis* (45). Likewise, simultaneous vaccination with APMV-1 and HEV appears to have a peculiar effect, that is, APMV-1 antibody production is enhanced while TAdV-3 antibody production is suppressed. Spleens also exhibit more pronounced hyperplasia of the white pulp and an increase in apoptosis (54).

Immunity

Active

Turkeys recovering from natural or experimental infections with TAdV-3 are refractory to challenge. Protection does not appear to be strain specific. Strains that cause less than 1% mortality induce sufficient immunity to prevent infection with those producing much greater mortality (17). Antibodies against TAdV-3 may be detected as early as 3 DPI by ELISA (63). The occurrence of life-long immunity is believed to be the result of persistent infection since viral DNA can be detected in numerous tissues up to 70 DPI despite high levels of circulating antibody (9). Periodic recrudescence may explain variation in antibody titers over time.

Cell-mediated immunity undoubtedly plays a role in protection against infection and lesion formation, but its role is not fully understood. Inoculation of turkeys with TAdV-3 causes an increase in splenic CD4+ T cells 4–6 DPI (32, 55, 61) and in circulating (48) and splenic (61) CD8+ T cells 8–10 and 16 DPI, respectively. A decrease in CD3 + CD8α + T cells in spleen and blood was also observed (32). Selective *in vivo* T cell depletion with cyclosporin A enhanced splenic lesion formation and viral replication in MSDV-infected pheasants (24), but the same effect has not been observed in turkeys (61).

Passive

Maternal antibodies can provide protection from the development of clinical HE up to six weeks of age and interfere with vaccination up to five weeks of age (21). However, in a commercial setting maternal antibodies typically decline enough by 3.5–4 weeks-of-age to permit vaccination with splenic TAdV-3 vaccines (18, 66).

Diagnosis

Isolation and Identification of Causative Agent

TAdV-3 diagnosis changed from traditional to molecular methods (14). Large virus-concentrations can be found in bloody intestinal contents or splenic tissue obtained from dead or moribund turkeys, or spleen from affected chickens and pheasants (reviewed in [50]). TAdV-3 can be propagated in naïve turkeys, preferably six weeks of age or older, by inoculation intestinal contents or splenic homogenates. Alternatively, MDTC-RP19 cells can be inoculated with filtrates of splenic material to isolate and propagate the viruses (43, 44).

As reviewed before (50), positive identification of HEV, MSD, and AASV can easily and inexpensively be accomplished through the use of an agar gel immunodiffusion (AGID) test. Viral antigen can be identified in frozen or formalin-fixed tissues using immunofluorescent or immunoperoxidase staining methods. Less commonly used methods for antigen detection include antigen-capture ELISA and *in situ* DNA hybridization. Standard (reviewed in [50]), nested (9), and real-time (7, 58) PCR assays for detection of viral DNA in fresh or frozen tissues have been developed. Real-time PCR may also be used to titrate TAdV-3 live vaccines (41). Drying of splenic material or DNA extracts on filter paper was shown to be an adequate method of storage (52). For rapid diagnosis in the field a one-step real-time fluorescence loop-mediated isothermal amplification (RealAmp) assay was developed (38).

Serology

TAdV-3 antibodies can be detected in plasma or serum about 2–3 weeks PI by AGID (reviewed in [50]). It is advisable to run both acute and convalescent sera if a diagnosis is to be made based on serology. Maternal antibodies may be detected using AGID, but this method generally lacks sufficient sensitivity beyond one week of

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age. More sensitive HEV-ELISA techniques have been developed (reviewed in [50]) and are commercially available. Recently an ELISA system using a recombinant N-terminal part of the hexon protein as an antigen was established providing comparable results with a commercially available system (39). Commercial kits are not available for MSDV or AASV antibody detection.

Differential Diagnosis

In turkeys, an enlarged mottled spleen without evidence of TAdV-3 detection or intestinal bleeding warrants consideration of lymphoid neoplasia, that is, reticuloendotheliosis or lymphoproliferative disease. Enlarged, congested spleens in turkeys are often mistakenly attributed to TAdV-3, but commonly result from bacteremia associated with organisms such as *E. coli*, *Salmonella* spp., *Pasteurella multocida*, and *Erysipelothrix rhusiopathiae*. Additional signs and lesions usually accompany infections with these agents. Gastrointestinal bleeding and mucosal hyperemia may be associated with acute viral (highly pathogenic avian influenza, HPAI; Newcastle disease, ND), bacterial (endotoxemia, ileus induced intestinal autointoxication), parasitic (coccidiosis), or toxic (heavy metals, sulfa drugs) conditions. However, these would rarely be observed without other signs, lesions, or a history consistent with the etiology. Finally, it is important to note that mucosal hemorrhage and sloughing is commonly seen as a result of rapid postmortem autolysis of the intestinal tract.

Pheasants and chickens that die acutely with signs of respiratory distress but without enlarged mottled spleens should be tested for other respiratory diseases including AI, ND, *Syngamus trachea* infestation, and specifically in the case of chickens, infectious laryngotracheitis and infectious bronchitis. Respiratory signs with splenic enlargement and congestion should prompt consideration of bacterial pathogens such as *E. coli*, *Salmonella* spp., and *Pasteurella multocida*. Carbon monoxide, carbon dioxide, and natural gas also should be considered in confinement operations. Splenic enlargement and mottling without demonstration of MSDV or AASV should warrant histopathologic evaluation for neoplastic diseases such as Marek's disease, lymphoid leukosis, or reticuloendotheliosis. Adult chickens with hepatomegaly and/or splenomegaly without mottling should be tested for hepatitis E virus if AASV is ruled out.

Intervention Strategies

Management Procedures

Effective prevention and control of TAdV-3 begins with best management practices, especially biosecurity protocols, because movement of infectious material from one flock to another is the most common means of transmission. To eliminate TAdV-3 from confinement facilities with concrete floors, all organic material should first be removed followed by thorough cleaning and disinfection. In multi-stage/multi-age commercial operations, total elimination of the virus is considered impractical. In such cases, vaccination remains the only effective means of disease control and prevention. Besides biosecurity, flocks should be housed at appropriate temperatures without temperature fluctuation, especially in spring and fall when day and nighttime temperatures may vary significantly, or when birds are moved into a new barn (reviewed in [50]).

Vaccination

Types of Vaccine

It appears that virulence is a stable characteristic because 30 years of continuous vaccine use in the field has yet to produce a verifiable reversion. Avirulent isolates of TAdV-3 have been successfully used as live, water-administered vaccines (17). Two forms of vaccine are currently in use for turkeys. The first is a crude homogenate prepared from spleens obtained from 6-week-old SPF turkeys inoculated with a splenic homogenate containing the Domermuth strain (Virginia Avirulent 1). The second is a commercially available cell culture product (22) which contains an adapted/attenuated virus originally derived from the Domermuth strain. Both vaccines appear to produce adequate seroconversion and protection (6). Only the cell culture vaccine is recommended for use in turkey breeders. Vaccination with a splenic vaccine induces almost immediate protection, and administered even in the face of a field outbreak with a virulent strain can halt the progression of fulminant disease (49). A third method of vaccine production involving the propagation of avirulent TAdV-3 in peripheral blood leukocytes has been described (64) and was temporarily used in Canada. A purified hexon subunit vaccine (65) and a recombinant fiber knob subunit vaccine also have been developed (51), the latter being commercially available where live vaccines are not permitted. In some countries only inactivated (i) TAdV-3-vaccines are commercially available. The application of an iTAdV-3-vaccine once or twice may not sufficiently protect against the infection with circulating strains, possibly due to a poor immune response (25). Recently, antigenic drift was observed in circulating field strains isolated from TAdV-3-vaccinated turkey flocks suggesting an evasion from vaccinal immunity (3).

Field Vaccination Protocols and Regimes

The vaccination schedule is dependent on the farm history and potential exposure as well as the type of vaccine being utilized and/or licensed in the respective

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country. Water vaccination is the method of choice in many countries where live vaccines are licensed and is usually performed between 3.5 and 6 weeks of age. Recent field studies showed that maternally derived antibodies may decline faster in some turkey lines, suggesting an earlier vaccination time point to reduce the risk of field infections and vaccine-induced immunosuppression (18). Interestingly, stress applied on the day of vaccination, for example, through the movement of birds, appears to enhance the response to vaccination, either because it stimulates cell-mediated immunity or permits more efficient viral replication (42). A seroconversion rate of 60% or higher three weeks after vaccination with splenic homogenate is a good indication of 100% protection. A second vaccination one week after the first should be used on farms where seroconversion rates have been historically low (66), especially if cell culture vaccine is used. This phenomenon is likely due to differences between vaccine types in terms of shed rate and subsequent auto-vaccination. No roll-over effect relative to virulence has been noted. Turkeys exposed to immunosuppressive agents prior to vaccination exhibit a reduced response (10). The virus titer per label dose may vary significantly between vaccines from different companies and even between vaccine lots of one company emphasizing the need for a more standardized evaluation of vaccine titers (41).

Live, avirulent, water-administered vaccines are also effective for controlling MSD of pheasants (19, 50), but none are commercially available in the United States. Vaccines for AAS of chickens have not been developed due to the sporadic, subclinical nature of the disease.

Treatment

In the case of an outbreak, barn temperatures may be increased if birds huddle or bunch up, and tilling and litter movement should be discontinued until birds recover to decrease the likelihood of secondary infections. Prior to the advent of effective vaccines, HE was treated by injection of convalescent antiserum obtained from healthy flocks at slaughter (15). Also, due to the immunosuppressive nature of TAdV-3 and the potential for multi-agent interactions, treatment for secondary bacterial infections such as colibacillosis often must be considered. For subsequent flocks on the same premises, correction of management deficiencies and vaccination for other primary agents that may interact with HE vaccine must be considered. Specific treatments have not been described for MSD of pheasants or AAS of chickens.

Quail Bronchitis

Willie M. Reed and Sherman W. Jack

Summary

Agent and Disease. Quail bronchitis (QB) is a naturally occurring, acute, highly contagious, fatal respiratory disease of young bobwhite quail (*Colinus virginianus*) caused by an avian adenovirus. It is a seasonal disease with mortality reaching as high as 30%.

Diagnosis. Respiratory symptoms with gross lesions consisting of mucus in trachea, congested lungs, and caseous air sacculitis are helpful in making clinical diagnosis. Isolation and identification of adenoviruses will confirm diagnosis.

Intervention. There are no specific treatments for QB. Provision of good ventilation and warmth in the houses, without overcrowding will help in preventing outbreaks. In addition to increased biosecurity measures, avoiding mixed age populations, and measures to prevent transmission will be helpful for the control.

Introduction

Quail bronchitis (QB) is a naturally occurring, acute, highly contagious, fatal respiratory disease of young bobwhite quail (*Colinus virginianus*). The disease is of major economic significance to game bird breeders and has a worldwide distribution (1, 2). Quail bronchitis is characterized by rapid onset and high morbidity and mortality, and mainly affects captive-reared birds. The etiologic agent is quail bronchitis virus (QBV). Quail bronchitis virus and chicken embryo lethal orphan (CELO) virus, both aviadenoviruses (Table 9.2), are considered to be the same agent and are not distinguishable using conventional techniques (3, 20). Both viruses produce similar disease and lesions in bobwhite quail and chicken embryos.

Few type I avian adenoviruses, other than QBV and CELO, have been evaluated for pathogenicity in bobwhite quail, with the exception of Indiana C adenovirus. Previous studies (11) have demonstrated that young bobwhite quail are susceptible to infection with Indiana

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C adenovirus, and the clinical disease and pathologic manifestations are indistinguishable from both naturally occurring and experimental infection with QBV.

Quail bronchitis virus is infectious for domestic poultry, including chickens and turkeys, as well as other avian species. The infection is generally asymptomatic, although there is evidence of seroconversion. Although QB/CELO virus has induced neoplasms in laboratory animals, there is no known public health significance (15).

History, Incidence, and Distribution

Quail bronchitis was first described by Olson (16) from a 1949 outbreak in West Virginia. A similar disease in quail had been reported as early as 1933 by Levine, however, and an agent similar to QBV was isolated by Beaudette in 1939. Following Olson's report, several outbreaks were reported in Texas in 1956–1957 and in Virginia in 1959 (4, 5). Infection occurred in three-week-old to mature bobwhite quail, with mortality in some pens reaching 80%. Chukar partridges on the game bird farm did not develop the disease. Circumstantial evidence indicated transmission of QBV from inapparently infected chickens or captive game birds other than quail to the affected bobwhite quail. The most recent outbreak of QB was 2012 in Minnesota, where the disease occurred in quail chicks ranging in age from 5 days to 8 weeks and suffered from respiratory distress and elevated mortality.

Since the early descriptions, QB has been frequently diagnosed as the cause of mortality in captive-reared bobwhite quail. The true incidence and distribution of infection are unknown, but asymptomatic infection in older birds is believed to be widespread. Infection had not been identified in wild bobwhite quail until 1981, when King et al. (14) reported antibodies against serotype 1 avian adenovirus in 23% of mature, free-ranging bobwhite quail collected from a research station. Quail bronchitis remains a seasonal problem in commercially produced quail, at least in the South-Eastern United States. Mortality may reach up to 30% before 6 weeks of age in Bobwhite quail reared for release in hunting preserves.

Etiology

Quail bronchitis is caused by an avian adenovirus. It contains a DNA genome and is icosahedral and nonenveloped, and ranges in size from 69–75 nm in diameter (6). Based on virus neutralization, QBV is fowl adenovirus A species in the genus *Aviadenovirus* (Table 9.2) and is indistinguishable from the Phelps strain of CELO virus (8, 15, 20). QBV/CELO serve as the type strain for fowl adenovirus A. Other techniques have been used to classify avian adenoviruses (e.g., physicochemical properties, hemagglutination, and restriction endonuclease mapping), but they have failed to further clarify the taxonomy of these agents. As with other adenoviruses, avian adeno-associated virus (AAAV) may occur with QBV (23).

Laboratory Hosts and Pathogenicity

Quail bronchitis virus is readily propagated in embryonating chicken eggs and in cultures of chicken kidney or liver cells. Although QBV will grow in chicken fibroblasts, this system is less suitable for cultivation because virus multiplication is poor. Propagation may be interfered with by concurrent AAAV infection (15, 23) or by maternal antibodies in the yolk of embryonating eggs (21, 22).

In most diagnostic laboratories, initial isolation is performed in embryonating chicken eggs, sometimes requiring several blind passages before typical lesions and mortality patterns develop. A common and proven route of inoculation is via the allantoic cavity. High yields of virus can be detected in allantoic fluid 48–96 hours postinfection (PI). Isolation and propagation of QBV using the yolk sac route in antibody-free embryonating eggs is also an effective method. Infection of the embryo by the yolk sac or allantoic cavity results in dwarfing, curling, and stunting of the embryo in 2–4 days. Examination of affected embryos reveals widespread congestion and hemorrhage and enlargement of the liver, with varying degrees of necrosis and hepatitis with intranuclear inclusion bodies.

Experimental inoculation of hamsters leads to various kinds of neoplasms, depending primarily on the route of inoculation. Subcutaneous inoculation results in fibrosarcomas, hepatomas, or hepatic carcinomas, and intracranial inoculation leads to the development of ependymomas (1, 15). Quail bronchitis virus/CELO has not been found to be oncogenic in mice or chickens (15).

Pathobiology and Epidemiology

Natural and Experimental Hosts

Bobwhite quail are the principal species that develop clinical signs and mortality due to infection with QBV. Clinical disease has been reported in Japanese quail. Chickens and turkeys may be experimentally infected but develop few or only mild clinical signs. Inapparent infections of chickens are suggested by serologic evidence (19, 20).

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Transmission, Carriers, and Vectors

Quail bronchitis is highly contagious, as demonstrated by explosive morbidity and mortality in susceptible flocks. Most signs are seen in quail younger than six weeks of age. Although not experimentally documented, transmission is probably by aerosol. However, fecal–oral or mechanical transmission has been documented for other avian adenovirus, and QBV has been isolated from cecal tonsil during experimental infections (12). Serologic evidence of infection of other gallinaceous birds may suggest that even though they fail to develop clinical signs, these species may serve as a vector for QBV.

Incubation Period, Signs, Morbidity, and Mortality

Quail bronchitis is often a catastrophic disease of captive-reared bobwhite quail, which is manifested by respiratory distress that leads to death in young quail. Morbidity and mortality from field cases frequently exceed 50% and may be much higher in flocks affected at younger than 3 weeks of age. In experimental infections of 1-week-old quail, mortality began 2 days following intratracheal infection and subsided by day 9 (7). Mortality in quail inoculated at 3 weeks of age occurred between 6 and 11 days PI. Death is uncommon in birds older than 6 weeks of age.

Frequently, the first reported sign in the flock is a sudden increase in mortality. Closer inspection, however, frequently reveals sick birds that demonstrate decreased feed consumption, ruffled feathers, huddling under brooders, wing droop, open-mouthed breathing, "snicks," and nasal–ocular discharge. Following infection, signs may develop as early as 2 days but generally develop in 3–7 days. Severity of infection varies depending on the age at which the bird is infected. Quail bronchitis is most severe in quail less than three weeks of age. Older birds frequently are asymptomatic but develop antibodies to group I/serotype 1 adenovirus. This suggests that survivors may be immune to subsequent virus exposure, but the persistence of these antibodies and the level of immunity have not been investigated. Antibodies against QBV have been identified in recently hatched quail that did not exhibit adverse signs of infection. These antibodies were lost at between four and six weeks of age, suggesting that there were maternal antibodies.

Gross Lesions and Histopathology

The principal lesions of QB are in the respiratory tract (9). Nasal–ocular discharge also may be noted. Opacity and filling of the trachea by pale, moist, necrotic, and sometimes hemorrhagic exudate is common (Figure 9.13A). On cross section, the mucosa is markedly thickened (Figure 9.13B). Similar exudate may be found in the anterior air sacs. Histologically, tracheal lesions may include epithelial deciliation, cell swelling, karyomegaly, necrosis, desquamation, and leukocyte infiltration (Figure 9.13C). Basophilic, intranuclear viral inclusions are common in intact or desquamated tracheal epithelium. Electron microscopic changes are similar to those seen histologically but also demonstrate phagocytosed viral particles.

In the lungs, red, consolidated areas surround the bronchial hilus (Figure 9.13D). On section, bronchi frequently contain exudate similar to that in the trachea, indicative of a necrotizing, proliferative bronchitis. Inflammatory exudates consisting of lymphocytes, heterophils, and fluid may extend into the surrounding pulmonary parenchyma, but the intensity of the leukocyte response varies and may be confounded by secondary bacterial infections. Histologically, bronchial changes are similar to those in the trachea, except that bronchi may demonstrate more epithelial proliferation. Most lesions are associated with large basophilic intranuclear inclusions (Figure 9.13E).

Lesions in the liver include multifocal pale, pinpoint to 3 mm necrotic foci. Histologically, these foci are characterized by hepatocellular necrosis, infiltrated to varying degrees by lymphocytes and fewer heterophils. Inclusion bodies are occasionally seen in hepatocytes adjacent to necrotic foci and/or biliary epithelium.

Lesions occur in the spleen and cloacal bursa but can be difficult to identify in quail less than three weeks of age. The spleen may be mottled and slightly enlarged. Histologically, affected spleens have multifocal, often extensive zones of necrosis, characterized by lymphocytolysis with increased fibrillar eosinophilic intercellular material, with minimal leukocyte infiltration. Adenoviral inclusions are rare in the spleen. Histologic lesions of the cloacal bursa include necrosis of lymphocytes, frequently accompanied by generalized lymphoid depletion and follicular atrophy. Intranuclear viral inclusions are common in bursal epithelium. Experimentally, some quail also develop necrotizing pancreatitis associated with adenoviral inclusions.

Immunity

The duration of immunity in QB is not known, but survivors of both naturally occurring and experimental infections were refractory to challenge with QBV for at least six months, and significant antibody levels developed in serum of quail following infection (2, 3, 16). Young chicks with maternal antibody also are refractory to challenge with QBV, but maternal antibody is not believed to prevent virus multiplication.

Diagnosis

In quail chicks, sudden onset of rales, sneezing, or coughing that spreads rapidly through the flock and results in mortality suggests QB. Excess mucus in the trachea, bronchi, and air sacs is added evidence of the disease. Severity of signs, rapidity of spread, and the presence of

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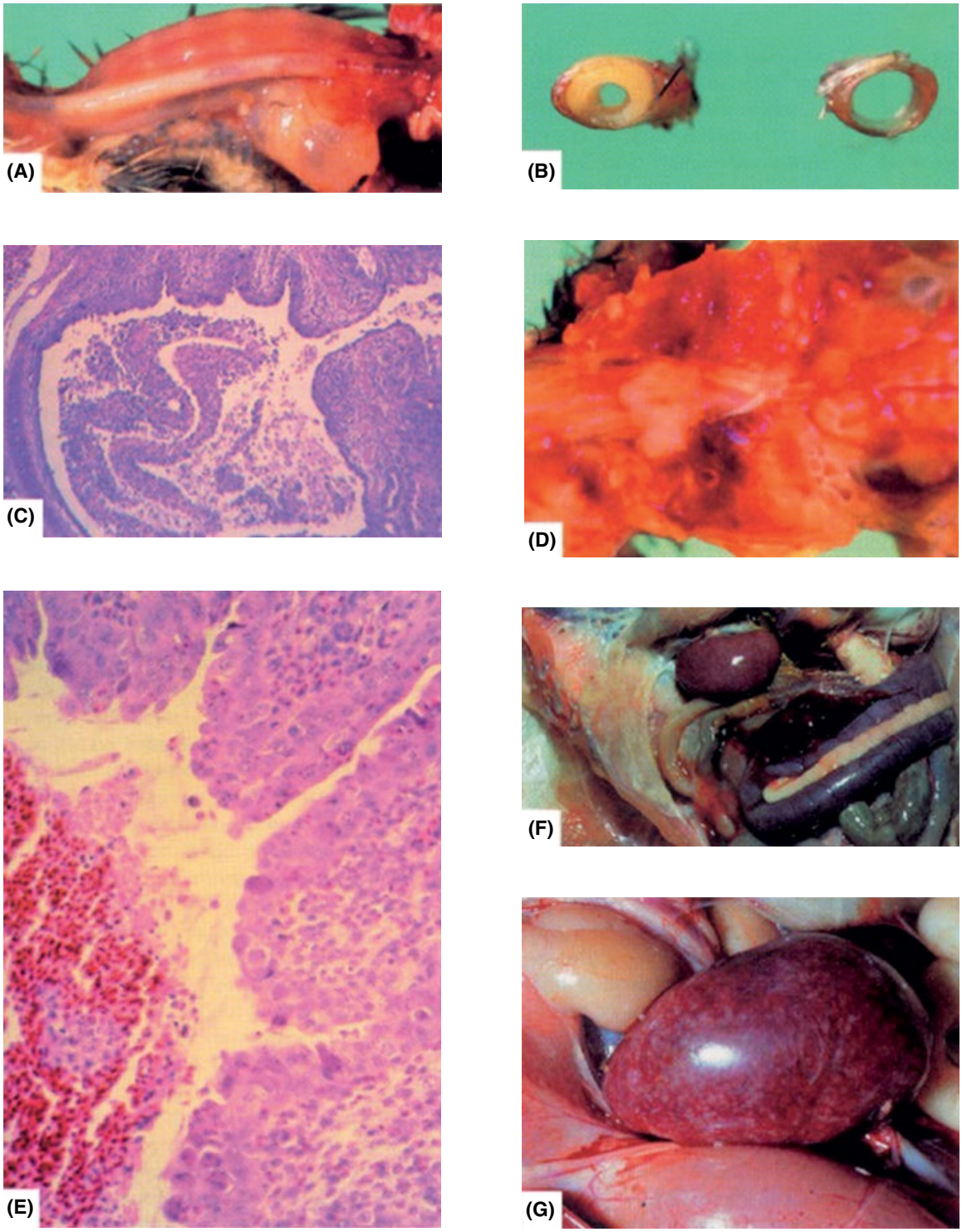


Figure 9.13 A–E Quail bronchitis (QB). (A) Trachea from a young quail chick infected with quail bronchitis virus (QBV). There is opacity of the trachea due to the presence of necrotic exudate. (B) Cross section of the trachea from a young quail chick infected with QBV. The mucosa of the section on the left is extremely thickened, causing partial obstruction, and the section on the right is minimally affected. (C) Microscopic section of trachea from QBV-infected quail. There is epithelial deciliation, cell swelling, necrosis, desquamation, and leukocyte infiltration. (D) Quail chick infected with QBV. The lungs are congested and contain red consolidated areas surrounding the bronchial hilus. (E) Microscopic section of pulmonary bronchus from a quail infected with QBV. There is epithelial cell proliferation, leukocyte infiltration, and luminal exudate. Basophilic intranuclear inclusions are within epithelial cells. (F and G) Hemorrhagic enteritis. (F) Turkey, seven weeks old. Duodenal loop is dark because of bloody contents (one section opened to show contents). Note splenic enlargement and mottling. There is also inflammation of a thoracic air sac (left) typical of acute colisepticemia, which often follows hemorrhagic enteritis virus (HEV) infection. (G) Markedly enlarged and mottled spleen in turkey with HEV infection. (Barnes)

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lesions are less marked in older quail. Isolation and identification of an agent indistinguishable from QBV (or CELO virus) confirms the diagnosis. Inoculation of 9– to 11-day embryonating chicken eggs via the chorioallantoic sac with suspensions of trachea, air sacs, or lungs has been used for isolation of the virus. Yates et al. (23) recommended suspensions of fecal samples or homogenates of the posterior small intestine (ileum) or colon. Jack et al. (12, 13) reported good success in isolating QBV from the liver of naturally infected birds and from the cloacal bursa and cecal tonsils of experimentally infected birds. Three to five blind passages are made with allantoamnionic fluid harvested from chilled eggs up to 6 days or more PI or earlier from embryos that died 24 hours PI or later or that exhibit signs of stunting in daily candling. According to Yates et al. (23), a few strains seem to require inoculation via the yolk sac in 5- to 7-day-old embryos.

Embryo mortality (increasing with number of passages), stunting, thickening of the amnion, necrotic foci, or mottling of the liver, and accumulation of urates in the mesonephros are typical changes caused by QBV or CELO virus. Neutralization of the isolated virus by specific QBV or CELO virus antiserum confirms identification of the virus and the diagnosis.

In general, information pertaining to isolation, propagation, and identification of CELO or any group I/serotype 1 avian adenovirus would be applicable to QBV. Yates et al. (23) noted preference for chick embryo kidney or kidney cell (CK) cultures, and Jack and Reed (10, 11) have described propagation of QBV in chicken embryo liver (CEL) tissue. The agar gel immunodiffusion (AGID) test may be used to place an isolated virus in the avian adenovirus group, but it does not identify the serotype. Serotype classification is based on virus neutralization (10). In the absence of virus isolation or with failure to isolate a virus, the AGID test, using stock antigen on paired sets of serum samples, may be of value. A markedly higher percentage of positive precipitin tests among samples collected during convalescence (2–4 weeks after initial signs) than among sera collected during the acute phase (first few days of signs) should add weight to a presumptive diagnosis of QB based on clinical observations.

Pulmonary aspergillosis may be differentiated from pox by the presence of caseous plugs in lungs or deposits in air sacs with pockets of grayish or greenish spore accumulations. Although bacterial infections might complicate the disease, none is known to cause rapid development of signs, lesions, and mortality typical of QB. DuBose (2) suggested that Newcastle disease might present a clinical picture similar in part to QB, but clinical Newcastle disease has not been described in bobwhite quail. Histologic identification of intranuclear inclusion bodies morphologically characteristic of adenovirus in tracheal or bronchial epithelium is highly suggestive of infection with QBV (9).

Treatment, Prevention, and Control

No specific treatment exists for QB. Increased warmth in the brooder house, adequate ventilation but no drafts, and avoidance of crowding are suggested supportive measures during an outbreak. Prevention is based on protecting susceptible quail from all possible sources of QBV or CELO virus. In addition to the usual sanitation procedures and measures to prevent entry of infectious agents onto the premises, care should be taken to keep adult quail, as well as other avian species, away from young quail. Control measures on a farm should be started immediately, when even a tentative diagnosis of QB has been made. In addition to general measures to prevent transmission from group to group, hatching operations may need to be deferred until two weeks after signs have disappeared to prevent an outbreak in the presence of highly susceptible young quail.

Attempted eradication of QBV from bobwhite quail on a large game bird farm was unsuccessful but may have been responsible for preventing losses and clinical QB over a two-year period (3). In that effort, 80% of the 10,000 quail hatched during the previous year died from the disease. In addition to measures described previously, older quail were marketed, and only survivors from hatches that had been affected at less than four weeks of age were kept for breeders. Virus-neutralization antibody at a high level was detected in three-month-old quail hatched two years later, but no signs of QB were detected in the intervening period up to the time the farm closed the following winter. Winterfield and Dhillon (17) used a type 1 adenovirus serotype in quail chicks as a vaccine against QB. The isolate, designated Indiana C virus, was isolated from chickens (18). It proved non-pathogenic for quail in a laboratory trial and was subsequently used on a farm where QB was endemic and losses were extensive. It was reported that the disease quickly subsided. In other studies (11), however, experimental inoculation of quail at one or three weeks of age resulted in mortality rates of 33–100%. In quail inoculated at six or nine weeks of age, mortality ranged from 0–10%. Gross and histologic lesions included necrotizing tracheitis and bronchitis with pneumonia, necrotizing hepatitis and splenitis, and lymphoid depletion of the cloacal bursa. Based on these findings, Indiana C appears to be highly pathogenic for bobwhite quail and is not recommended for use as a vaccine to prevent QB. More studies on potential use of vaccines to prevent QB are needed.

Cleaning, disinfection, and acquisition of quail chicks from a source without a recent history of QB appear to contribute to prevention. In addition, avoidance of cold stress, ammonia, and dusty environments also appears to minimize clinical problems of QB.

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Hemorrhagic Enteritis and Related Infections

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10

Pox

Deoki N. Tripathy and Willie M. Reed

Summary

Agent, Infection, and Disease. Fowlpox is a common disease of poultry seen in many countries. Caused by the fowlpox virus, a DNA virus belonging to the family Poxviridae, the disease is characterized by production losses and cutaneous lesions, and mortality especially when the birds have more generalized forms of the disease. Fowlpox virus genome encodes for a number of proteins some of which have immunomodulatory roles. Fowlpox is transmitted mostly through mechanical transmission from the contaminated poultry house environment, and insects are also thought to assist transmission.

Diagnosis. A diagnosis of fowlpox requires identification of typical gross lesions supported by histopathology for presence of cytoplasmic inclusion bodies; electron microscopy for the viral particles of typical morphology or polymerase chain reaction (PCR) amplification of specific region of the virus genome.

Intervention. Control is possible by vaccination of susceptible chickens with an attenuated live vaccine in areas where the disease has been prevalent. As the virus spreads slowly, vaccination of all susceptible birds should be considered when the initial signs become apparent.

Introduction

Definition and Synonyms

Pox is a common viral disease of commercial poultry (chickens and turkeys) as well as of pet and wild birds. Of the approximately 9,000 bird species, about 232 in 23 orders have been reported to have acquired a natural pox virus infection (10). Fowlpox is an economically important disease of commercial poultry because it can cause a drop in egg production, slow growth, and unexpected mortality. Pox is a slow-spreading disease characterized by the development of discrete nodular proliferative lesions on the non-feathered parts of the body (cutaneous form) or fibronecrotic and proliferative lesions in the mucous membrane of the upper respiratory tract, mouth, and esophagus (diphtheritic form). Concurrent systemic infections also may occur.

Economic Significance

Mortality in flocks exhibiting the mild cutaneous form of the disease is usually low. However, it may become high with generalized infection, especially when lesions are primarily diphtheritic or when the disease is complicated by other infections or poor environmental conditions. Under those conditions the economic losses can be significant. The systemic form of disease in canaries causes high mortality.

Public Health Significance

Avianpox is not of public health significance. It does not cause productive infection in mammalian species. However, a pox virus isolated from a rhinoceros (44) was characterized as fowlpox virus (FWPV).

History

The term fowlpox initially included all pox virus infections of birds, but now it is primarily used to refer to the disease in chickens. Initially, Woodruff and Goodpasture (94, 95) presented evidence that the virus particles (Borrell bodies) within the inclusion bodies (Bollinger bodies) were the etiologic agent of fowlpox. Later, Ledingham and Aberd (40) demonstrated that antisera produced against FWPV after immunization or following recovery from infection agglutinated suspensions of elementary bodies of FWPV.

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Etiology

Avianpox viruses infecting various avian species (canary, fowl, junco, mynah, pigeon, psittacine, quail, sparrow, starling, turkey, crow, peacock, penguin, alala, apapane, condor) are recognized within the *Avipoxvirus* genus in the family Poxviridae by the International Committee on taxonomy of viruses (http://ictvonline.org/virusTaxonomy.asp) (81, 82). Fowlpox virus (FWPV) is the type species of the genus. Because of its economic importance, more basic and applied studies have been done on it than on any other member of this genus.

Morphology

All avianpox viruses have a similar morphology. The mature virus (elementary body) is brick-shaped and measures about $330 \times 280 \times 200$ nm. The outer coat is composed of random arrangements of surface tubules (Figure 10.1A). Fowlpox virus and other avianpox viruses consist of an electron-dense, centrally located biconcave core or nucleoid and two lateral bodies in each concavity, and are surrounded by an envelope (Figure 10.1B).

Chemical Composition

The main components of FWPV are protein, DNA, and lipid. The virus has a particle weight of 2.04×10^{-14} g and contains 7.51×10^{-15} g protein, 4.03×10^{-16} g DNA, and 5.54×10^{-15} g lipid; nearly one-third of FWPV is lipid. Squalene as a major lipid component and elevation of cholesterol esters were detected in virus preparation from infected chick scalp epithelium (42, 93). The average weight of the inclusion body is about 6.1×10^{-7} mg, 50% of which is extractable lipids. The protein content/inclusion body is 7.69×10^{-8} mg, and the average weight of DNA/inclusion is 6.64×10^{-9} mg (53, 54).

Virus Replication

The cytoplasmic site of DNA synthesis and packaging within the infectious virus particle are characteristics of pox viruses. Related information on replication of pox viruses may be found elsewhere (12, 48).

Fowlpox virus contains genes that encode for a DNA ligase, ATP-GTP binding protein, uracil DNA glycosylase, DNA polymerase, DNA topoisomerase, DNA processing factor, and replication-essential protein kinase (4). In addition, FWPV possesses a gene that encodes for the DNA repair enzyme, CPD photolyase that repairs UV-induced damage to the DNA by using visible light as a source of energy (70, 71).

Replication of avianpox viruses appears to be similar in dermal or follicular epithelium of chickens, ectodermal cells of the chorioallantoic membrane (CAM) of developing chicken embryos, and embryonic skin cells. Differences in the host cell and virus strain, however, may be reflected in the time scale of replication and virus output.

Biosynthesis of FWPV in dermal epithelium involves two distinct phases: a host response characterized by marked cellular hyperplasia during the first 72 hours and synthesis of infectious virus from 72–96 hours postinfection (PI) (16, 17).

The replication of viral DNA in dermal epithelium begins between 12 and 24 hours PI and is followed by the appearance of infectious virus later. Epithelial hyperplasia between 36–48 hours PI ends in a 2.5-fold increase in cell numbers by 72 hours PI. The rate of viral DNA synthesis is low during the first 60 hours of infection. Enhancement in the rate of viral DNA synthesis occurs between 60 and 72 hours PI concomitantly with a sharp decline of cellular DNA synthesis. Between 72 and 96 hours PI, the synthesis of viral DNA becomes progressively more prominent, and no further hyperplasia is observed (15, 16). The genome of FWPV contains six genes with putative protein modification functions. These include three serine/threonine protein kinase (PK), one tyrosine PK, a metalloprotease, and a tyrosine/serine protein phosphatase. These are involved in phosphorylation of virus proteins during virion assembly, viral protein processing, and virion morphogenesis (4).

Based upon the sequence analysis, FWPV encodes homologues of at least 31 known vaccinia virus structural proteins (4), and the majority of them are associated with the intracellular mature virus particle (IMV). Of these proteins, 12 are located within the core and 7 are associated with the membrane. Three proteins also are associated with extracellular enveloped virions (EEVs). In addition, homologues of five proteins, which represent two conserved pox virus gene families and have putative structural functions, are present in FWPV. Also, FWPV contains homologues of pox virus A-type inclusion (ATI) proteins. These inclusions protect mature virions from environmental insults and assist in prolonged survival in nature. Stability of the virus in the environment may be further supported by the presence of photolyase and glutathione peroxidase (4, 71).

Ultrastructural studies have focused on the morphogenesis of the virus in various developmental stages that lead to mature virions (6, 7). After adsorption to and penetration of the cell membrane by FWPV, and within one hour after infection of dermal epithelium or two hours after infection of CAM, there is uncoating of the virus before synthesis of new virus from the precursor material. Few small virus factories containing crescents and few isolated immature viruses (IV) are observed at 12 hours PI. The virus factories increase in size and contain more IV by 16 hours PI. Although the majority of viral particles appear as IV between 16 and 66 hours PI,

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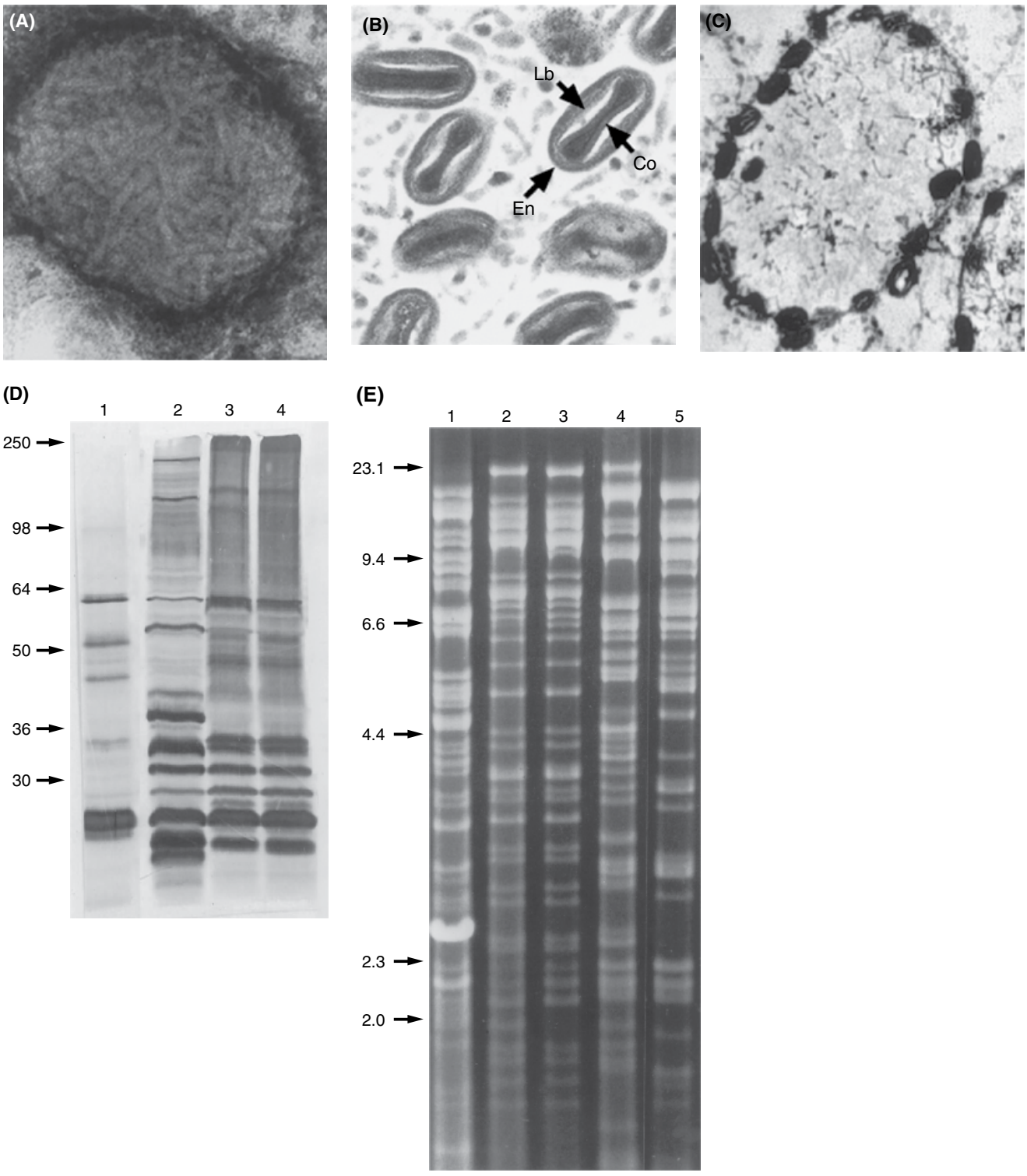


Figure 10.1 (A) Negatively stained fowlpox virus (FWPV) showing random distribution of surface tubules. (B) Ultrathin section of cutaneous pox lesion from a naturally infected dove showing virus particles of typical pox virus morphology. Co = core, Lb = lateral bodies, En = envelope (Basgall). (C) Ultrathin section of diphtheritic fowlpox lesion from a chicken showing an A-type inclusion body in which virus particles of typical pox virus morphology are distributed around the periphery of the inclusion body. (D) Strain variation in antigenic composition by immunoblotting of soluble antigens of avianpox viruses. Antigens prepared from cells infected with FWPV strains: 101 (lane 1), Ceva (lane 2), Minnesota (lane 3), and Nebraska (lane 4). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose. Viral antigens were detected by reaction with chicken anti-FWPV serum. (E) Agarose gel electrophoretic analysis of the genomes of a field isolate of (1) mynahpox, (2 and 3) a vaccine and field isolate of fowlpox, respectively, (4) a field isolate of canary pox, and (5) a vaccine isolate of quailpox virus after cleavage with HindIII. The scale on the left-hand side of the gel represents the position of the l HindIII fragments (kb).

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all subsequent steps of morphogenesis also may be observed. Only a few isolated IMV are detected by 47 hours PI, some of which appear completely or partially wrapped and in the process of becoming intracellular enveloped virus (IEV). Intracellular mature virus particles are also found in clusters associated with membranes. Accumulation of viral particles near the plasma membrane suggests that FWPV exits the cell mostly by budding (12). Inclusion bodies are present at 72 hours after infection of dermal epithelium and at 96 hours after infection of the CAM (5). The A-type inclusions may contain virions within or toward the periphery (Figure 10.1C).

Susceptibility to Chemical and Physical Agents

Fowlpox virus is known to withstand 1% phenol and 1:1,000 formalin for nine days but is inactivated by 1% caustic potash when freed from its matrix. Heating at 50°C for 30 minutes or 60°C for 8 minutes also inactivates the virus (5). Trypsin has no effect on the DNA or whole virus. When desiccated, the virus shows marked resistance. It can survive in dried scabs for months or even years.

Strain Classification

Avianpox viruses are antigenically and immunologically distinguishable from each other, but varying degrees of cross relationships exist. Antigenic characterization of immunogenic proteins by immunoblotting (Figure 10.1D) and genomic characterization by restriction fragment length polymorphism (RFLP) of DNA (Figure 10.1E) have been useful to some extent in detecting minor differences among the strains tested.

A monoclonal antibody has been used to differentiate FWPV strains. Several major and minor immunogenic polypeptides of FWPV strains have been resolved by immunoblotting, which reveals antigenic differences among the vaccine and field strains of FWPV. Similar differences are observed between FWPV and viruses isolated from the wild birds (33–35).

Genomic Differences in Avianpox Viruses

Similar to other pox viruses, the FWPV genome is composed of a single linear double-stranded DNA molecule with a hairpin loop at each end. Although the overall genomic organization of FWPV appears to be similar to that of other members of the Poxviridae family, some genomic rearrangement has occurred. The electrophoretic profiles of restriction enzyme-digested FWPV and vaccinia virus DNAs are distinct. Although the DNA of fowlpox, pigeonpox, and juncopox virus have similar genomic profiles in the RFLP analysis, restriction endonuclease analysis profiles of quailpox, canarypox, and mynahpox virus DNA as well as Hawaiian isolates reveal marked differences from those of FWPV.

The FWPV genome contains a central coding region and two identical, inverted terminal repeat (ITR) regions of 9,520 bp at both termini. The complete sequence of the genome of a vaccine-like strain of FWPV has been determined (4). It contains 288,539 bp, and encodes for 260 putative genes of 60–1,949 amino acids in length. The genome of a tissue culture passaged FPV strain FP9 is approximately 260 kb in size (38). Based upon homologies with other viral or cellular genes, 101 open reading frames (ORFs) of FWPV have been assigned similar or putative functions. The nucleotide composition of FWPV is 69% A + T, which is uniformly distributed over the entire length of the genome. Six small regions with higher G + C content (50%) are located in the terminal genomic regions. Fowlpox virus genome is composed of 31 genes in the ankyrin repeat family, 10 genes in the N1R/p28 family, and 6 genes in the B22R family. The B22R ORFs alone comprise 12% of the viral genome. Because fewer ankyrin genes were found in the genome of FWPV after extensive passage in tissue culture, it is likely that in other avianpox viruses the number of ankyrin repeat genes also may vary. Since pox virus ankyrin repeat genes have been associated with host range functions, loss or disruption of many of these genes may be associated with the narrowing of host range.

Interestingly, the genomes of the majority of field isolates causing outbreaks of fowlpox in vaccinated flocks contain an integrated, nearly intact provirus copy of reticuloendotheliosis virus (REV) In contrast, only variable-length, REV long terminal repeat (LTR) remnants are present in the genome of all FWPV vaccine strains (32, 64, 67). These remnants are also retained, presumably after the loss of the REV provirus, by a minor proportion of each field strain population.

The 365 kbp canarypox virus genome (87) contains 328 potential genes in the central region and in the 6.5 kbp inverted terminal repeats.

Nucleotide sequence analysis reveals many similarities as well as genomic differences with FWPV. For example, canarypox virus encodes 39 genes for which any homologue is absent from or fragmented in FWPV. An intact and highly conserved homologue of cellular ubiquitin gene is disrupted in FWPV, for instance. While the gene encoding for CPD-photolyase in FWPV is also present in the canarypox virus (CNPV), the REV sequences present in the FWPV genome are absent in canarypox virus (19, 32, 64, 67).

Complete nucleotide sequences of two viruses from South Africa, from a penguin and pigeon (50) revealed that they are closely related to each other and more

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closely related to FWPV than CNPV. Nucleotide sequences of genomes of condorpox virus (CDPV), psittacinepox (PAPV), apapanepox (APPV), and Hawaiian Goose (HGPV) show their differences from each other but are more closely related to CNPV (85). Nucleotide sequences of two avianpox viruses isolated from marine birds, pacific shearwaters (*Ardenna* spp) showed the highest degree of similarity with CNPV but significant differences from each other (58).

Immunomodulatory and Non-Essential Genes

The functions of the proteins encoded for by more than half of the genes of FWPV are not known. Some putative genes and the probable functions of their encoded proteins are briefly described here:

- A homologue of the eukaryotic transforming growth factor β (TGFβ), a multifunctional peptide that stimulates connective tissue growth and differentiation, is encoded within the FWPV genome. Because TGFβ also exhibits a range of immunomodulatory effects, including suppression of cellular and humoral immune mechanisms, the FWPV version may have a role in suppression of the host immune response and/or cell growth and differentiation.
- Two ORFs encoding proteins similar to the cellular β nerve growth factor (β-NGF) have been identified in the FWPV genome. These proteins may play some part in inhibiting antiviral immune responses in virus-infected cutaneous and respiratory tract infections.
- Four ORFs of FWPV show similarity to the CC (beta) class of small soluble chemokines. CC chemokines are known to attract T lymphocytes and natural killer (NK) cells to sites of infection. These CC chemokine homologues could function as antagonists and cause a broad-range inhibition of normal CC chemokine function during host antiviral immune responses.
- Three genes encoding proteins homologous to G-protein-coupled receptors are present in the FWPV genome. It is likely that the encoding proteins may bind to chemokines involving cell signaling that affect viral replication and pathogenesis in the host.
- An ORF of FWPV encodes a putative IL-18 binding protein. Because IL-18 homologues have been found to inhibit IL-18 dependent gamma interferon production, it may have an anti-inflammatory function during FWPV infection.
- An ORF of FWPV with homology to semaphorins is likely to be associated with immunomodulatory function.
- The FWPV genome has eight ORFs that encode proteins similar to C-type lectins NKG2 and CD94 proteins present on NK cells and CD69 located on the surface of lymphocytes. C-type lectin cellular NK cell receptors bind class I major histocompatibility complex antigens and promote or inhibit immune activity through intracellular signaling pathways. It is likely that the expression of these proteins in FWPV-infected cells interferes with normal immune surveillance or host responses.
- Five homologues of serine proteinase inhibitors (serpins) encoded by FWPV may be associated with host-range functions involving anti-inflammatory activity and/or regulation of cellular apoptosis in specific cells (4).

One of the characteristic features of avianpox virus infection is cellular hyperplasia of affected tissue. In this regard, a gene encoding a protein similar to epidermal growth factor (EGF) is present in the genome of FWPV. Although this virus protein is not essential for virus replication, it may influence virulence, stimulate cell proliferation, and contribute to the hyperplasia observed in infected tissues. Furthermore, a homologue of the T10 gene that encodes a protein expressed at high levels in epithelial cells in the trachea, esophagus, and lung of vertebrates is present in the FWPV genome. This T10 homologue may be required to extend the virus's host range to epithelial cells of the respiratory tract (4).

The FWPV genome contains an ORF that encodes for a putative protein with similarity to the protein encoded by Marek's disease virus and fowl adenovirus, indicating its role in avian host range function. Interestingly, a natural dual viral infection of trachea by FWPV and herpesvirus has been reported previously (24). Because homologues of FWPV open reading frames were detected in the genome of Marek's disease virus (14), the likelihood of exchange of genetic material from one virus to another and emergence of a genetically and antigenically different virus is possible. In this regard, integration of full-length REV in the field isolates of the FWPV genome indicates an event of natural genetic engineering in viruses. The FWPV genome also contains a homologue of the glutathione peroxidase gene whose product may provide protection from oxidative stress, allowing efficient replication of virus under environmental conditions. Interaction of this enzyme with other proteins (e.g., photolyase) may have a synergistic effect on prolonging the survival of the virus in the poultry environment.

Hemagglutination (HA) activity has been detected in a few strains of pigeonpox virus and in one strain of FPV (26). Although such HA activity was not detected in most avianpox viruses, the nucleotide sequence of a putative HA gene has been identified in the genome of FWPV. A similar nucleotide sequence is also present in the DNA of other strains of FWPV, but functional HA activity could not be demonstrated when using chicken red blood cells. In preliminary studies, the HA gene appears to be nonessential for virus replication in tissue

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culture, but its functional role is not known at this time. Similarly, the thymidine kinase (TK) gene is nonessential for virus replication. Consequently, it has often been used for insertion of foreign genes to create recombinant FPV. Because TK has been associated with virulence, the resulting TK-deleted recombinants are less virulent than the parent FWPV.

Laboratory Host Systems

Birds

A substantial degree of host specificity exists among avianpox viruses, especially those that infect wild birds. For example, a pox virus from a flicker (*Colaptes auratus*) revealed strict host specificity (36) when several species of wild and domestic birds were tested for susceptibility. Similarly, avianpox virus strains isolated from various species of thrushes (Turdidae) did not protect chickens against FWPV. Differences in host susceptibility also were observed when a pox virus isolated from parrots was inoculated into susceptible parrots and chickens. Although it was more pathogenic for parrots than chickens, it did not provide protection against FWPV. Furthermore, vaccination of chickens with either fowlpox or pigeonpox virus vaccine did not provide protection against challenge with psittacinepox virus (11).

A pox virus from a Canada goose (*Branta canadensis*) could be transmitted to domestic geese but not to chickens or domestic ducks. Sparrows and canaries were highly susceptible to a pox virus isolated during an outbreak in sparrows but produced a mild, local cutaneous reaction in chickens, turkeys, and pigeons (28). Chickens and pigeons were found to be refractory to infection with an avianpox virus isolated from a buzzard (*Accipiter nisus*). In an aviary housing more than 100 birds of a variety of species, only Rothchild's mynahs (*Leucopsar rothschildi*) were infected with an avianpox virus. The virus, however, was pathogenic for starlings in the surrounding area but did not infect chickens. Mynahs and starlings are members of the family Sturnidae, and starlingpox has been reported to be specific for birds in that family (39). Pox virus strains isolated from magpies (*Pica pica*) and great tits (*Parus major*) did not infect young chickens. Pox virus isolated from cutaneous proliferative lesions of a greater hill mynah (*Gracula religiosa*) produced severe necrotizing and proliferative lesions in chickens and bobwhite quail previously vaccinated with fowlpox, pigeonpox, or quailpox viruses (55, 56). In an outbreak of avianpox, canaries and house sparrows were affected, although ten species of passerine birds were housed within the facility (20).

Three isolates of pox viruses from Hawaiian forest birds (alala and apapane species) produced mild lesions in chickens. Similarly, two avianpox viruses (Hawaiian goose and palila) produced only a localized lesion in susceptible chickens. The lesion persisted for a short duration and the birds were not protected against challenge with FWPV (35).

Studies on the differentiation of fowl, canary, turkey, and pigeonpox viruses based on pathogenicity for chickens, turkeys, pigeons, ducks, and canaries have been summarized (43). Canaries are highly susceptible to canarypox virus but show resistance to turkeypox, fowlpox, and pigeonpox viruses. Pigeonpox virus produces a mild infection in chickens and turkeys but is very pathogenic for pigeons. Susceptibility of ducks to turkeypox virus and not to FWPV has been suggested for differentiation of these two closely related viruses.

Avian Embryos

Nine- to 12-day-old developing chicken embryos can be used for initial isolation and propagation of avianpox viruses by CAM inoculation (see under "Diagnosis").

Cell Culture

Avianpox viruses can be propagated in cell cultures of avian origin (e.g., chicken embryo fibroblasts, chicken embryo dermis and kidney cells, and duck embryo fibroblasts). A permanent cell line QT35 of Japanese quail origin as well as the chicken liver cell line LMH (31) can support growth of some avianpox viruses after adaptation. While avianpox virus infections of mammalian cells are believed to be abortive, in a study, Syrian baby hamster kidney (BHK-21) cells (89) were found permissive for three avianpox virus strains.

Cytopathic Effects

Characteristic cytopathic effect (CPE) produced by the avianpox viruses in chicken embryo fibroblasts and QT35 cells is characterized by an initial phase of rounding of the cells followed by a second phase of degeneration and necrosis.

Plaque Formation

Differences in the plaque-forming ability of avianpox viruses have been observed. Plaques are evident by 3–4 days PI in quail cells with certain avianpox viruses after adaptation. Adaptation of the virus in cell culture may be necessary because not all strains produce plaques.

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Pathobiology and Epidemiology

Incidence and Distribution

Fowlpox is worldwide in distribution in commercial chickens. The incidence, however, is variable. In high-density areas where multiple-age birds are raised under confined conditions, the disease tends to persist for a long time despite preventive vaccinations. Fowlpox viruses have been isolated in all regions of the United States from previously vaccinated chicken flocks experiencing high mortality due to the diphtheritic form and/or cutaneous form of pox. Cross-protection studies revealed that some of these isolates have a limited immunologic relationship to strains of pox viruses used in commercial vaccines, indicating that currently available vaccines may not be fully effective in providing adequate protective immunity against challenge with these variant pox viruses (22). Clinical veterinarians have reported high prevalence of the disease in various areas of the United States, particularly in the south. It can be a problem in layers in the Midwest and other areas. The disease occurs in a relatively small proportion of layer and breeder flocks and only affects a relatively small percentage of birds within a flock, mostly as dry pox but occasionally as wet pox. It is not uncommon to find a few individual hens with dry pox lesions around their head, comb, and wattles in multiple flocks. The disease occasionally may turn into a flock problem if there is a vaccine or vaccination failure or a preceding immunosuppressive infection with Marek's disease virus (MDV) and/or chicken infectious anemia virus (CIAV). In certain regions of the United States (South Carolina, North Carolina, Virginia, Maryland, Delaware) the disease has low prevalence and moderate significance.

In Europe, the disease is reported to be increasing in prevalence although its significance is moderate. Fowlpox disease occurs sporadically in chickens and turkeys in countries such as Australia, Zealand, Papua New Guinea, and Fiji and is controlled by vaccination. Flocks located in tropical areas are frequently under challenge with FWPV and even two vaccinations appear to be insufficient to prevent a high incidence of cutaneous and diphtheritic infection. Countries where the flocks are infected with FWPV containing REV provirus appear to exhibit a high prevalence of fowlpox in layers and breeders as well as in long-lived broilers (6–16 weeks of age), for example, in China.

Natural and Experimental Hosts

Fowlpox and turkeypox virus infections are economically important diseases in domestic poultry. Among companion birds, avianpox virus infections most often occur in blue-fronted Amazon parrots and in large aviaries of canaries where the disease is likely to be enzootic because of intimate contact. Therefore, canarypox and psittacinepox are especially significant for aviculturists because the disease can result in high losses in a short time. Severe outbreaks of quailpox in pen-raised quails have been reported.

Because of convenience, reasonable cost, and ready availability, susceptible chickens of various ages are used widely as experimental hosts in biological characterization of avianpox virus isolates. Most of the pathogenesis studies, however, have been conducted with FWPV. Pathogenesis of FWPV infection in chickens inoculated intradermally or intratracheally was similar with only minor differences. In chickens infected intradermally, the virus was first detected in the skin at the inoculation site on day two and in lungs on day four, followed by detectable viremia on day five PI. In chickens infected intratracheally, the virus was first detected in the lungs on day two, followed by viremia on day four PI. The virus was recovered from the liver, spleen, kidney, and brain of birds of both groups (63). In chickens inoculated intravenously, miliary nodules were observed in the kidneys at 10–18 days PI in addition to cutaneous lesions and diphtheritic lesions on the mucous membrane of the upper respiratory tract. Characteristic microscopic changes including inclusion bodies were observed in the epithelial cells of renal tubules 4–14 days PI, and in the epithelial reticular cells of the thymic medulla 4–10 days PI (77).

Transmission

Pox virus infection occurs through mechanical transmission of the virus to the injured or lacerated skin. Mechanical transmission of turkeypox virus from infected toms to turkey hens through artificial insemination has been reported. Insects also serve as mechanical vectors of the virus, resulting in ocular infection. The virus may reach the laryngeal region via the lacrimal duct to cause infection of the upper respiratory tract (21). In a contaminated poultry environment, the aerosol generated by feathers and dried scabs containing pox virus particles provide suitable conditions for both cutaneous and respiratory infection. Cells of the mucosa of the upper respiratory tract and mouth appear to be highly susceptible to the virus as initiation of infection may occur in the absence of apparent trauma or injury. Mosquitoes can infect a number of different birds after a single feeding on a bird infected with avianpox virus. Eleven species of Diptera have been reported as vectors of avianpox virus. The mite (*Dermanyssus gallinae*) also has been implicated in the spread of FWPV.

In some flocks, the virus may persist for extended periods. This is common in large multiple-age complexes. Kirmse observed persistent cutaneous lesions of avianpox virus infection in a yellow-shafted flicker over a

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period of 13 months during which intracytoplasmic inclusions were demonstrable in the lesions (36).

Incubation Period

The incubation period of the naturally occurring disease varies from about 4–10 days in chickens, turkeys, and pigeons and is about 4 days in canaries.

Clinical Signs

The disease may occur in one of the two forms, cutaneous or diphtheritic, or both. In addition, a systemic form of infection with high mortality is usually seen in canaries. The cutaneous form of the disease is characterized by the appearance of nodular lesions on the comb, wattle, eyelids, and other non-feathered areas of the body (Figures 10.2A and 10.2C). Cutaneous eye lesions interfere with the bird's ability to find food and water. In the diphtheritic form (wet pox), cankers or diphtheritic yellowish lesions (Figure 10.2B) occur on the mucous membranes of the mouth, esophagus, or trachea with accompanying coryza-like mild or severe respiratory signs similar to those caused by infectious laryngotracheitis virus infection of the trachea (Chapter 5). Lesions in the corner of the mouth and on the tongue, throat, and upper part of the trachea interfere with eating, drinking, and breathing. In pullets coming into lay and in older birds, the disease often runs a slow course accompanied by unthriftiness and reduced egg production. In canaries, clinical signs include respiratory distress; loss of feathers and or/scaly skin on the head, neck, and back; weight loss; and high mortality.

Morbidity and Mortality

The morbidity rate of pox in chickens and turkeys varies from a few birds being infected to involvement of the entire flock if a virulent virus is present and no preventive measures have been taken. Birds affected with the cutaneous form of the disease are more likely to recover than those with the diphtheritic form involving oral mucosa and the respiratory tract.

The effects of pox in chickens usually include emaciation and poor weight gain; egg production is temporarily retarded if layers are infected. The course of the mild cutaneous form of disease is about 3–4 weeks, but if complications are present, the duration may be considerably longer. With virulent strains of FWPV, both primary and secondary cutaneous lesions may persist for more than four weeks. In such cases, cutaneous lesions around the eyes or diphtheritic lesions in the mouth and upper respiratory tract interfere with normal functions resulting in significant mortality.

In turkeys, the retardation of growth development of market birds is of greater financial importance than mortality. Blindness due to cutaneous eye lesions and starvation cause most of the losses. If pox occurs in breeding birds, decreased egg production and impaired fertility may result. In uncomplicated mild infections, the course of the disease in a flock may be 2–3 weeks. Severe outbreaks often last 6, 7, or even 8 weeks.

Flock mortality in chickens and turkeys is usually low, but in severe cases it may be high. In pigeons and psittacines, morbidity and mortality rates are similar to those in chickens. Pox in canaries can cause mortality as high as 80–100%. In a natural outbreak of canarypox virus mortality in excess of 65% occurred in a flock of 450 canaries.

Pathology

Gross

The characteristic lesion of the cutaneous form of pox in chickens is epithelial hyperplasia involving the epidermis and underlying feather follicles, with formation of nodules that first appear as small white foci and then rapidly increase in size and become yellow. In chickens infected intradermally, a few primary lesions appear by the fourth day. Papules are formed by the fifth or sixth day. This is followed by the vesicular stage, with formation of extensive thick lesions (46). Adjoining lesions may coalesce and become rough and gray or dark brown. After about two weeks or sometimes sooner, lesions have areas of inflammation at the base and become hemorrhagic. Formation of a scab, which may last for another 1–2 weeks, ends with desquamation of the degenerated epithelial layer. If the scab is removed early in its development, there is a moist, seropurulent exudate underneath covering a hemorrhagic granulating surface.

Attenuated vaccine viruses produce localized lesions, which are mild in comparison to the severe ones due to pathogenic strains. The secondary lesions produced by pathogenic strains may persist for several weeks (80).

In the diphtheritic form, slightly elevated, white opaque nodules or yellowish patches develop on the mucous membranes of mouth, esophagus, tongue, or upper trachea. Nodules rapidly increase in size and often coalesce to become a yellow, cheesy, necrotic, pseudodiphtheritic, or diphtheritic membrane (Figure 10.2B). If the membranes are removed, they leave bleeding erosions. The inflammatory process may extend into sinuses, particularly the infraorbital sinus (resulting in swelling) and also into the pharynx and larynx (resulting in respiratory disturbances) and esophagus. It is not uncommon to find cutaneous as well as diphtheritic lesions in the same bird. In canarypox virus infection, gross lesions may include thickened eyelids and small nodules on the skin of the head and neck, enlarged thymus, mild to severe

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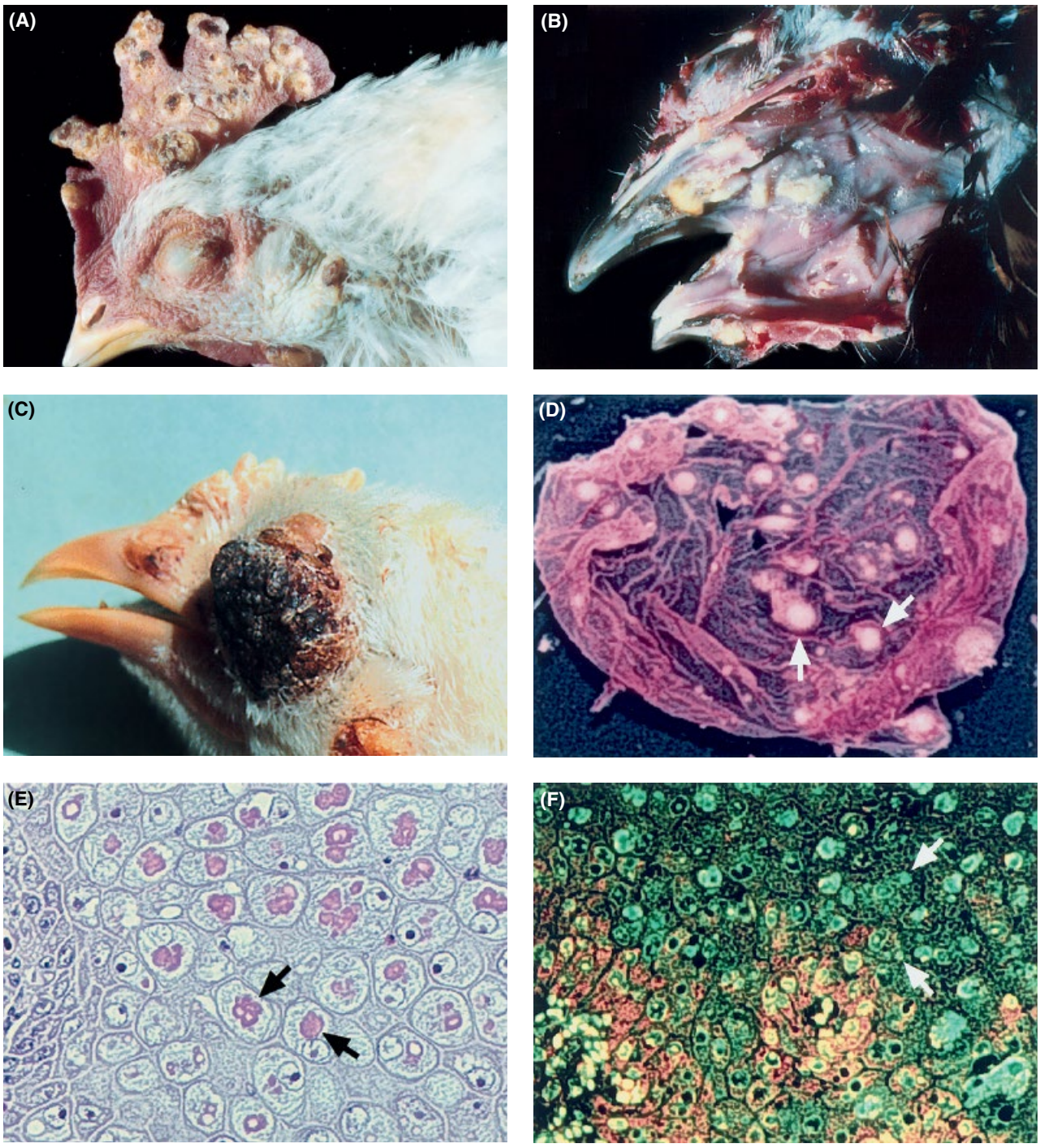


Figure 10.2 (A) Cutaneous fowlpox virus (FWPV) lesions on the comb of a chicken (Shivprasad). (B) Diphtheritic FWPV lesions in the mouth of a chicken (Shivprasad). (C) Cutaneous FWPV lesion on the eye and nostrils of an experimentally infected chicken. (D) Pocks (arrows) produced by FWPV on the chorioallantoic membrane of developing chicken embryo. (E) Microscopic examination of a cutaneous lesion produced by canarypox virus. Eosinophilic cytoplasmic inclusion bodies (arrows) are present in most of the infected cells. Infected cells are enlarged, and some infected cells have lost their nuclei. (F) Microscopic examination of a section of "pock" (as seen in D) stained with acridine orange (AO). Cytoplasmic inclusion bodies containing DNA stain green (arrows) with AO. (*For color detail, please see the color section*.)

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consolidation of the lungs, and exudates in the sinuses and trachea (62).

The first indication of pox in turkeys is appearance of minute yellowish eruptions on the dewlap, snood, and other head parts. They are soft and easily removed in this pustular stage, leaving an inflamed area covered with a sticky serous exudate. The corners of the mouth, eyelids, and oral membranes are commonly affected. Lesions enlarge and become covered with a dry scab or a yellow–red or brown wart-like mass. In young poults, the head, legs, and feet may be completely covered with lesions, which may even spread to the feathered parts of the body.

In some cases, avianpox virus infection may be characterized by cutaneous, diphtheritic, systemic, and oncogenic manifestations (86), while in others the infection may be localized and characterized by the presence of small, pale, firm nodules in some internal organs. For example, in a natural pox virus infection in Galapagos doves (*Nesopelia g. galapagoensis*) small (1–6 mm), pale, firm nodules in the lungs (45) were characterized by lobulated and non-lobulated nodular foci, located mainly in the airways, originating from primary and secondary bronchi. Similarly, in a 3-month-old fledging Andean condor no lesions were found on the entire skin. However, the oral cavity, esophagus, and crop had multifocal raised yellow plaques. Most internal organs including heart, lungs, liver, kidney, small intestine, pancreas, and spleen had single to multiple soft white nodules ranging in size from 0.2–0.8 cm in diameter (33).

Microscopic

The most important feature of infection (whether the lesion is cutaneous, diphtheritic, systemic, or from infected CAM) is hyperplasia of the epithelium with enlargement and ballooning of infected cells, as well as associated inflammatory changes. Characteristic eosinophilic A-type cytoplasmic inclusion bodies (Bollinger bodies) are observed by light microscopy (Figure 10.2E) in infected cells (95). Because pox viruses are the largest among viruses, the elementary bodies can be observed in smears prepared from the lesions after staining by the Gimenez method (82) or with Wright's stain.

Histopathologic changes of tracheal mucosa include initial hypertrophy and hyperplasia of mucus-producing cells, with subsequent enlargement of epithelial cells that contain eosinophilic cytoplasmic inclusion bodies (Figure 10.2E). These inclusion bodies stain green with acridine orange, indicating DNA in the inclusions (Figure 10.2F). Inclusion bodies may be present in various stages of development, depending on the time after infection, and may occupy almost the entire cytoplasm, with resulting cell degeneration. Often, clusters of epithelial cells resembling a papilloma may be observed (76). Rarely, the cloaca and cloacal bursa had thickening of epithelium and interfollicular epithelial layers with typical eosinophilic intracytoplasmic inclusions (51).

Ultrastructure

Ultrastructural features of avianpox viruses are briefly described in the virus replication and diagnostic sections. Because of their large size, typical morphology, and characteristic ultrastructural details, diagnosis of avianpox viruses is relatively easy both under electron microscopy by negative staining or in ultrathin sections (Figure 10.1A–C).

Diagnosis

In spite of antigenic, genetic, and biological differences, pathogenesis of these viruses presents many similarities, which help in rapid diagnosis of the disease. Tentative diagnosis based on clinical signs and lesions, supported by histopathology, is the method of choice to confirm the diagnosis in many laboratories. Details on various diagnostic methods are available elsewhere (81, 82).

Microscopy

Tissue sections from cutaneous or diphtheritic lesions are processed by conventional methods or by using a solution that fixes and dehydrates the tissues simultaneously followed by hematoxylin–eosin (H&E) staining for detection of cytoplasmic inclusions (Figure 10.2E). Cutaneous lesions typical of avianpox (Figure 10.2A, C) and diphtheritic lesions in upper respiratory and oral mucosa (Figure 10.2B) must be confirmed by either histopathology (presence of cytoplasmic inclusions as shown in Figure 10.2E) or by virus isolation. Histopathological examination is also a widely used method for the diagnosis of avianpox virus infection.

Electron microscopy can be used for the demonstration of virus particles in lesions and exudate by negative staining or in ultrathin sections (Figure 10.1A–C).

Isolation and Identification of Virus

Bird Inoculation

Avianpox viruses can be transmitted to susceptible birds by applying a suspension of the lesion material from infected birds to their scarified comb or denuded feather follicles of the thigh, or by the wing-web stick method. Fowlpox virus can be transmitted readily to susceptible chickens, with typical cutaneous lesions developing in 5–7 days.

Avian Embryo Inoculation

Sterile preparations of clinical samples such as cutaneous or diphtheritic lesions can be used for inoculation onto the CAM of 9- to 12-day-old developing SPF chicken embryos. Five to seven days after inoculation, the CAM is examined for pock lesions (Figure 10.2D). In

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some cases the pock lesions may be very minute or there may be slight thickness of the CAM due to very low concentration of the virus in the inoculum. In such cases a second embryo passage is advisable.

Cell Culture

Primary cell cultures of chicken embryo or kidney or secondary cell cultures of avian origin can be used for virus isolation. In this regard, secondary cell lines, for example, QT35 and LMH, will support the growth of some strains.

Serology and Protection Tests

Both cell-mediated and humoral immunity following vaccination or naturally occurring infection provides protection (47, 57, 66). Cell-mediated immunity (CMI) develops earlier than the humoral antibody response. In a study in which both responses were measured, high levels of anti-FWPV antibodies were detected by enzyme-linked immunosorbent assay (ELISA). Seroreactive polypeptides (B cell antigens) of FWPV antigen with molecular weights of 44.5, 66.5, 75, 90.5, and 99 kDa were detected by western blotting analysis. Also significant increases in CMI responses were observed in inoculated chickens as determined by lymphocyte proliferation assay, cytotoxicity assay, and T cell immunoblotting. The predominant T cell antigen of FWPV detected had a molecular weight of 66.5 kDa (57).

ELISA is the test of choice for evaluation of immune response to monitor the vaccination response of birds. Cross-protection tests generally are used to determine immunogenicity of fowl and pigeonpox vaccines. For this purpose, at least 20 SPF chickens are vaccinated according to manufacturer's directions. An additional 20 nonvaccinated and isolated birds of the same source and age are kept as controls. At three weeks after vaccination, vaccinated and control birds are challenged with a different strain of FWPV capable of causing clinical signs of pox in the control birds. The challenge virus may be applied to the skin of denuded feather follicles of the thigh, to scarified comb, or by the wing-web method at a site opposite that used for vaccination. The birds should be examined for takes (see "Immunization"). For satisfactory immunization, at least 90% of the controls should have lesions and at least 90% of the vaccinated birds should not.

Cross-protection tests for the antigenic relationship of the avianpox viruses generally are not practical for routine diagnosis but may be necessary for their biological characterization (13, 35, 56, 64, 92).

Immunodiffusion

Immunodiffusion has been used for the identification of fowlpox and pigeonpox viruses and to differentiate antibody responses due to fowlpox and from those of other avian viral diseases. Although the test is simple to perform, its sensitivity is low and because of cross-reacting antigens differential diagnosis of strains may not be easy. Because precipitating antibodies are detectable for only a short duration after infection, serum must be collected at the appropriate time, usually within 15–20 days after onset of infection. An agar gel precipitation test was used to determine the antibody responses of birds against fowlpox in Nigeria (2).

Passive Hemagglutination

A passive hemagglutination test (79) detects antibodies in the serum of FWPV-infected chickens earlier than the immunodiffusion test. Although this test is very sensitive, its use has been limited because it requires sheep or horse red blood cells for sensitization with soluble pox virus antigens. Furthermore, differentiation of viruses is not possible because of cross-reacting antigens.

Neutralization

Virus neutralization in cell culture or chicken embryos may be used; however, this procedure is not convenient as a routine diagnostic test.

Fluorescent Antibody, Immunoperoxidase, and ELISA

Direct or indirect immunofluorescence or immunoperoxidase tests reveal specific staining of intracytoplasmic inclusions in virus-infected cells. In this regard, immunoperoxidase reaction can be done conveniently with the formalin-fixed infected tissue sections. Such stained slides can be stored for several days without loss of staining.

Immunoblotting

Immunogenic proteins of vaccine and field strains of FWPV can be compared by immunoblotting. Although common antigens are detected (Figure 10.1D), strains can be differentiated to some extent by the presence or absence of unique proteins of differing electrophoretic mobilities (60), Monoclonal antibodies also have been used to characterize field isolates and vaccine strains of FWPV (65).

Molecular Methods

Restriction Endonuclease Analysis of Avianpox Virus DNA

Restriction fragment length polymorphism (RFLP) can be used for comparing the genomes of avianpox viruses by examination of the relative mobilities of restriction endonuclease-generated fragments of their DNAs. The genetic profiles of FWPV strains are similar, with a high proportion of co-migrating fragments, although most strains could still be distinguished by the presence or

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absence of one or two DNA fragments (Figure 10.1E). The characteristic electrophoretic profile of restriction endonuclease-digested DNA has facilitated comparison of other members of the *Avipoxvirus* genus. In this regard, genomic profiles of fowlpox, quailpox, canarypox, and mynahpox viruses are distinct. Similarly, the Hawaiian bird pox viruses, alalapox and apapanepox, have genetic differences that distinguish them from each other as well as from FWPV (30, 34, 60 83).

Genomic Fragments as Diagnostic Probes

Selected genomic fragments or oligonucleotides designed from the published sequences of FWPV have been used as probes in detecting FWPV-specific DNA in the test samples. Crude DNA isolated from the skin or diphtheritic lesion is transferred to a solid surface (e.g., nitrocellulose membrane) and then hybridized with either a cloned fragment or an oligonucleotide which has been radioactively labeled (usually with ^{32}P dCTP) or with a nonradioactive substance (e.g., digoxigenin). This procedure is sensitive and specific and can be used in mixed infections. For example, a dual FWPV and infectious laryngotracheitis virus infection was confirmed using virus-specific genomic probes (24).

Polymerase Chain Reaction (PCR)

Genomic DNA sequences of various sizes can be amplified by polymerase chain reaction (PCR) using specific primers from FWPV or CNPV nucleotide sequences. In the case of mixed infections, fragments of different sizes can be amplified in a single PCR, using pathogen-specific primers. For example, the diphtheritic form of fowlpox and infectious laryngotracheitis produce similar clinical signs and tracheal lesions, accurate diagnosis can be done by using virus-specific primers.

Currently, PCR is being used to differentiate vaccine and field strains of FWPV because the majority of the latter contain intact REV provirus, whereas the vaccine strains contain only REV LTR sequences (3, 9, 15, 25, 30, 32, 41, 52, 69 72–74). In this regard, formalin-fixed tissue sections of avianpox virus-infected samples (e.g., CAM, skin) can be used to isolate the viral genome. Such DNA is used to amplify specific genomic fragments in a PCR reaction. Nucleotide sequences of the amplified fragments can be determined for differentiation of strains and evaluation of their phylogenetic relationships. In considering a virus strain as a putative vaccine, its antigenic, genetic, and biologic evaluation may be required. Because antigenic and biologic characterizations are time consuming, genetic evaluation could be done rather easily. In this regard, to determine the genetic relationship of a vaccine strain of fowlpox vaccine strain, selected genomic fragments were amplified by PCR; the nucleotide sequence of the amplicons was determined and compared with other respective sequences (84).

As all avianpox viruses produce A-type inclusion bodies, primers for amplification of this gene have been frequently used. Similarly, P4b gene is highly conserved, and has been used frequently to discriminate among avianpox viruses. In this regard, analysis of a 578 bp PCR amplified P4b gene fragment has been used to discriminate avianpox viruses. After analysis of sequences from several avianpox viruses, three clades, that is, FWPV or FWPV-like in clade A and CNPV, CNPV-like viruses in clade B and clade C representing psittacines have been described (29). Further, subclades within the clades have been observed. This approach has been used in many cases for phylogenic characterization of avianpox viruses in Egypt, South Africa (1, 49, 50), and other countries. In addition to 4b gene, analysis of nucleotide sequences of DNA polymerase gene has been used in some recent studies. Since complete nucleotide sequences of several avianpox viruses have been determined, nucleotide analysis of other conserved genes will assist in characterization of these viruses.

Differential Diagnosis

Because fowlpox and infectious laryngotracheitis virus can produce similar tracheal lesions in chickens, the diphtheritic form of fowlpox in chickens with associated respiratory signs must be differentiated from infectious laryngotracheitis. In the case of infectious laryngotracheitis (Chapter 5), intranuclear inclusions are detected in the tracheal epithelium. Cutaneous lesions caused by pantothenic acid or biotin deficiency in young chicks or by T-2 toxin (18, 96) could be mistaken for pox lesions. Similarly, diphtheritic pox lesions in doves and pigeons may be mistaken for lesions caused by *Trichomonas gallinae*, which are diagnosed by microscopic examination of smears or by culture.

Intervention Strategies

Management Procedures

Because of its genetic make-up and inherent stability, FWPV can persist in scabs in the poultry environment and become a source of infection for young susceptible replacement birds. The greater frequency of the disease is perhaps due to closer confinement of chickens, especially in large multiple-age complexes. Such conditions provide opportunity for the transmission of FWPV from bird to bird as well as through aerosol. Close confinement and unclean houses increase the opportunity for spreading the disease. Mosquito control has been implemented as an adjunct to vaccination for some turkey flocks in high-risk areas in Australia. Indirect strategies for prevention and control involve the reduction of moisture in the environment such that the insect vectors are minimized.

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Immunization

Vaccines of fowlpox and pigeonpox virus origin are routinely used for vaccination of chickens and turkeys in areas where the disease is endemic. These should contain a minimum concentration of 10^5 EID_{50}/ml to establish satisfactory takes for good immunity (91). Fowlpox and pigeonpox virus vaccines labeled "chick embryo origin" are prepared from infected CAM. Fowlpox virus vaccine labeled "tissue culture origin" is prepared from infected chicken embryo fibroblast cultures.

Vaccination essentially produces a mild form of the disease. Directions for use of vaccine as supplied by the producer should be followed explicitly. In some operations vaccination with one or two strains of FWPV vaccine may be essential for prevention of clinical infection. Often many operations in the United States use both fowlpox and pigeonpox virus vaccines in an attempt to afford optimal protection. Vaccine should not be used in a flock affected with other diseases or in generally poor condition. All birds within a house should be vaccinated on the same day. Other susceptible birds on the premises should be isolated from those being vaccinated. If pox appears in a flock in an initial outbreak with only a few birds affected, non-affected birds should be vaccinated. Proper administration of vaccine prevents the disease.

A vaccine vial should be opened immediately before use. Only one vial should be opened at a time, and the entire contents should be used within two hours. After the vaccine is prepared, the vaccinator's hands should be washed thoroughly. Vaccine should contact the bird only at the site of immunization. Extreme precautions should be taken not to contaminate other parts of the bird, the premises, or miscellaneous equipment. All contaminated vaccine equipment, unused vaccine, or empty vials should be decontaminated, preferably by incineration.

Fowlpox Vaccine

Fowlpox vaccine is commonly applied by the wing-web method to 4-week-old chickens and to pullets about 1–2 months before egg production is expected to start. It is also used to revaccinate chickens held for the second year of egg production. The vaccine is not to be used on hens while they are laying. In Europe, the vaccine is administered at 8–12 weeks of age. Attenuated FWPV vaccines of cell culture origin can be used effectively on chicks as young as one day of age and have been used at times in combination with other vaccines.

Turkeys may be vaccinated by the wing-web method, but the virus may spread and infect the head region. The site of choice for vaccination is about midway on the thigh. Initially, turkeys are vaccinated when they are 2–3 months old, but those to be used as breeders should be revaccinated before production. Revaccination at 3- to 4-month intervals during the laying season might be of some advantage, depending on the level of risk. Fowlpox vaccine is not to be used on pigeons.

In recent years, outbreaks of fowlpox have occurred in all regions of the United States and other countries in chickens that had been vaccinated with either fowlpox or pigeonpox virus vaccines, indicating their inability to provide adequate immunity (75). Often combined fowlpox and pigeonpox virus vaccines have been used in chicken flocks with variable results. In this regard, field isolates of FWPV from vaccinated flocks show variable pathogenicity in chickens. Most of the virus strains isolated during outbreaks in previously vaccinated chicken flocks contain full-length REV in their genome. A comparison *in vivo* of a field strain of FWPV, its genetically modified progeny (in which all REV sequences were deleted), and a rescue mutant (in whose genome the REV provirus was inserted in its previous location) indicated that elimination of the provirus sequences correlated with reduced virulence (68).

Pigeonpox Vaccine

Pigeonpox vaccine contains live nonattenuated, naturally occurring virus from pigeons. If used improperly, the vaccine can cause a severe reaction in these birds. The virus is less pathogenic for chickens and turkeys.

Pigeonpox vaccine may be applied by the wing-web method and can be used on chickens of any age. It is generally applied to chickens at four weeks of age and about one month before egg production is expected to start. When birds younger than four weeks are vaccinated, they should be revaccinated before the start of production. Birds held for the second year of production should be revaccinated.

Turkeys can be vaccinated at any age by the wing-web or thigh stick methods. Day-old poults can be vaccinated if necessary, but it is better to wait until they are about eight weeks old so that a better immune response is obtained. Revaccination may be necessary and advisable during the growing period. Turkeys retained as breeders should be revaccinated. Safety and efficacy of vaccination of 1-day-old turkey poults with fowlpox virus vaccine by subcutaneous route has been determined (59).

Pigeons can be vaccinated by the wing-web method. The vaccine can be applied by the feather follicle method, but this is not generally employed. Differences in the immunizing properties of pigeonpox vaccines have been observed.

Canarypox Vaccine

Vaccination of birds at weaning age is suggested, and booster vaccinations are recommended every 6–12 months and 4 weeks prior to laying or vector season.

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Quailpox Vaccine

A live vaccine of quailpox virus origin was available commercially in the past. Quailpox virus does not appear to provide adequate protection against FWPV infection (23, 92).

Vaccine Takes

The flock should be examined about 7–10 days after vaccination for evidence of takes. A "take" consists of swelling of the skin or a scab at the site where the vaccine was applied and is evidence of successful vaccination. Immunity normally develops 10–14 days after vaccination. If the vaccine is properly applied to susceptible birds, the majority of the birds should have "takes". In large flocks, at least 10% of the birds should be examined for takes. The lack of a take could be the result of the vaccine being applied to an immune bird, use of a vaccine of inadequate potency (after the expiration date or subjected to deleterious influences), or improper application.

Prophylactic Vaccination

Immunization against pox consists of vaccinating susceptible birds prior to the time the disease is likely to occur. Usually this is done during spring and summer in areas where the disease occurs in fall and winter. However, in large complexes containing multiple-age birds and in tropical climates, where the disease may occur throughout the year, vaccination may be performed at any time when warranted without regard to the season.

In ovo Vaccination

Although studies on *in ovo* administration of FWPV vaccines to 18-day-old chicken embryos have provided encouraging results, it is not being used widely.

Recombinant FWPV Vaccines

Potential of FWPV as a Polyvalent Vaccine

Avianpox viruses have contributed significantly in the development of virology, immunology, vaccinology, and viral vector biology. The pox viruses have some unique features (e.g., cytoplasmic site of multiplication, large genome, and unique viral enzymes and transcription system), which allow the expression of foreign genes in a faithful manner. Thus, a large variety of genes encoding antigenic proteins of specific pathogens have been inserted into the genome of FWPV. Because of its large genome size with several nonessential loci, genes from more than one pathogen can be inserted into its genome to create a polyvalent vaccine.

Several nonessential regions, including some in the terminal inverted repeats, have been identified in the FWPV genome and have been used in the generation of recombinant viruses. Experiments with a recombinant FWPV co-expressing Newcastle disease virus (NDV) fusion and hemagglutinin neuraminidase genes and infectious laryngotracheitis virus (ILT) glycoprotein B gene indicated that this vaccine could be used as a potential vaccine for prevention of NDV and ILT by a single immunization (73). With available genetic information only on FWPV and CNPV, development of polyvalent recombinant vaccines for prevention of poultry disease appears feasible. In spite of limited genetic information and antigenic information available on other avianpox viruses, their genomes also can be manipulated toward the development of a new generation of effective poultry vaccines.

Commercially Available Recombinant FPV Vaccines

Several live FWPV-vectored vaccines, for example, Newcastle disease–fowlpox vaccine for subcutaneous or wing-web stab immunization of 1-day-old chickens, is available commercially. Similarly, a recombinant FWPV vaccine expressing genes of ILT virus also has become available. Two recombinant FWPV vaccines expressing H5 hemagglutinin gene of avian influenza virus (8) have been used by the poultry industry for several years in Mexico with highly encouraging results. Details on avianpox virus-vectored vaccines have been reviewed by Weli and Tryland (90).

There has been a concern that immunity generated against FWPV after initial vaccination may prevent the subsequent use of the same recombinant virus containing gene(s) of different pathogens as an immunizing agent. To circumvent this problem, other antigenically distinct avianpox viruses can be considered as vaccine vectors for re-immunization. Although FWPV and CNPV have been used as vectors for recombinant vaccines, other avipoxviruses, for example, quailpox, psittacinepox, sparrowpox, and condorpox, as well as those isolated from Hawaiian endangered birds, also can be considered as potential vectors. Studies with avianpox viruses from endangered Hawaiian forest birds show that these viruses are genetically, antigenically, and biologically different because they produce only mild localized lesions in chickens.

Avianpox Viruses as Expression Vectors for Genes from Mammalian Pathogens

The natural host range of avianpox viruses is limited to avian species. However, these viruses can initiate an abortive infection *in vitro* in cell lines of nonavian origin. Although infectious progeny virus is not produced, foreign antigens are synthesized authentically, processed,

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and presented on the cell surface. In this regard, expression of the rabies virus glycoprotein in recombinant FWPV and canarypox viruses (78) provided a great impetus toward the use of avianpox viruses for the development of vaccines for both man and animals. A canarypox virus-vectored vaccine expressing rabies virus glycoprotein G for use in cats and canarypox virus-vectored distemper vaccine for dogs is currently available commercially. Similarly, a recombinant canarypox virus vaccine expressing antigens of West Nile virus has been licensed for use in equine species.

Treatment

No specific treatment exists for birds infected with avianpox viruses.

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11

Avian Reovirus Infections

Jacob Pitcovski and Sagar M. Goyal

Summary

Agent, Infection, and Disease. Avian reoviruses (ARVs) belong to the Reoviridae family in the genus *Orthoreovirus*. They are nonenveloped viruses composed of a double concentric icosahedral capsid with an external diameter of 80–85 nm that encloses 10 double-stranded RNA genome segments. The virus is ubiquitous among domestic poultry and other avian species, and possible cross-species infection is suggested. The ARV replicates in the gut of avian species and pathogenic strains can affect tendon and liver. There appears to be a wide range of pathogenicity among isolates, but most are harmless. In broilers and turkeys, the virus can cause a significant problem of arthritis/tenosynovitis, which is characterized by swelling of the hock joints and lesions in the gastrocnemius tendons. Avian reoviruses are also associated with other avian diseases, such as stunting–malabsorption syndrome, myocarditis, hepatitis, and respiratory and enteric diseases but their exact role in these conditions is unclear.

Diagnosis. Avian reoviruses can be propagated relatively easily in chick embryos and in cell culture. Diagnostic methods include gross and histopathological lesions, serum neutralization (SN), enzyme-linked immunosorbent assay (ELISA), and reverse transcription-polymerase chain reaction (RT-PCR).

Intervention. Prevention of infection in chickens is mainly by vaccination of maternal flocks to: (1) eliminate infection of breeders that might transfer the virus to progeny; and (2) transfer of maternal antibodies that will protect the hatched chick for the first days of life, which is their most susceptible period. Attenuated and inactivated vaccines, using classical or autogenous strains, are available and it is believed that vaccines composed of a mixture of representative strains that resemble the field strains might confer wide protection.

Introduction

Avian reovirus (ARV) is ubiquitous in commercial poultry, especially in broilers. It is frequently isolated from both healthy and diseased chickens from the gastrointestinal and respiratory tracts (98). The name "reovirus" is an acronym for "respiratory, enteric orphan," since they were first isolated from these sites in humans with no apparent association with disease. Avian reovirus belongs to the genus *Orthoreovirus*, which is one of 15 genera of the Reoviridae family. Poultry diseases associated with ARVs usually result in low mortality but high morbidity and significant economic losses. Though the most important clinical and economical disease is viral arthritis and tenosynovitis (36), ARV induces immunosuppression and is also associated with infection of the liver, heart, and intestine (95). Despite heavy vaccination of breeding hens, offspring are not fully protected, possibly due to the many variants with broad antigenic diversity.

Public Health Significance

There are no reports of ARV as being zoonotic agents.

History

Avian reovirus was first isolated in 1954 from the chicken respiratory tract (19) and later in 1957 from a case of synovitis in chickens (123). The changes in the tendons and tendon sheaths associated with the virus were termed tenosynovitis or viral arthritis in 1967 (11). The virus was eventually identified in 1972 as a reovirus (123). The first commercially available live vaccines were developed in 1983 by 235 serial passages of S1133 strain of ARV in embryonating chicken eggs and 100 additional passages in chicken embryo fibroblast cells (37). Since then, this strain has been extensively used in live attenuated vaccines. More details regarding the history of ARV may be found in the previous editions of *Diseases of Poultry*.

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Etiology

Classification

Reoviridae consists of two subfamilies (Sedoreovirinae and Spinareovirinae) and 15 genera. Viruses in the Spinareovirinae subfamily are characterized by the presence of 12 spikes or turrets at the icosahedral vertices of the virus, which help the exit of nascent mRNA synthesized by core-associated enzymes. Orthoreovirus is one of nine genera under Spinareovirinae, the members of which infect only vertebrates and are spread by respiratory or fecal–oral routes. There are six species in the *Orthoreovirus* genus, namely mammalian orthoreovirus (MRV), avian orthoreovirus (ARV), baboon orthoreovirus, Nelson Bay orthoreovirus, piscine orthoreovirus, and reptilian orthoreovirus.

The reovirus genome consists of segmented, double-stranded RNA (dsRNA). Reovirus RNA is usually regarded as noninfectious. The virus encodes several proteins, which are needed for replication and conversion of the dsRNA genome into positive sense RNAs. The virion has a buoyant density in CsCl of 1.36–1.39 g cm^{-3}. Virus infectivity is moderately resistant to heat, organic solvents, and nonionic detergents.

Although MRVs and ARVs share many biological characteristics, they differ in host range, pathogenicity, coding capacity, and other biological properties (51). The structure and biology of ARVs have been reviewed in detail (5). Since reoviruses of turkeys (TRV), ducks (DRV), and geese (GRV) have distinct molecular characteristics from chicken strains, the use of ARV is often used for chicken-derived reoviruses only (51). Recently, it has been proposed to divide TRVs into TERV (turkey enteric reovirus) and TARV (turkey arthritis reovirus).

Structure and Morphology

Avian reovirus is a nonenveloped virus composed of a double concentric icosahedral capsid with an external diameter of 80–85 nm (Figure 11.1). The innermost protein layer of reovirus particles has an internal diameter of approximately 50–60 nm that surrounds the RNA polymerase and 10 linear dsRNA genome segments with sizes of 1–4 kilobases (kb). Virus structure and genome segments that code for each of the proteins are shown in Figure 11.2. The dsRNA segments are divided into three size classes: L1–L3 (large) encoding λa, λb, and λC structural proteins, M1–M3 (medium) encoding μA and μB structural proteins, and S1–S4 (small) encoding σA, σB, and σC structural proteins. At least 12 primary translation products are expressed, of which 10 structural proteins



Figure 11.1 Avian reovirus particles under the electron microscope. Diameter of the double-shelled virions is 70–80 nm. Phosphotungstic acid negative stain. (Courtesy of R.C. Jones)

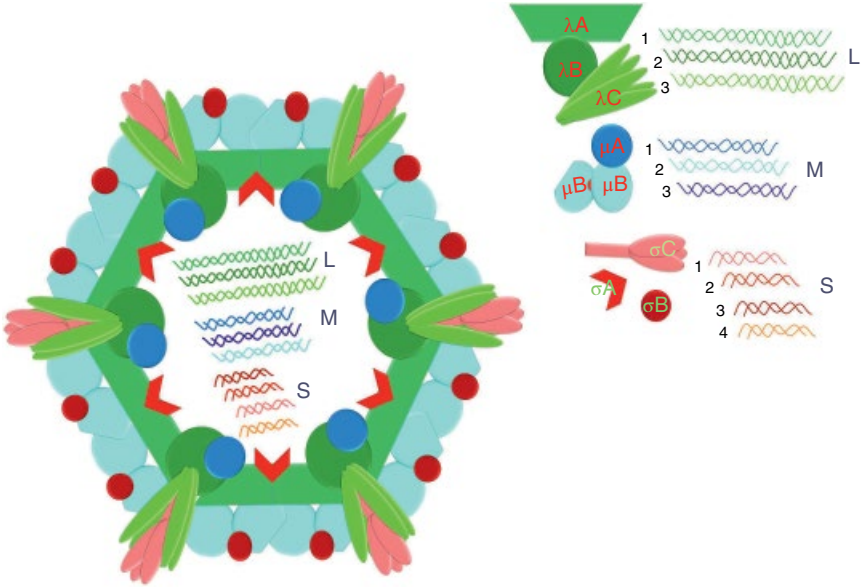


Figure 11.2 Avian reovirus (ARV) structure. Avian reovirus is a non-enveloped virus, with a capsid of an icosahedral symmetry of 85 nm. L, M, and S are double-stranded RNA fragments. All other components are the viral structural proteins. Each RNA fragment encodes a protein of the same color. (Courtesy of Ehud Shahar)

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are incorporated into the virion. Eight are primary translation products and two originate by post-translational cleavage of μB. Several non-structural proteins are expressed by segments M and S (5, 120). The inner core shell of the virus is formed mainly by the λA protein, in addition to λB, μA, and σA which are minor components of the viral core. Also, μB and σB form most of the outer capsid. λC extends from the inner core to the outer capsid of the virion. σC, and a minor capsid protein, is the viral cell-attachment protein and elicits reovirus-specific neutralizing antibodies.

Replication

The replication of avian reoviruses is illustrated in Figure 11.3. The virus attaches to cellular receptors via the σC viral protein followed by receptor-mediated endocytosis. Although the nature of the cell receptor has not been identified, it is estimated that a single chicken embryo fibroblast cell presents 1.8×10^4 receptor sites for ARV (28). Following penetration into the host cell, the virus enters intracellular vacuoles where uncoating takes place (45). Following uncoating, viral cores are released into the cytoplasm. All ten viral mRNA are transcribed using the negative strand as template, catalyzed by virus-encoded dsRNA-dependent RNA polymerase. All transcripts are monocistronic (except S1 fragment that expresses p10, p17 and σC) and are produced in similar amounts (5). This is followed by assembly of the virion within cytoplasmic inclusions (also termed viral factories or viroplasms); the cores are assembled and then coated by outer-capsid polypeptides to generate mature virions (5).

Susceptibility to Chemical and Physical Agents

Avian reoviruses are stable between pH 3.0 and pH 9.0 but are inactivated at 56 °C in less than one hour (50). Avian reoviruses are relatively resistant to disinfectants (79, 84). Reoviruses survived for at least 10 days on feathers, wood shavings, egg shells, and feed (101). In drinking water the virus was detectable for at least 10 weeks with little loss of infectivity. Turkey arthritis reovirus (TARV), TERV, and CARV can survive in poultry litter and drinking water at room temperature (~25 °C). In autoclaved dechlorinated tap water, those three viruses were able to survive for 9 to 13 weeks. In autoclaved litter, the viruses survived for 6 to 8 weeks and in nonautoclaved litter, the survival was for only 6 to 8 days (80).

Avian reoviruses lose infectivity rapidly when suspended in salt-free neutral pH buffer or acidic buffer, and when the viral suspensions are subjected to cycles of freezing

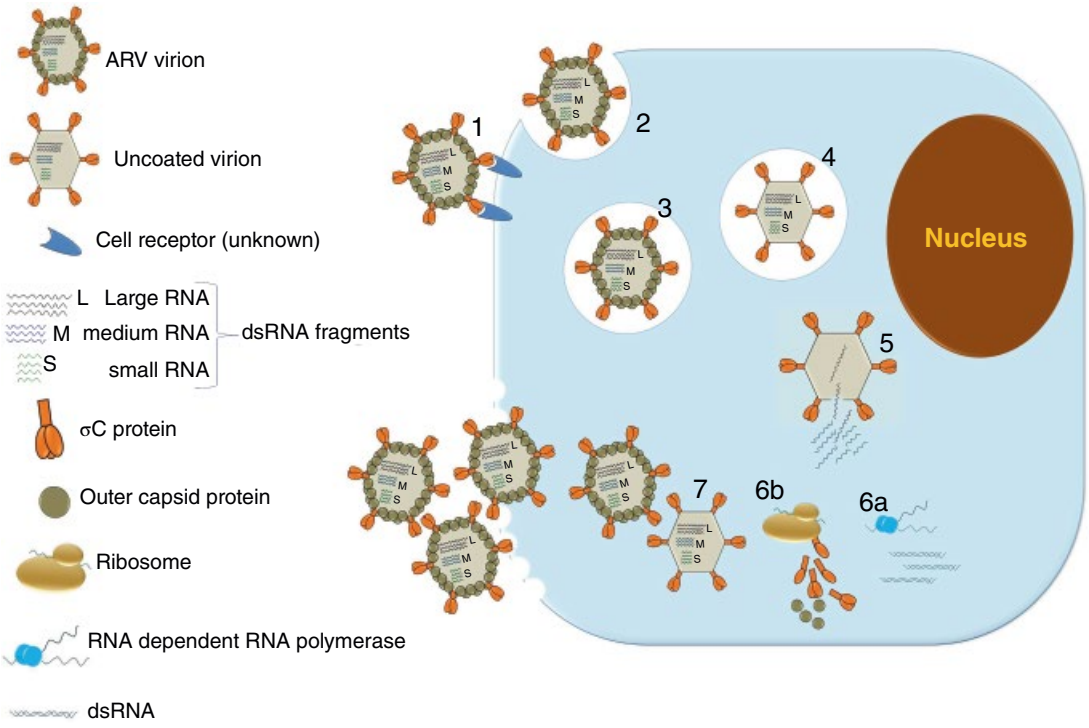


Figure 11.3 Avian reovirus replication cycle. All stages are taking place in the cell cytoplasm: (1) attachment of virion to cell receptor via capsid protein sigma C, (2) cellular-receptor mediated viral endocytosis, (3) uptake by endosome, (4) un-coating of virion (endosome-acidification dependent), (5) release of mRNA into cytosol and transcription of all viral mRNA segments, (6) viral mRNA is used as (a) templates for synthesis of complementary RNA; (b) translation into viral proteins at the ribosomes, (7) virion assembly, and (8) virion exit followed by cell lysis. (Courtesy of Ehud Shahar)

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and thawing. However, purified ARV virions are stable for several months when stored at 4°C in 0.15 M salt buffer at neutral pH (28). The ARVs lose their outer capsid polypeptides upon incubation at 40°C, which may be correlated with the body temperature of their chicken hosts (39.5°C).

Strain Variation

Traditionally, ARVs are classified according to their serotypes or relative pathogenicity for chickens (128). Inoculation of specific pathogen free (SPF) chickens with antigenically similar viruses by various routes, demonstrated strain differences based on relative pathogenicity and virus persistence (112). Based on disease signs, mortality, weight depression, tissue lesions, invasiveness, and viral persistence in chickens, the isolates were classified as being of low, intermediate, or high pathogenicity. Highly pathogenic isolates persisted longer in tissues of infected birds and elicited a rapid and prolonged antibody response. All isolates induced equal frequencies of mortality in chicken embryos.

In the last decade, reoviruses that are antigenically and genetically distinct from vaccine strains have been isolated from chicken and turkey flocks (25, 72, 81, 103, 104, 119, 121). The pathogenicity of Chinese ARVs has changed in recent years making disease control more complex (135).

The pathogenesis of four TERVs (NC/SEP-R44/03, NC/98, TX/98, and NC/85) and one ARV (strain 1733) was tested by infecting SPF poults (89). The TERV isolates were from turkey flocks experiencing poult enteritis and were genetically distinct from previously reported ARVs. No lesions were found in tissues from poults inoculated with the virulent chicken-origin strain although viral antigen was detected in the bursa of Fabricius and the intestine. The TERVs displayed similar tissue tropism but showed substantial differences in the severity of the lesions produced. Poults inoculated with NC/SEP-R44/03 or NC/98 had moderate to severe bursal atrophy, whereas poults inoculated with TX/98 or NC/85 presented mild to moderate bursal lymphoid depletion. In another study, the pathogenicity of three TARVs (TARV-MN2, TARV-MN4, and TARV-O'Neil) and one TERV was compared. The O'Neil strain of TARV was the most pathogenic causing tenosynovitis and clinical lameness followed by TARV-MN2 and TARV-MN4. The TERV-MN1 did not cause tenosynovitis (104).

Strain Classification

Antigenicity

λB and σC proteins of the outer capsid of ARV induce neutralizing antibodies, (125) (for location in virus structure see Figure 11.1); λB induces broadly specific neutralization (125). σC functions in the identification and attachment of the virus to the target cell (75), and is the most variable protein in the reovirus (8, 65, 67). Hence, σC has been found suitable for comparison among strains. As σC induces neutralizing antibodies (125), inefficiency of vaccines in the past decade may be explained, at least partially, by differences in σC between vaccine and field strains (25).

Immunogenicity

Humoral Immune Response. Avian reoviruses possess group- and serotype-specific antigens (94). Neutralizing antibodies can be detected 7–10 days following infection. Maternal antibodies can afford protection to 1-day-old chicks against natural and experimental infections (70). Level of protection conferred by antibodies is related to serotype similarity, virus virulence, host age, and antibody titer (91, 125).

Cell-Mediated Immunity. Recovery from reovirus infection involves both B and T cell activity but protection is predominantly B cell-mediated (60). The experimental suppression of T cell-mediated immunity resulted in increased mortality in reovirus-infected birds, but the relative severity of tendon lesions was unaffected (42). CD8+ T cells may play a major role in the pathogenesis and/or reovirus clearance in the small intestine (108). T cells and plasma cells were the predominant inflammatory cells in the synovium. In the acute phase, T cells, mostly $CD8^+$, were present in low numbers. Most activity was in the subacute phase with increased numbers of $CD4^+$ and $CD8^+$. Aggregates of cells, IgM-positive B cells and plasma cells, were also present. The chronic stage was characterized by large numbers of $CD4^+$ cells (90).

Interferon production induced by ARV has been demonstrated *in vitro* and *in vivo*. The attenuated strain S1133 induced interferon in chick embryo cell cultures, and *in vivo* in the lungs but not in other tissues (17, 18). A pathogenic reovirus elicited the production of interferon detectable in serum samples (17, 18). Viral σA is considered to play a key role in the resistance to antiviral activity of interferon. In mice, inactivated ARV can induce IFN-dependent isotype switching leading to IgG2a antibody responses (126).

Genetic and Molecular

In addition to serotyping and pathotyping, ARVs can also be characterized by molecular methods, usually by comparison of σC-encoding gene and its protein product (67). Although phylogenetic studies classify ARVs into various groups and lineages, meta-analytically there is no identical pattern, which suggests that different ARV genome segments may evolve in an independent manner (44, 67).

Nucleotide sequences of S-class genome segments of ARVs isolated over a 23-year period from different hosts,

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pathotypes, and geographic locations were compared (67). The S1 genome segment, encoding σC, showed noticeably higher divergence than the other S-class genes. Genetic analysis of the σC-encoding gene of ARV isolates enable grouping of field strains into 3–6 genotyping clusters (25, 119). Only part (in one report as low as 22%) of the ARV isolations belonged to the same genotyping cluster as the vaccine strains (S1133, 1733, and 2048) (115). In addition, a new genotype cluster was identified (72).

Phylogenetic analysis of sequence data of twelve TARV, seven in Pennsylvania (over 99% similarity with each other) and five in Minnesota, all isolated in the last decade, were characterized by sequencing of σC gene (117). Comparison of these isolates and additional 25 ARV strains retrieved from GenBank revealed five genotyping clusters (cluster 1–5). All twelve that were recently isolated were in genotyping cluster 2. Comparison of amino acid sequences of the Pennsylvania TARV isolates with ARV vaccine strains (S1133, 1733, and 2048) in cluster 1 revealed that there was less than 56% similarity in amino acid sequences. In northern China eleven ARV field strains were identified from chickens with viral arthritis and reduced growth (135). Comparative analysis of the σC nucleotide and amino acid sequences demonstrated that almost all isolates were closely related to ARV S1133, and clustered in the first genetic lineage.

Pathogenesis

Upon infection of the gastrointestinal tract, some ARV strains invade multiple organs of chickens and establish persistent infections, one manifestation of which is the development of arthritis in the tendons of the chickens. Reovirus isolated from intestinal contents of broiler chickens with malabsorption syndrome produced a transient, but significant, depression in body weight gain when inoculated orally into one-day-old chicks (92). In addition, clinical signs and tissue lesions similar to those observed in field cases were also produced. Avian reoviruses can be transmitted vertically via the egg as well as horizontally following the ingestion of infected fecal material (1). Inoculation of one-day-old SPF chicks with an avian arthrotropic reovirus (strain R2) by oral, subcutaneous, footpad, and intra-articular routes, produced arthritis/tenosynovitis with synovial hyperplasia and lymphocytic infiltration in all birds. One-day-old broiler chicks from vaccinated or unvaccinated breeder flocks were challenged with a virulent form of the vaccine strain. Chicks derived from the vaccinated flock, which had maternal antibodies, had reduced tenosynovitis lesions by about 50%. The rate of virus recovery from the hock joints, however, was similar in both groups (57). The possible epidemiological importance of persistent virus in the joints of clinically protected chicks is not known.

Pathobiology and Epizootiology

Diseases in Chickens

Avian reoviruses are ubiquitous among poultry and are associated with a number of diseases in chickens including arthritis/tenosynovitis, runting-stunting syndrome (RSS), hepatitis, myocarditis, hydropericardium, osteoporosis, malabsorption syndrome (MAS), immunosuppression, respiratory and enteric diseases as well as central nervous system disease (112). Since ARVs are easy to grow *in vitro* they are often considered as the cause of a clinical condition from which they are isolated, although they may actually play little or no part in it. Reports of diseases caused by ARV appear from most poultry-producing areas worldwide, mainly as a disease of broilers. The diseases caused by ARVs in chickens are described below. Since arthritis is the most important economically, and there is clear association between virus and viral arthritis, this clinical outcome will be described in more detail.

Arthritis and Tenosynovitis

Viral arthritis/tenosynovitis in poultry is one of the pathological manifestations of infection caused by different serotypes and pathotypes of ARV (51). Avian reovirus may produce the disease by itself or in combination with other agents, such as *Mycoplasma synoviae* or *Staphylococcus* spp. (36). This may be a cause of differences in the nature and severity of reovirus-induced disease expression. Viral arthritis is an economically important disease of chickens, which mainly affects meat-type chickens but has also been diagnosed in commercial layers and layer breeders (56, 95). Breeder flocks that develop viral arthritis just prior to the onset of or during egg production may be characterized by lameness as well as increased mortality, decreased egg production, suboptimal hatchability/fertility, and vertical transmission of virus to progeny. Shedding of virulent reovirus vertically by a breeder flock may affect progeny and cause severe losses.

In the last decade, reported cases of arthritis have been increasing worldwide (see Economic losses section). Morbidity and condemnations classified as "synovitis" can reach up to 100% of the flock. The disease in chickens is controlled by vaccination (see Vaccination section). The nature of the disease following reovirus infection is dependent upon host age, immune status, virus pathotype, and route of exposure. Birds are most susceptible to ARV at a young age (96). In addition to differences in tissue tropism among strains, a range of virulence exists, from high to virtually harmless.

In commercial chickens arthritis and nonarthritis reoviruses were different when characterized by virus neutralization (92). Recently, some investigators have

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started using the terms CARV (for arthrotropic strains causing chicken arthritis) and CRV (reovirus strains causing other diseases in chickens). In addition to causing arthritis, the CRVs have the potential to induce other pathological changes in chickens including ruptured gastrocnemius tendons, pericarditis, myocarditis, hydropericardium, uneven growth, and mortality (51). In recent outbreaks, genetically variant viruses were detected (see Strain Variation).

Incidence and Distribution. Reovirus infections are prevalent worldwide in chickens, turkeys, and other avian species. Viral arthritis is observed primarily in chickens and turkeys (56, 81, 104). Over the decade there has been a surge of viral arthritis in the United States (13, 72), Canada, Peru, Chile, Brazil, Europe (40, 119), and Israel (25). The virus is also commonly found in the digestive and respiratory tracts of clinically normal chickens and turkeys (51), which makes it difficult to interpret the relationship between virus isolation and disease.

Economic Losses. Clinical outbreaks of viral arthritis caused by ARV can inflict serious economic losses, especially if they appear close to the marketing time of the broilers (4–6 weeks of age). Mortality can be severe due to aortic ruptures and euthanasia of lame birds. Condemnations due to tendon ruptures can also be significant at processing. Once a breeder flock starts shedding virulent reovirus vertically and the progeny is affected, losses can be severe and may last for a long period. Morbidity and condemnations classified as "synovitis" can reach up to 100%, especially following infection with variants that are genetically distinct from the vaccine strains. In tenosynovitis-affected broiler flocks, the economic losses are due to increased mortality (72), general lack of performance including diminished weight gains, poor feed conversions, uneven growth rates, reduced marketability of affected birds (16), and condemnation and downgrading of carcass (119). In severe cases of viral arthritis, early marketing may reduce the economic losses.

Natural and Experimental Hosts

Although reoviruses have been found in many avian species, chickens and turkeys are the only recognized natural or experimental hosts for reovirus-induced arthritis (51). Although clinical signs of arthritis/tenosynovitis are similar in both chickens and turkeys, there is increasing molecular evidence that reoviruses from chickens (ARV) and turkeys are genetically distinct from each other and with those isolated from ducks and geese. Avian reoviruses share only 55–80% nucleotide identities with TARVs based on sequence analysis of S1, S3, and S4 gene segments (82). Experimental evidence of TARVs infecting chickens with some pathology has been reported (106).

Age-associated Resistance

Reovirus-induced arthritis can be readily reproduced in one-day-old chickens that are free of maternal antibody. Birds inoculated at one day or one week of age were more susceptible to reovirus-induced disease than those inoculated at two weeks, indicating an age-associated resistance. The virus is able to infect older birds but the disease generally is less severe and the incubation period is longer (52).

Coinfections

The pathogenicity of reovirus isolates was shown to be enhanced by coinfection with *Eimeria tenella* or *E. maxima* although the outcome depended on the reovirus strain used (99). Exposure to infectious bursal disease virus or some dietary regimes increased the severity of tenosynovitis resulting from infections with ARV (111). Reoviruses also may exacerbate disease conditions caused by other pathogens including chicken anemia virus (76), *Escherichia coli*, and common respiratory viruses (97). A coinfection of reovirus and *Cryptosporidium baileyi* produced systemic infection (31).

Transmission

Reoviruses are transmitted by horizontal and vertical routes although there is considerable variation in the ability of various strains to spread laterally (35). The virus is excreted from the intestinal and respiratory tracts although it is generally shed from the intestine for longer periods, suggesting fecal contamination as a primary source of contact infection. One-day-old chickens were more susceptible to reovirus introduced via the respiratory route than by the oral route (96). Virus may persist for long periods in cecal tonsils and hock joints, particularly in birds infected at a young age (74). This implicates carrier birds as potential sources of infection. Arthritis/tenosynovitis can also be reproduced in turkeys by inoculating TARV via oral, intratracheal, and footpad routes (104). The possibility of avian reoviruses entering through broken skin in the foot and localizing in the hock joint was demonstrated using a trypsin-sensitive strain of ARV (1).

The ARVs can also be transmitted vertically. Following oral, tracheal, and nasal inoculation of 15-month-old breeders, virus was present in chicks from eggs laid 17, 18, and 19 days postinfection (PI) (77). Also, reoviruses were present in chicken embryo fibroblast cells prepared from embryonating eggs derived from experimentally infected hens. Avian reovirus was isolated from liver, intestine, and hock joints of chicks that were hatched

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from eggs laid by virus-infected SPF hens between 5 and 17 days PI, although no virus was isolated from cloacal swabs from these hens (2). It should be noted that the rate of egg transmission was low in all of these studies.

Incubation Period

The incubation period differs depending upon the virus pathotype, age of host, and route of exposure (95). For 2-week-old chickens, the incubation period varied from 1 day (footpad inoculation) to 11 days (intramuscular, intravenous, intrasinus inoculation). The incubation period following intratracheal inoculation and contact exposure was 9 and 13 days, respectively (86). Mature birds inoculated by oral and respiratory routes showed the presence of virus at 4 days PI. The rate of virus isolation was greatly reduced by 2 weeks, and no virus was present at 20 days PI. There was frequent localization of virus in the flexor and extensor tendons of the pelvic limb, although gross lesions were not evident. Footpad inoculation of 1-day-old chickens with an arthrotropic reovirus (R2) produced a more rapid progression of disease than either the oral, subcutaneous, or articular routes (47). When infected by the oral route, the virus was found in the epithelium of the intestine and bursa of Fabricius within 2 to 12 hours PI followed by virus distribution in a wide range of tissues, including the hock joint, within 24–48 hours (54). Some strains cause microscopic inflammatory changes in the digital flexor and metatarsal extensor tendons without the development of gross lesions (51)

The development of joint lesions is a slow process and thus arthritis is usually not seen in birds before 4–7 weeks of age (34). In view of increased resistance with age, the development of lesions at maturity is difficult to explain. Morbidity can be as high as 100% while mortality is generally less than 6%. Viral arthritis in turkeys is predominantly seen in male birds 14 weeks or older (118). However, it is not known if there is an age-linked resistance to infection as seen in chickens. Recently, cases of arthritis were detected in young turkeys at 6–8 weeks of age and virus was isolated from the tendons of poults as young as 1-week of age (Porter, Mor, Goyal, unpublished data).

Clinical Signs

In infected chickens, lameness usually appears at 4–5 weeks of age although the infection may have occurred soon after hatching. In acute infections, some chickens also may be stunted.

Gross

The most severely affected birds exhibit swollen hock joints and enlargement in the area of the gastrocnemius or digital flexor tendons. Rupture of the gastrocnemius tendon may occur in heavy birds (55). In severe cases, birds are immobilized and may be recumbent close to drinkers. The typical uneven gait in bilateral rupture of the tendon results from the inability of the bird to mobilize the metatarsus. The latter is often accompanied by ruptured blood vessels.

In naturally infected chickens, gross lesions consist of swelling of the gastrocnemius, digital flexor, and metatarsal extensor tendons. The first lesion is evident by palpation just above the hock and may be readily observed when feathers are removed (Figure 11.4).

The affected joints usually feel warm. The rupture of gastrocnemius tendon is often perceived as a greenish discoloration of the skin due to extravasation of blood. Removal of the skin at necropsy will reveal the broken end of the tendon (56) (Figure 11.5).

Enlargement of the shank below the hock may suggest swelling of the digital flexor tendons; however, necropsy usually reveals that the swelling is due to sigmoid folding of the flexor tendons, which have ruptured at the level of the hock itself, together with gelatinous fluid. Swelling of footpad and hock joint are less frequent. The hock usually contains a small amount of straw-colored or blood-tinged exudate. In a few cases, there is a considerable amount of purulent exudate resembling that seen with mycoplasma synovitis. Early in infection, there is marked edema of the tarsal and metatarsal tendon sheaths (Figure 11.4). Petechial hemorrhages are frequent in the synovial membranes above the hock.

Inflammation of tendon areas progresses to a chronic-type lesion characterized by hardening and fusion of tendon sheaths. Small, pitted erosions develop in the articular cartilage of the distal tibiotarsus. These erosions enlarge, coalesce, and extend into underlying bone. An overgrowth of fibrocartilaginous pannus develops on the articular surface. Condyles and epicondyles are frequently involved (59). The diaphysis of the proximal metatarsal of the affected limb is also enlarged.

Microscopic and Ultrastructural

In chickens inoculated with an arthrotropic strain of ARV lymphocytes were characterized in the tarsal joint synovium (90). Cryostat sections of whole joints taken from 2–35 days PI were analyzed using monoclonal antibodies directed against B lymphocytes, T lymphocytes, and chicken Ia antigen. The results suggested that the types, numbers, and activation level of lymphocytes in tarsal joints were similar but not identical to those seen in rheumatoid arthritis. Histologic changes were usually the same in both natural and experimental infections (89). During the acute phase (7–15 days following footpad inoculation), edema, coagulation necrosis, heterophil accumulation, and perivascular infiltration are seen along with hypertrophy and hyperplasia of synovial cells,

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Figure 11.4 Edema of digital flexor tendon sheaths in a broiler chicken. (A) Swelling of the right hock joint (left). (B and C) Inflammatory changes in the hock joint including fibrinotic exudation, adhesions, and hemorrhages due to reovirus. (Courtesy of Avishai Lublin) (*For color detail, please see the color section*.)

Figure 11.5 Gastrocnemius tendon rupture in 7-week-old broilers infected with reovirus. (A) Normal gastrocnemius tendons complex in the hock joint, (B) tears, (C) looseness, and (D) separation of the tendons. (Courtesy of Avishai Lublin) (*For color detail, please see the color section*.)

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Figure 11.5 (Continued)

infiltration of lymphocytes and macrophages, and proliferation of reticular cells. These latter lesions cause parietal and visceral layers of the tendon sheaths to become markedly thickened. The synovial cavity is filled with heterophils, macrophages, and sloughed synovial cells. Periostitis is characterized by increased osteoclasts activity. During the chronic phase (starting at 15 days PI), the synovial membrane develops villous processes, and lymphoid nodules are seen. After 30 days, inflammatory changes become more chronic with an increase in fibrous connective tissue and a pronounced infiltration or proliferation of reticular cells, lymphocytes, macrophages, and plasma cells.

Similar inflammatory changes develop in the tarsometatarsal and hock joint areas. Development of sesamoid bones in the tendon of the affected limb is inhibited. Some tendons are replaced completely with irregular granulation tissue. At 54 days PI, orally infected birds show chronic fibrosis of tendon sheaths, with fibrous tissues invading tendons and resulting in ankylosis and immobility. Linear growth of cartilage cells in the proximal tarsometatarsal bone becomes narrow and irregular. Erosions on the hock joint cartilage are accompanied by a granulation pannus. Osteoblasts become active and lay down a thickened layer of bone beneath the erosion. Osteoblastic activity is present on the condyles, epicondyles, and accessory tibia, producing osteoneogenesis and subsequent exostosis (89).

Ultrastructurally, the gastrocnemius tendon and sheath in broilers infected at one day of age were characterized by degenerative changes in fibroblasts including cytoplasmic vacuolization, membrane disruption, loss of ribosomes from the endoplasmic reticulum, and generalized mitochondrial and cellular disruption (42). Lesions found in the heart include an infiltration of heterophils between myocardial fibers, but it is not clear whether this is pathognomonic of reovirus infection (89). The pathogenicity of ARVs for day-old chicks revealed the arthrogenic potential for many strains and ability to cause marked hepatic necrosis (53).

Respiratory Tract Infections

In general, ARVs are not considered as primary causes of respiratory disease in poultry although the first reported avian reovirus caused a mild respiratory disease in baby chicks (78). Another isolate was unable to cause respiratory disease by itself, but in combination with a strain of *Mycoplasma gallisepticum* of low pathogenicity, respiratory signs and lesions were observed.

Enteric Infections

Enteric disease in chickens can occur due to several well-defined pathogens including reovirus. The disease is characterized by delayed growth, low uniformity, lethargy, and watery diarrhea (122). Intestinal content samples from healthy flocks and flocks with diarrhea were tested for astrovirus, coronavirus, reovirus, and rotavirus. About 80% of the tested flocks were positive for one or more of these viruses with coronavirus being the most common (56.4%) and reovirus being the least common (5.6%) (69). In another study it was determined that despite replicating in the intestinal epithelium and causing small intestine lesions including denuding of the villi, none of the tested ARV viruses caused weight gain depression (109). Altogether, it seems that reovirus alone cannot induce intestinal lesions.

Additional Disorders

Occasionally, reovirus produces generalized infections, with several organs affected. In broiler chickens sudden death and starvation were associated with hepatitis, ascites, hydropericardium, pale kidneys, and depleted bursas. Reoviruses were isolated consistently from the tissues of the affected birds. Possibly other factors may interact with the reoviruses to induce these clinical outcome (2).

Reoviruses can experimentally cause hepatitis in immunosuppressed chicks (60). Another disorder associated with reovirus is hydropericardium that leads to poor weight gain, increased feed conversion ratios, and condemnations at the processing plant (3).

Several studies have suggested that reoviruses play a secondary role in runting and stunting syndrome (RSS) that is characterized clinically by growth retardation, lameness, poor feathering, and shank depigmentation. Gross lesions consist of enlarged proventriculus, pancreatic atrophy, and bone abnormalities. Histopathologic changes include hepatitis, nephritis, myocarditis, pericarditis, catarrhal enteritis, pancreatic necrosis, and encephalomalacia.

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Diseases in Turkeys

Arthritis and Tenosynovitis

The sudden appearance of reoviral arthritis in US turkeys is speculated to have been due to reassortment of strains, which produced an arthrotropic virus specific for turkeys (118). However, turkey reovirus-associated arthritis is a well-known phenomenon (87). TARV-associated cases of turkey arthritis resurfaced in the United States as reovirus was isolated from the gastrocnemius tendons and tibiotarsal joint fluid of lame, greater than 12 weeks old, male turkeys in the Midwest in 2011. Affected birds had swollen hock joints and were lame. Morbidity varied from 5–7% and mortality, including culling, was between 2% and 30%. Hens were less severely affected.

Since then, the problem of arthritis/tenosynovitis in 12–18-week-old turkeys has been increasing in the United States and other countries. The lame birds are recumbent with wing tip bruises ("wing walkers"), uni- or bilateral swelling of the hock (tibiotarsal) joints (Figure 11.6), and increased fluid in the tendon sheath and hock joint. In some chronic cases, rupture of gastrocnemius and/or digital flexor tendon has been observed (Figures 11.7 and 11.8).



Figure 11.6 Turkey arthritis reovirus infection. Enlargement of hock (intertarsal) joint of 16-week-old male turkey. (Courtesy of Robert E. Porter)

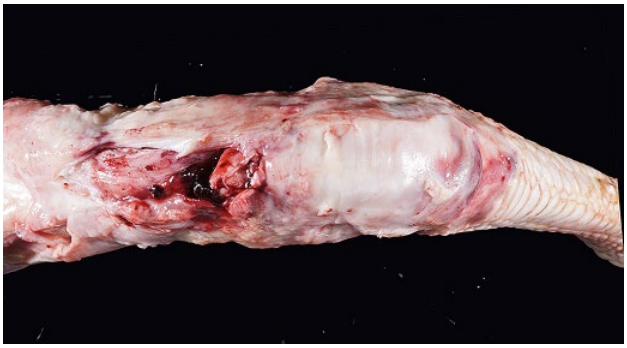


Figure 11.7 Turkey arthritis reovirus infection. Hock joint with skin removed reveals periarticular fibrosis with rupture and hemorrhage of gastrocnemius tendon of 16-week-old male turkey. (Courtesy of Robert E. Porter)



Figure 11.8 Turkey arthritis reovirus infection. Digital flexor tendon is stretched and curled in 16-week-old male turkey. (Courtesy of Robert E. Porter)

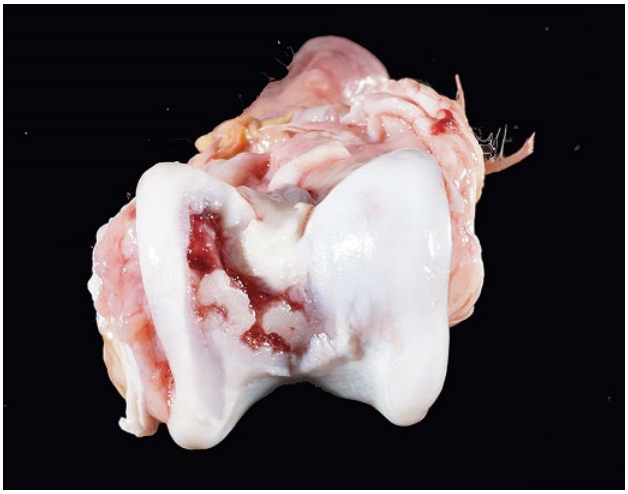


Figure 11.9 Turkey arthritis reovirus infection. Chronically infected male turkeys occasionally show erosion of articular cartilage of the distal tibiotarsus. (Courtesy of Robert E. Porter)

TARV was isolated from gastrocnemius and digital flexor tendon of affected birds (81). Using these viral strains, experimentally reproduced tenosynovitis in turkeys was observed thus fulfilling Koch's postulates; only TARVs produced lameness/tenosynovitis in turkeys and not CARV or TERVs (turkey enteric reoviruses) (104, 106). Lately, another change has been seen in the clinical picture of some cases, for example, cartilage erosions, which have not been previously described (Figure 11.9).

In addition, clinical lameness has been observed in young turkeys at the age of 6–8 weeks. In some instances, lameness affects 35–70% of a flock resulting in huge economic losses to turkey producers from excessive culling, diminished carcass quality, and reduced market weights in addition to raising animal welfare concerns. TARVs have a unique ability to produce gastrocnemius tenosynovitis in turkeys. Administration of TARV O'Neil strain through the oral or intratracheal route is a reproducible model to study pathogenesis of TARV infection,

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and there is a difference in the pathogenicity of various TARV strains (104).

Oral inoculation of TARV-O'Neil in one-week-old turkey poults produced lymphocytic tenosynovitis at four weeks postinoculation (106). However, clinical lameness was first displayed at 8 weeks of age while ruptured gastrocnemius tendons with progressive lameness were seen at 12–16 weeks of age. Histologic inflammation scores of tendons at each time point were significantly higher in the virus-inoculated group than in the control group ($p < 0.01$). Lesions began as lymphocytic tenosynovitis with mild synoviocyte hyperplasia at four weeks of age and progressed to fibrosis as the birds aged. These results demonstrate the potential of TARV to infect young turkeys and to produce subclinical tenosynovitis that becomes clinically demonstrable as the turkeys age.

The location and extent of virus replication as well as the cytokine response induced by TARV-O'Neil during the first two weeks of infection after oral inoculation of one-week-old male turkeys were characterized as well (105). Viral copy number peaked in jejunum, cecum, and bursa of Fabricius at 4 days PI but increased dramatically in leg tendons at 7 and 14 days PI. Minimal number of copies were detected in internal organs and blood during the same period. The virus was detected in cloacal swabs at 1–2 days PI but it peaked at 14 days PI indicating enterotropism of the virus and its early shedding in feces. Elevation of IFN-α and IFN-β was observed in the intestines at 7 days PI and a prominent T helper-1 response (IFN-γ) at 7 and 14 days PI. IFN-γ and IL-6 were elevated in gastrocnemius tendons at 14 days PI. Elevation of antiviral cytokines in intestines occurred at 7 days PI along with a significant decline of viral replication. T helper-1 response in intestines and leg tendons was the dominant T-helper response. These results suggest a possible correlation between viral replication and cytokine response in early infection of turkeys with TARV.

Enteric Disease

Enteritis in turkey poults is a major cause of morbidity and mortality and turkey reoviruses are associated with this condition (48, 49, 85, 129). However, turkey reovirus can also be detected in apparently healthy turkey poults, and some of these isolates experimentally have been found to be pathogenic and produce enteritis and destruction of intestinal villi, while others are nonpathogenic or of low pathogenicity (15). Gastrointestinal transit time in reovirus-infected turkeys was found to be significantly longer than in normal turkeys (26).

Turkeys infected with enteric reovirus developed diarrhea with pasty (fecal covered) vent feathers and vent skin that was necrotic and hyperemic. Necropsy revealed distension of small intestines and ceca with watery contents and gas (85). Microscopically, intestines showed mild crypt enterocyte hyperplasia with lymphocytic infiltration in the submucosa at 1–2 weeks PI (89). Reovirus was present in intestinal contents of about 40% of chicken with poult enteritis syndrome and in turkey flocks (89), respectively. In the United States, a condition in young turkeys called poult enteritis and mortality syndrome (PEMS) caused major losses to the turkey industry in the 1990s. Its main features include stunting and poor feed utilization resulting from enteritis (4). In the more severe form, runting, immune dysfunction, and up to 100% morbidity and mortality are reported. The etiology of the disease is not completely understood, but enteropathogenic *E. coli*, coronavirus, astrovirus, and reovirus have been isolated from such cases. It was experimentally shown that while PEMS reovirus isolate ARV-CU98 did not induce fulminating PEMS in turkey poults, it was able to cause some of the typical clinical signs including intestinal alterations and significantly lowered liver and bursa weights (33). The implied involvement of the three viruses in PEMS led to the development of a multiplex real-time RT-PCR for the detection of turkey coronavirus, astrovirus, and reovirus (110). In an electron microscopic study of viruses associated with PEMS in California turkeys from 1993 to 2003, reoviruses, birnaviruses, and adenoviruses were less common than rota-like and small round viruses (129). Various combinations of turkey astrovirus, rotavirus, and reovirus infections were inoculated in 3-day old poults (110). The most marked signs of enteric disease were found when all three agents were present, with reovirus being the least pathogenic component. Field evidence supported the experimental findings that the presence of two or three different viruses in combination are likely to affect the dynamics of enteric disease in turkeys (48).

Myocarditis

Turkey reoviruses have also been associated with myocarditis as demonstrated by isolation of virus from the heart of a 17-day-old turkey poult showing increased fluid in epicardium and myocardium along with macrophages and plasma cells infiltration within necrotic myocardium (89, 107). A retrospective study of cases of myocarditis associated with reovirus from 1991 to 2009 concluded that reovirus was a likely cause, with vitamin E deficiency also contributing to the condition (20).

Immunosuppression

The potential for reoviruses to cause immunosuppression in turkeys has been described. Following infection of groups of poults with four reoviruses of turkey-origin there were variable degrees of cloacal bursal atrophy and lymphoid depletion in bursa and spleen, depending on the strain. No such lesions were produced with virulent chicken strains, although viral antigen was detected in the intestine (14, 89).

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Diseases in Ducks and Geese

Duck reoviruses (DRVs) have been isolated from several species of ducks, including mallards, healthy Pekin ducks, and diseased ornamental ducks. All DRVs share a common group antigen with chicken reoviruses. However, two duck reovirus strains that were examined by immunoprecipitation and virus neutralization were antigenically distinct from standard chicken strains (32). Reovirus isolated from Pekin ducks were able to cause microscopic lesions of tenosynovitis in SPF chicks. Specific ELISAs for detecting antibodies to duck reoviruses have been developed, including a σB-σC test using proteins that are expressed in *E. coli* (133). No satisfactory vaccines are available but a baculovirus-expressed σC alone or with expressed σB appears to be a good candidate (61).

Muscovy duck reovirus (MDRV) typically affects young ducklings at 2–4 weeks of age, causing diarrhea, difficulty in movement, and high morbidity. Mortality can be 10% or higher in ducklings. In 2002, a new disease emerged in Muscovy ducks and geese in China, which was named as hemorrhagic necrotic hepatitis and the virus isolated from these cases was named as the novel duck reovirus (NDRV) (10, 132). Based on the σC gene sequence a separate classification for MDRV was suggested (61, 134).

Goose reovirus (GRV) is the causative agent of arthritis in geese. The disease is characterized by splenitis with miliary necrotic foci during the acute phase, and epicarditis, arthritis, and tenosynovitis during the subacute/chronic phase (88). Clinical signs usually appear at 2–3 weeks of age and persist for 3–6 weeks. The virus isolated from several organs of the diseased birds reproduced the disease in young goslings. Serological evidence of reovirus infection in wild geese has been reported, but with no clear association with disease (43).

Recently, the complete genome sequences of DRVs and GRVs have been reported (12, 124, 132). Molecular sequencing of the σC gene has shown that DRV and GRV belong to a different species of orthoreoviruses although they share a common ancestor with the chicken virus.

Diseases in Other Avian Species

The ARVs have also been isolated from cases of enteritis in wild birds including pigeons, grey parrots, quail, and pheasants (27, 83, 93, 100). Serological surveys have shown the presence of antireovirus antibodies in several other avian species, for example, ostriches (*Struthio camelus*) (7), rockhopper penguins (*Eudyptes chrysocome*) (58), bean geese (*Anser fabalis*), and white-fronted geese (*Anser albifrons*) (43). All these reoviruses share a common group antigen with chicken reoviruses. Their importance as pathogens in the host species has not been determined (51). More details regarding reovirus isolation from other species may be found in the previous editions of *Diseases of Poultry*.

Diagnosis

Diagnosis of viral arthritis can be made on the basis of clinical signs and gross and histologic lesions. However, those signs are not pathognomonic and some joint lesions may resemble those caused by *Mycoplasma synoviae*, staphylococci, or other bacteria. Involvement of metatarsal extensor and digital flexor tendons (Figure 11.4), and sometimes heterophil infiltration in the heart, assist in differentiating reovirus arthritis from similar disease caused by other agents (41). Nevertheless, demonstration of virus in clinical material is required in order to confirm the cause. This can be accomplished by virus isolation and/or RT-PCR.

Detection of Virus

Virus Isolation

As avian reoviruses are ubiquitous and mostly not pathogenic, isolation of virus from the intestine alone is likely to be meaningless in interpreting the cause of joint lesions. Virus isolation from hock joint tissues can be taken as an evidence of a causal relationship. However, the virus cannot be isolated from joints in advanced stages of infection. The tissues of choice for virus isolation are hypotarsal sesamoid bone with the tendons that pass through it, synovial membrane, and articular cartilage. Specimens can be stored at 4 °C temporarily or for longer periods at −20 °C or below.

Reoviruses propagate readily in embryonating chicken eggs following inoculation via yolk sac or chorioallantoic membrane (CAM) with mortality occurring after 3–5 and 7–8 days postinoculation, respectively. The virus can also be propagated in various cell cultures including Vero, BHK-21, Crandell-Reese feline kidney (CRFK), Georgia bovine kidney (GBK), rabbit kidney (RK), porcine kidney (PK-15), LMH (ATCC CRL-2113), Japanese quail (QT35), and chicken lymphoblastoid cells as well as in primary kidney cells from 2- to 6-week-old chickens and in primary embryo liver cells. Chicken embryo fibroblasts are suitable but the virus often requires adaptation. Chicken-origin cell cultures infected by reovirus are characterized by the formation of syncytia, which may occur as early as 24–48 hours, followed by degeneration of the monolayer and giant cells floating in the medium. Infected cells exhibit intracytoplasmic inclusions that may appear either eosinophilic or basophilic. After isolation, the virus can be identified by electron microscopy, immunofluorescence, or by RT-PCR and sequencing.

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For isolation from joints, tissue samples are more likely to yield virus than swabs. Pieces of hypotarsal sesamoid bone with the tendons that pass through it, synovial membrane and articular cartilage are the tissues of choice. Joint material should be taken from infected as well as apparently healthy birds as the number of clinically affected birds in a flock may be relatively small but virus may be detected before lesions have developed.

Localization of Virus in Tissues

Lesion-associated reovirus proteins or nucleic acid can be detected in formalin-fixed tissue using immunohistochemical procedures (114) or *in situ* hybridization (64). The probes used in various nucleic-acid methods are based on genes or gene-regions that are relatively conserved among different strains. Demonstration of reovirus antigens in cryostat sections of snap-frozen tendon sheaths and other tissues by fluorescent antibody staining is a rapid alternative method of diagnosis in the early stages of infection (57).

Molecular Methods

RT-PCR, with the advantages of being a rapid, specific, and sensitive method, has become universally used for general identification of ARV (68). RT-PCR have been developed for screening of vaccines (6), simultaneous detection of multiple avian viruses (9), and for universal detection of all ARVs or reference strains of chickens, pheasants, and turkeys (116). A one-step RT-PCR for the detection of turkey reoviruses was recently described (82). A rapid, specific, and sensitive TaqMan real-time RT-PCR, which uses a primer-probe set from the conserved region of σ4 genome segment, was developed (29). Genetic characterization of ARV isolates is performed by sequencing, mostly of σC gene, followed by bioinformatics analysis (see Strain Variation). Full genomic characterization of newly emerging ARV strains may be performed by using next-generation sequencing (NGS).

Determination of Pathogenicity

In spite of extensive molecular studies, there are no recognized markers for pathogenicity of avian reoviruses. Arthrotropic potential of a reovirus obtained from an affected joint can be confirmed by inoculation into the footpad of 1-day-old susceptible chicks. If pathogenic, the virus will induce a pronounced inflammation of the footpad within 72 hours (73). A more natural but prolonged effect may be achieved after oral infection.

Serology

Determination of anti-ARV antibody levels is a useful indicator of immunity following vaccination or infection. The ELISA commercial systems are widely used for assessing reovirus antibody levels on a flock basis (66). These tests are efficient for the detection of antibodies to ARV but not to TERV and TARV. ELISA methods were developed using whole virus antigen, and recombinant σC and σB (66) or σA in monoclonal antibody-based competitive ELISAs (63). A significant correlation was found between the level of anti-ARV antibodies in sera as determined by ELISA and virus neutralizing antibodies (46). Since reovirus infections are widespread among commercial flocks and there might be more than one serotype simultaneously, the diagnostic value of serological profiling is often difficult to interpret with the currently available ELISA kits. The use of variable regions of σC from four prototypes of ARVs or two non-structural proteins as antigen in ELISA, enabled the differentiation of infected from vaccinated chicks (DIVA) (23, 131), respectively. Serotype differences among virus isolates is routinely determined by virus neutralization test (128).

Prevention and Control

Biosecurity

The ARV is ubiquitous in chicken farms with prevalence of up to 100% (122). Maintaining freedom from infection in modern, intensively housed chicken flocks is difficult but biosecurity procedures can reduce prevalence of infections. These procedures include elimination of contaminated food and water that lead to ARV infections being spread through the fecal–oral route, avoiding multi-age farms that can circulate the virus between older and younger birds and cleaning out and disinfection of affected barns. Avian reovirus is relatively stable but multi-component disinfectants are considered to be effective inactivating agents (80).

Vaccination

The control of viral arthritis is facilitated mainly by vaccination of breeders to induce high levels of neutralizing antireovirus antibodies. Those antibodies are expected to prevent infection of the breeder flock that might be followed by vertical transmission of the virus to offspring via the yolk sac (51), and to deliver maternal-derived antibodies to the progeny (38, 113). The most susceptible age for pathogenic reovirus infection is in the first few days of life (98). Maternal antibody half-life in the chick is about 5 days, and at 10–15 days of age the level of maternal antibody is nonprotective (21). Appropriate vaccination of broiler breeders can contribute to achieve high effective antibody levels at hatch and prolong the period of protection (39).

Vaccination can be performed by live attenuated or inactivated vaccines. The live attenuated vaccines that are in use worldwide are based on S1133 strain of ARV

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that was developed in 1983. Until recently, most inactivated vaccines were derivatives of S1133, such as strains 2177, 1733, and 2408. All these strains are almost identical to each other in the amino acid sequence of σC that induces the production of neutralizing antibodies. However, in the last decade, following the emergence of new antigenically different variants, inactivated autogenous vaccines are used, which greatly reduces the prevalence of viral arthritis (102). Those autogenous vaccines need to be updated on a regular basis because of the emergence of variant strains of the virus. Since many variants are circulating in the field, it is necessary to determine, based on sequence and serological studies, which of the isolates will be used as a representative autogenous vaccine and confer the widest protection against the circulating genotypes (25). Vaccination with a combination of representatives of genotypic groups can provide broad protection against a range of field viruses (73).

There is no consensus on vaccination regime but combination of vaccination with live attenuated virus followed by inactivated vaccine was found to induce the highest levels of antibodies (127). Live vaccines should be administered only prior to the onset of egg production in order to prevent trans-ovarian transmission of the vaccine virus (22). The common protocols for vaccination of broiler breeders include 1–3 live attenuated vaccines up to 12 weeks of age followed by 1–3 inactivated vaccines. Live and inactivated vaccines are administered via intramuscular or subcutaneous routes. The vaccination is expected to protect against tenosynovitis as well as the malabsorption syndrome.

The generally accepted method of assessing the efficacy of reovirus vaccines is by challenge of 1-day-old chicks via the footpad method using the autogenous virulent virus three weeks post vaccination. Effective vaccines will reduce footpad swellings but sometimes the results are difficult to interpret (71).

Several novel approaches to reovirus vaccination have been reported. *In ovo* vaccination of ARV in complex with antibodies was found safe (30). Numerous studies describe the use of subunit vaccines that induce virus neutralization antibodies, including σC and σB expressed in a baculovirus (62) and σC expressed in yeast, plants or *E. coli* (24, 130).

For turkeys, use of polyvalent autogenous vaccines in breeders has greatly reduced the prevalence of turkey viral arthritis strains of reovirus. Commercial vaccines for turkeys are not available.

Conclusions

Reoviruses are common among domestic poultry and other avian species. The virus is the principal cause of several diseases, mainly arthritis/tenosynovitis in chickens and turkeys. Reoviruses have also been isolated from apparently healthy birds, and serum antibodies are often found in both ill and healthy birds. Apart from tenosynovitis in chickens and turkeys, reovirus is considered as the cause of other clinical diseases, but a clear relationship to reovirus infection cannot be definitively established. There appears to be a wide range of pathogenicity among isolates, but most are probably harmless. There may be differences in tissue tropism, although all appear to replicate in the gut, and pathogenic strains affect the liver. The serologic or molecular relationship of reoviruses from minor poultry and exotic species to the tenosynovitis strains is unknown, but the potential for cross-species infection is suggested. Commercial vaccines are available against chicken reoviruses but not against viruses of other species including turkeys. Recently, a large number of variants have been revealed in both chicken and turkey reoviruses, making their control difficult with an increasing reliance on autogenous vaccines. In the era of molecular biology and deeper understandings of the genetics and immunological aspects of reovirus, a vaccine that will confer protection against a wide-range of variants maybe a possible task.

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12

Viral Enteric Infections

Introduction

Yehia M. Saif

Our knowledge of enteric viruses has increased substantially in the last three decades. Several factors have contributed to this progress. The realization that pathogens other than bacteria must be an important component of the etiology of enteric disease has fueled the search for other infectious agents with specific emphasis on viruses. The availability of diagnostic tools was another incentive. Most useful among these tools was direct and immune electron microscopy and increasing the use of next generation sequencing.

The morphologic identification of viruses by electron microscopy from gut contents paved the way for attempts to purify, cultivate, and develop diagnostic reagents and to further characterize these agents. Another technique that was used successfully earlier to identify some viruses in fecal samples is electropherotyping of genomic RNA. This technique is useful for detection and differentiation of double-stranded RNA viruses, such as rotaviruses and reoviruses.

A significant finding reported in the earlier studies was the presence of a variety of viral species that could be present in different combinations in the gastrointestinal (GI) tract of young poultry. The availability of specific pathogen free (SPF) poultry was of major value in studies of these viruses. In the last decade, more diagnostic technologies such as reverse transcriptase-polymerase chain reaction (RT-PCR) became available and are used routinely in some laboratories to detect enteric viruses in gut contents. Removal of inhibitors from fecal material has increased the utility of the test. In addition, the use of internal controls has improved the test specificity.

Most enteric viral infections occur in the first three weeks of life. The clinical signs and lesions induced by the different viruses have similarities; hence, it is difficult to attribute a specific enteric disease to a given virus unless laboratory studies are initiated to identify the causative agent(s). In addition, the presence of different combinations of viruses could result in varied disease presentations. In general, high morbidity and low mortality exist when only one virus is detected, but mortality could be high when several viruses are present. An example of the significant economic impact of these combinations is the condition that was designated poult enteritis and mortality syndrome that was identified in the United States.

Different terms have been used in the literature to describe different conditions/syndromes of enteric disease. Unfortunately such descriptions are not instructive and are confusing at best because these descriptions do not refer to a specific etiology or etiologies. Because diagnostic tools are available for most enteric viral infections, it is preferable to designate these conditions as enteritis with reference, when available, to the specific infectious agents involved.

Diarrhea is a common manifestation of the disease, and the GI tract is usually distended with gas and liquid/frothy contents. Different viruses replicate at different parts of the GI tract and at different sites on the villi. Epidemiologic studies indicated that most of these viruses do not persist for long in the birds. Unfortunately, many of the enteric viruses are uncultivable, which hampers research and diagnostics.

Little evidence of egg transmission of enteric viruses exists, and there are gaps in our knowledge of the epidemiology of the infections. Active immunity apparently plays a role in limiting the disease, but the benefits of passive immunity are limited to the first few days of life. No commercial vaccines are available for most of these infections.

Enteric viruses are commonly the cause of most of the primary insults to the GI tract of young poultry. This provides other agents, especially bacteria, with the milieu to replicate, attach, and penetrate cells, leading to further damage. It has been shown that during the course of

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several enteric viral infections, bacteria adhere and form a membrane on the surface of the villi. Counteracting secondary bacterial involvement is probably the reason for the reported effectiveness of antibiotic treatment of some cases of enteric disease initiated by viruses in young birds.

The GI tract has the largest surface area in the body, and it is continually exposed to a variety of insults and stimulations. In food animals, the integrity of the GI tract is of paramount importance. Efficient utilization of nutrients depends on a healthy GI tract, and this is especially true for the young of the species. Damage to the GI tract early in life could result in irreversible damage to the flock.

The progress made in the last three decades has been remarkable, but gaps remain in our knowledge of enteric viruses and new enteric viruses continue to be detected as evidenced by the recognition of parvoviruses in chickens and turkeys in this chapter. Further information that could lead to novel methods for control of these infections should be significant.

Significant new information has been reported since the last edition of *Diseases of Poultry* for coronaviruses, rotaviruses, reoviruses, astroviruses, and enteroviruses, which has necessitated updating these subchapters. However, no new information or cases of toroviruses have been reported and the reader is referred to the 12th edition of *Diseases of Poultry* for information.

Turkey Coronavirus Enteritis

James S. Guy

Summary

Agent, Infection, and Disease. Turkey coronavirus (TCV) is the cause of enteritis in young turkeys and decreased egg production in turkey breeder hens. TCV is shed in feces of infected birds and spreads horizontally through ingestion of feces and feces-contaminated materials. Turkey coronavirus has been identified in most turkey producing regions of the world.

Diagnosis. The preferred diagnostic tests for TCV include: (1) isolation of TCV in 15-day embryonating turkey eggs, (2) detection of TCV antigen or RNA in intestines or bursa of Fabricius of clinically-affected birds, or (3) serologic detection of TCV-specific antibodies using immunofluorescence procedures or enzyme-linked immunosorbent assays.

Intervention. Elimination of TCV from contaminated premises by depopulation, cleaning, and disinfection is the preferred control strategy. No licensed vaccine is available.

Introduction

Turkey coronavirus (TCV) is the cause of an acute highly contagious enteric disease of turkeys characterized by depression, anorexia, diarrhea, and decreased weight gain. Bluecomb disease, transmissible enteritis, and coronaviral enteritis are synonyms of TCV enteritis of turkeys.

History

In 1951, Peterson and Hymas (69) described an enteric disease of turkeys that initially was referred to as bluecomb disease. In 1971, Adams and Hofstad (2) propagated a virus from bluecomb disease-affected turkeys using embryonating chicken and turkey eggs, and experimentally reproduced enteric disease using this embryo-propagated virus; this virus subsequently was identified as a coronavirus (65, 73). Additional information on historical aspects of this disease may be found in prior editions of *Diseases of Poultry*.

Etiology

Classification

Turkey coronavirus is classified as a member of the Coronaviridae (22). The Coronaviridae comprise a large family of RNA-containing viruses that infect a wide variety of avian and mammalian species (76). The Coronaviridae is in the order Nidovirales, an order composed of viruses having linear, nonsegmented, positive-sense, single-stranded RNA genomes with similar genomic organization and nested sets of subgenomic mRNAs (22, 76). The coronavirus genome consists of an RNA molecule that is approximately 28 kilobases in size (76). Coronaviruses possesses four major structural proteins referred to as spike (surface) glycoprotein (90–180 kilodaltons [kDa]), integral membrane protein (20–35 kDa), small envelope protein (12.5 kDa), and nucleocapsid protein (50–60 kDa) (76). Some coronaviruses also contain a fifth major structural protein, the hemagglutinin-esterase (120–140 kDa) (76).

Coronaviruses have been subdivided into four genera based on serological and nucleotide sequence analyses (11, 22, 76). Infectious bronchitis virus (IBV) and TCV are members of the *Gammacoronavirus* genus (16–18, 22, 33).

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Turkey coronavirus has been shown to be closely related to IBV based on antigenic and nucleotide sequence analyses (5, 8, 9, 12, 18, 26, 35, 42, 46–49, 80). Studies have indicated a high degree of sequence identity between integral membrane protein, nucleocapsid protein and polymerase (ORF1b) genes of TCV and IBV, but only limited sequence identity between TCV and IBV spike glycoproteins (5, 8, 9, 12, 18, 42, 47, 48, 80).

Morphology

Coronaviruses are roughly spherical, pleomorphic, enveloped particles, with diameters of 60–200 nm (76). They possess a characteristic surface structure composed of long (12–24 nm), widely spaced, club-shaped peplomers (Figure 12.1). These peplomers give virions the distinctive appearance of a solar corona, hence the name coronavirus.

Chemical Composition

The turkey coronavirus genome consists of a linear, nonsegmented, single stranded RNA molecule that is approximately 28 kilobases in size (11, 12, 26). Turkey coronavirus, like IBV, possesses spike (surface) glycoprotein, integral membrane, small envelope, and nucleocapsid proteins (8, 9, 12, 18, 26, 47, 48). However, the size and structural properties of these proteins have not been determined. The virus lacks a hemagglutinin-esterase protein (26).

Virus Replication

Turkey coronavirus replicates primarily in enterocytes in the jejunum and ileum (10, 66, 72), and epithelium of the bursa of Fabricius (35, 62). Viral antigens in intestinal enterocytes were found predominately in enterocytes lining the upper one-half to two-thirds of intestinal villi (Figure 12.2) (10, 35, 72). In the bursa of Fabricius, viral antigens are found in both follicular and interfollicular epithelium of the bursa of Fabricius (Figure 12.3); viral

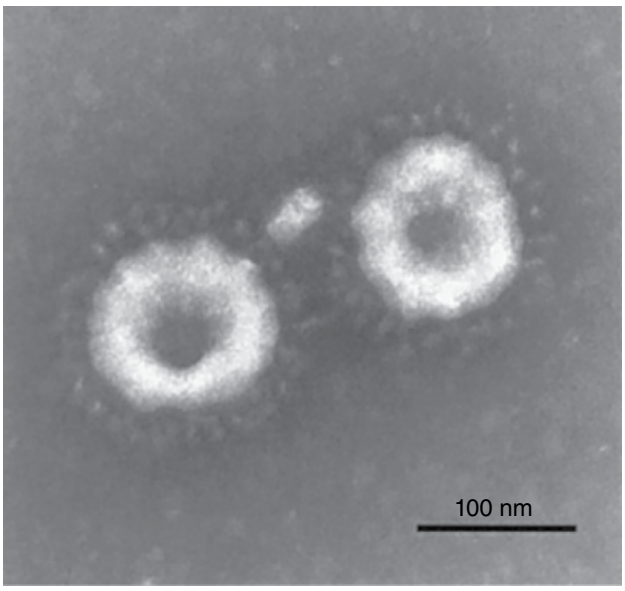


Figure 12.1 Negative contrast electron micrograph of turkey coronavirus.

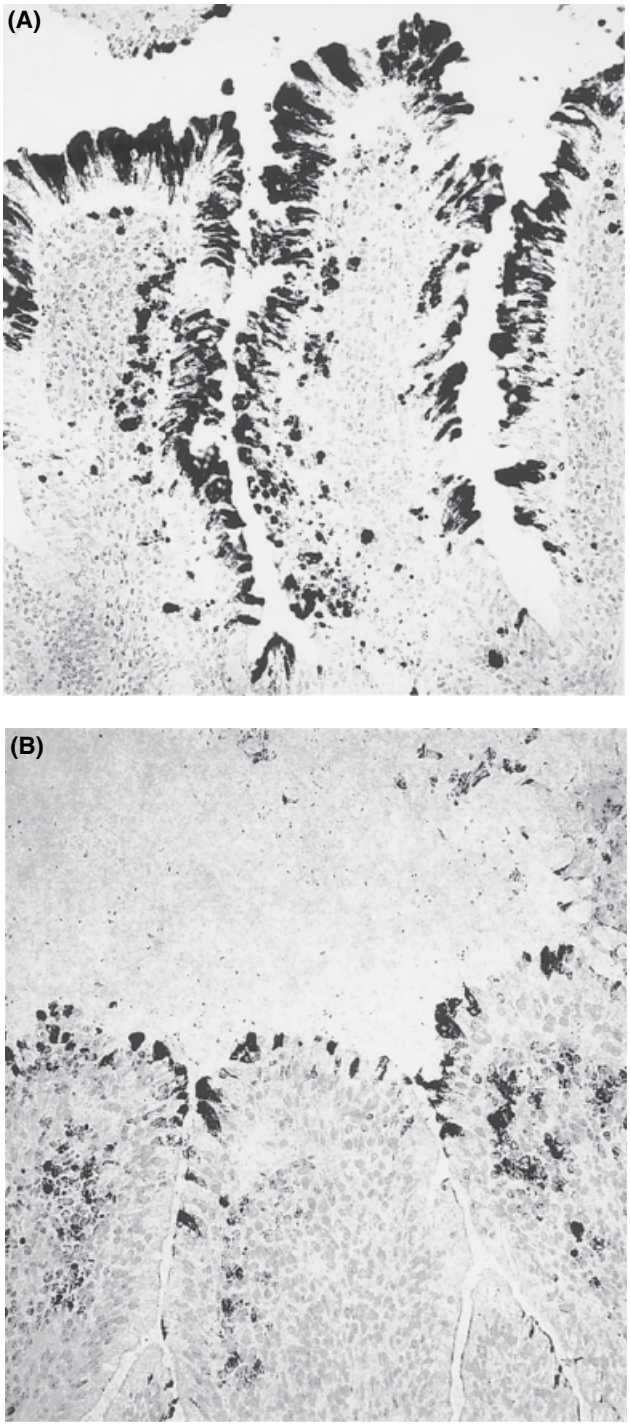


Figure 12.2 Immunoperoxidase staining of intestinal tissues from turkey coronavirus (TCV)-infected turkey. (A) Cecum, 1 day postinfection (DPI). (B) Ileum, 14 DPI. 350×.

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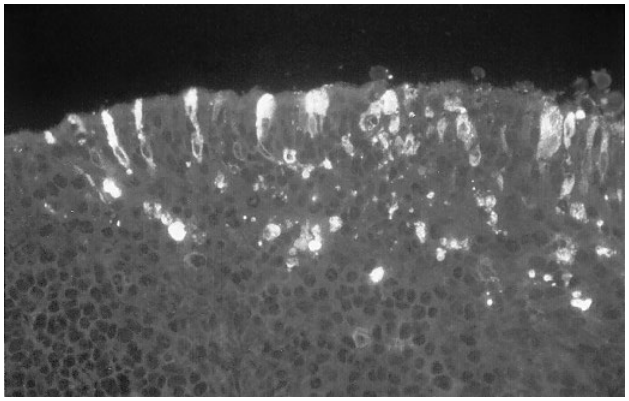


Figure 12.3 Immunofluorescent staining of bursa of Fabricius of turkey coronavirus (TCV)-infected turkey, 2 days postinfection (DPI). Note that staining is localized to bursal epithelium. ×240.

antigens are not found in bursal lymphoid follicles (35). In inoculated embryos, virus replication occurs exclusively in intestinal epithelial cells and bursa of Fabricius (72); virus replication has not been detected in allantoic, yolk, or amniotic membranes.

Thin-section electron microscopy of intestines from TCV-infected embryos and poults (4, 72) has shown that TCV replication occurs in the cytoplasm. Turkey coronavirus acquires its envelope by a process of budding through membranes of the endoplasmic reticulum and Golgi apparatus; virus particles accumulate in cisternae of the endoplasmic reticulum.

Susceptibility to Chemical and Physical Agents

Turkey coronavirus was demonstrated to be stable at pH 3 at 22°Cfor 30 min, and resistant to 50 °C for 1 hour, even in the presence of 1 M magnesium sulfate (23). Chloroform treatment at 4 °C for 10 minutes readily inactivated the virus.

Turkey coronavirus remained viable when stored in intestinal tissues at −20 °C or lower for over five years (59). The virus was shown to survive in buildings and ranges for extended periods of time even after turkeys were removed from these premises (59). Storage of the virus for 10 days at 21.6 °C resulted in inactivation, but the virus survived for up to 20 days when stored at 4 °C (31). Saponified cresol and formaldehyde were shown to be effective disinfectants for eliminating TCV from contaminated buildings (68).

Strain Classification

Antigenic analyses based on cross-protection studies, cross-immunofluorescence, and enzyme-linked immunosorbent assays (ELISA) indicate close antigenic relationships between TCV isolates obtained from different geographical locations (35, 42, 46). Similarly, nucleotide sequence analyses of TCV proteins of different isolates have demonstrated that these viruses are genetically very similar (8, 9, 42, 44, 48, 54).

Antigenic differences between TCV strains recently were demonstrated based on serum-virus neutralization studies (44). Three TCV isolates were selected for antigenic analyses based on differences within their spike glycoprotein genes; cross-neutralization assays indicated that these isolates comprised distinctly different serotypes.

Laboratory Host Systems

Turkey coronavirus strains can be propagated in embryonated chicken eggs (more than 16 days of incubation) and embryonated turkey eggs (more than 15 days of incubation) by inoculation of the amniotic cavity (2, 34). In inoculated embryos, virus is recovered only from intestines and bursa of Fabricius (59).

Attempts to propagate TCV in a variety of avian and mammalian cell cultures generally have been unsuccessful (24, 59). Cell culture propagation of TCV using a human rectal adenocarcinoma (HRT) cell line has been reported; however, this has not been corroborated by other investigators (21, 35, 41).

Pathogenicity

Studies have not been done to examine differences in virulence among different isolates of TCV.

Pathobiology and Epizootiology

Incidence and Distribution

Turkey coronavirus has been identified in turkeys in the United States, Canada, Brazil, Italy, the United Kingdom, France, Poland, Turkey, and Australia (17, 18, 20, 54, 59, 63, 81). The virus has been identified in most turkey producing regions of the United States.

Natural and Experimental Hosts

Turkey coronavirus affects turkeys of all ages; however, clinical disease most commonly is observed in young turkeys during the first few weeks of life. Turkeys are believed to be the only natural host for TCV. Pheasants, sea gulls, coturnix quail, and hamsters are refractory to infection (39, 59). Chickens once were believed to be refractory to TCV infection (70, 78); however, recent studies indicate otherwise (29, 36, 43). In two separate studies, specific pathogen free chickens did not exhibit clinically apparent disease after experimental inoculation with TCV; however, susceptibility to TCV infection was demonstrated

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by seroconversion and detection of virus and viral antigens in intestinal tissues and bursa of Fabricius (36), and by detection of virus is in intestinal contents (43). In a more recent study, TCV was detected in both upper respiratory tissues and intestinal tissues of experimentally infected chickens (29).

Transmission, Carriers, Vectors

Turkey coronavirus is shed in feces of infected birds and spread horizontally through ingestion of feces and feces-contaminated materials. Turkey coronavirus generally spreads rapidly through a flock and from flock to flock on the same or neighboring farms. Mechanical movement of the virus may occur by people, equipment, vehicles, and insects. Darkling beetle larvae and domestic house flies have been shown to be potential mechanical vectors of TCV (13, 84). Wild birds, rodents, and dogs also may serve as mechanical vectors. There is no evidence that TCV is egg transmitted; however, poults may become infected in the hatchery via contaminated personnel and fomites such as egg boxes from infected farms.

Turkey coronavirus is shed in droppings of turkeys for several weeks after recovery from clinical disease (10). The virus was detected in intestinal contents of experimentally inoculated turkeys for up to six weeks postinoculation (PI) by virus isolation, and up to seven weeks PI by a reverse transcriptase-polymerase chain reaction (RT-PCR) procedure (10).

Incubation Period

The incubation period may vary from 15 days, but typically is 2–3 days.

Clinical Signs

In field cases, clinical signs occur suddenly, usually with high morbidity (59, 60). Birds exhibit depression, anorexia, decreased water consumption, watery diarrhea, dehydration, hypothermia, and weight loss. Droppings typically are green to brown, watery, frothy, and may contain mucus and urates. Flocks infected with TCV experience increased mortality, growth depression, and poor feed conversion compared with uninfected flocks. Mortality is variable in affected flocks; high mortality may occur depending on the age of the birds, concurrent infection, and management practices.

Experimental studies using egg-adapted strains of TCV indicate that TCV infection results only in mild disease and moderate growth depression; mortality generally is negligible (37, 43, 60, 64).

Turkey coronavirus infection of turkey breeder hens during production is a potential cause of decreased egg production (6, 7, 59). In experimental studies, TCV infection of turkey breeder hens resulted in transient drops in egg production (6, 7); however, combined infection with TCV and turkey astrovirus resulted in prolonged and more severe drops in egg production compared with hens infected with TCV alone (7).

Pathology

Gross

Gross lesions are seen primarily in intestines and bursa of Fabricius. Duodenum and jejunum generally are pale and flaccid; ceca are distended and filled with watery contents. Emaciation, dehydration, and atrophy of the bursa of Fabricius may be observed.

Microscopic

Microscopic lesions are observed in intestines and bursa of Fabricius of TCV-infected turkeys. In intestines, microscopic lesions in experimentally infected turkeys consist of decreased villous length, increased crypt depth, and decreased intestinal diameter (3, 30). The columnar epithelium of intestinal villi changes to a cuboidal epithelium and these cells exhibit a loss of microvilli. There is a decrease in number of goblet cells, separation of enterocytes from lamina propria, and infiltration of lamina propria with heterophils and lymphocytes. Epithelial repair is evident beginning at 5 days PI, and complete by 21 days PI (3, 30). By 5 days PI, columnar epithelium with microvilli begins to replace cuboidal cells, and goblet cells begin to reappear (30).

In the bursa of Fabricius, changes in epithelial cells are evident by 2 days PI and consist of epithelial necrosis and hyperplasia (37). The normal pseudostratified columnar epithelium of the bursa of Fabricius is replaced by a stratified squamous epithelium (Figure 12.4). Intense heterophilic inflammation is observed within and subjacent to the epithelium. Moderate lymphoid atrophy of bursal follicles is observed.

Ultrastructural

Ultrastructural changes in intestines of TCV-infected turkeys are confined to epithelial cells (4, 72). Ultrastructural changes include loss of microvilli, disruption of the terminal web region, degeneration of mitochondria, dilation of cisternae in the endoplasmic reticulum, increases in intracellular lipid, excessive sloughing of cells at villous tips, and shortening of villi. Coronavirus particles (80–140 nm in diameter) are observed within cisternae of the endoplasmic reticulum (72).

Pathogenesis of the Infectious Process

Turkey coronavirus replicates preferentially in enterocytes lining the apical portions of intestinal villi and in epithelium of the bursa of Fabricius (24, 37, 72). The site

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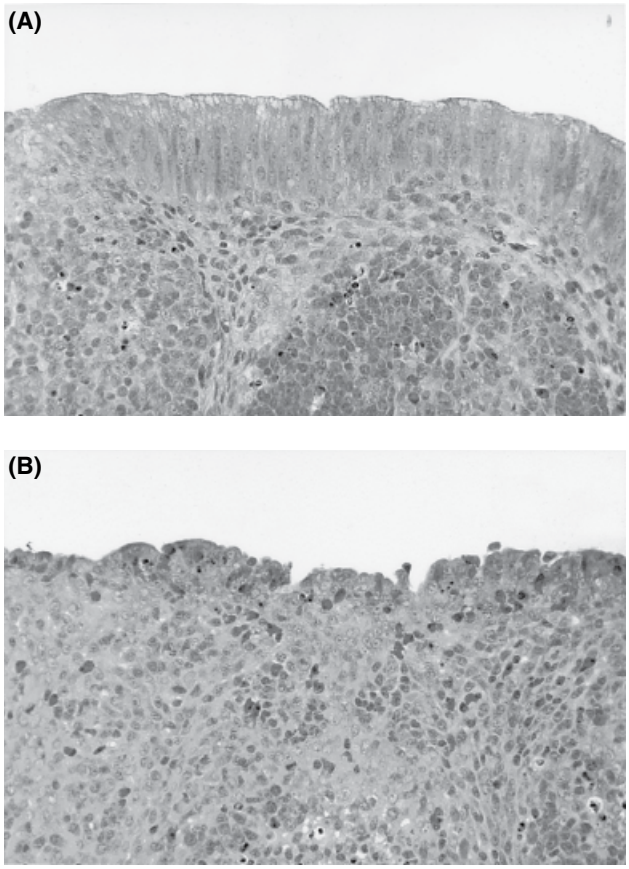


Figure 12.4 Histopathologic changes observed in bursa of Fabricius of turkey coronavirus (TCV)-infected turkey. (A) Sham-inoculated control. Note that epithelium consists of pseudostratified columnar epithelium. (B) TCV-infected turkey, 4 days postinfection (DPI). Note epithelial cell necrosis and hyperplasia with heterophilic inflammation. ×240.

of intestinal TCV infection suggests that the virus may cause diarrhea in a manner similar to other enteric coronaviruses (55, 72). Malabsorption, maldigestion and diarrhea likely result from TCV-induced destruction of villous epithelium; however, the virus may exert its effects in a more subtle manner through alterations in the physiology of these cells (72). Turkey coronavirus also may exert its effects by altering the normal intestinal flora (61).

Severe disease characterized by high mortality was a common feature of early descriptions of TCV infection (bluecomb disease) and early experimental studies using inocula composed of crude fecal/intestinal homogenates (59). More recent experimental studies using embryo-propagated TCV indicate that mortality due to TCV infection usually is negligible, at least under laboratory conditions (37, 43, 60, 64). Management practices, crowding, and secondary infections may exacerbate the effects of TCV infection and result in increased losses. Antibiotics have been shown to reduce mortality in TCV-infected flocks, most likely because they control secondary bacterial infections (69, 77).

Experimental studies with TCV and an enteropathogenic strain of *Escherichia coli* provide evidence suggesting an interaction between TCV and bacteria in the development of severe clinical disease (37, 64). In these experiments, young turkeys inoculated with only TCV developed moderate growth depression without significant mortality, and turkeys inoculated with only enteropathogenic *E. coli* did not develop clinically apparent disease. However, turkeys dually inoculated with TCV and enteropathogenic *E. coli* developed severe growth depression and high mortality.

Immunity

Active

Turkeys that recover from TCV infection are resistant to subsequent challenge (28, 59, 71). Turkeys that survived experimental TCV infection at 4 days of age showed no clinical signs when challenged at 11 and 22 weeks of age (71). Field observations indicate that flocks that recover from TCV infection are resistant to subsequent infection (70).

The nature of protective immunity in recovered birds is not fully understood. Specific secretory IgA, humoral, and T-cell mediated immunity have been demonstrated in recovered turkeys (28, 50, 51, 56–59). Specific secretory IgA was shown to persist in intestinal secretions and bile of recovered turkeys for at least six months (56). Concentrations of specific secretory IgA antibodies in feces of TCV-infected turkeys peaked at 3–4 weeks PI and disappeared at approximately 6 weeks PI (51).

Passive

Poults passively immunized against TCV by subcutaneous inoculation of serum from immune birds were not protected from challenge (71). Poults from immune and nonimmune breeder hens were equally susceptible to TCV challenge (82).

Diagnosis

Isolation and Identification of Causative Agent

Diagnosis of TCV infection generally requires laboratory assistance as other enteric pathogens of turkeys may cause similar clinical signs and lesions. Laboratory diagnosis may be achieved based on virus isolation, electron microscopy, serology, detection of viral antigens, or detection of viral RNA (45).

Virus isolation may be accomplished by inoculation of embryonated chicken or turkey eggs (see Laboratory

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Host Systems) with suspensions of intestinal contents, dropping samples, or tissues (intestines, bursa of Fabricius) from suspect infected turkeys (34). Clinical samples should be homogenized in an appropriate diluent, such as minimal essential medium, clarified by centrifugation, and filtered through a 0.45 μm filter. Embryonated chicken eggs (greater than 16 days of incubation) or turkey eggs (greater than 15 days of incubation) are inoculated by the amniotic route and returned to the incubator. Embryonated turkey eggs are the preferred substrate, as the relative sensitivity of chicken embryos to TCV has not been determined. After incubation for 2–5 days, the presence of TCV in embryo intestines may be determined by electron microscopy, immunohistochemical detection of viral antigen or RT-PCR detection of viral RNA (45).

Diagnosis based on electron microscopy requires the identification of virus particles having typical coronavirus morphology. However, coronaviruses must be distinguished from cell membrane fragments in feces that may resemble coronaviruses. Definitive identification may be accomplished by immune electron microscopy (45).

Immunohistochemical diagnosis is based on detection of TCV antigens in tissues using either immunofluorescent antibody (FA) or immunoperoxidase (IP) procedures. Both direct and indirect FA procedures have been described for detection of TCV antigens using frozen tissue sections (10, 66–68, 72). The direct FA procedure is an excellent diagnostic approach with respect to simplicity and speed of diagnosis. However, sensitivity and specificity of this procedure are dependent upon the quality of the antiserum used to prepare the fluorochrome-conjugated antibody, and once this reagent is produced it has a relatively short shelf life. The direct FA procedure was shown to detect TCV antigens in experimentally infected turkeys from 1–28 days PI (68).

Indirect FA and indirect IP procedures have been described for detection of TCV antigens in tissues of infected turkeys (10); these procedures utilized TCV-specific monoclonal antibodies (mAb) and frozen tissue sections. These mAb-based immunohistochemical procedures detected TCV antigens in intestines and bursa of Fabricius of experimentally infected turkeys as early as 1 day PI, and as late as 42 days PI. They were shown to have high specificity (greater than 92%), but low sensitivity (61–69%) compared with virus isolation.

A direct IP procedure was described for detection of TCV antigens in formalin-fixed paraffin-embedded tissues (14). This procedure utilized a biotin-conjugated antibody prepared using antibodies from hyperimmunized chickens. The direct IP procedure was shown to be highly specific (93%) and highly sensitive (85%) compared with detection by indirect FA.

Different RT-PCR tests have been developed for detection of TCV RNA in dropping samples and intestinal contents of infected turkeys (10, 83). These RT-PCR procedures have been shown to be highly sensitive and highly specific. In one study, RT-PCR was shown to detect TCV RNA in experimentally infected turkeys as early as 1 day PI and as late as 49 days PI; sensitivity and specificity of the RT-PCR procedure was 93% and 92%, respectively, compared with virus isolation (10). Multiplex RT-PCR procedures also have been developed that allow simultaneous detection of TCV and other enteric viruses (53, 75, 79).

A real-time RT-PCR procedure recently was described for detection and quantitation of TCV RNA in infected turkeys (19). Additionally, a reverse-transcriptase loop-mediated isothermal amplification procedure for detection of TCV RNA has been described (15). These detection procedures were demonstrated to be rapid, highly sensitive, and specific methods for TCV detection (15, 19).

Serology

Detection of TCV-specific antibodies most commonly is accomplished using the indirect FA procedure. Antigen for this procedure consists of either frozen sections of TCV- infected embryo intestines (66) or epithelium exfoliated from bursae of Fabricius of infected turkeys (32). Frozen tissue sections are prepared from intestinal tissues of TCV-infected turkey embryos, 24–48 hours after inoculation with embryo-adapted TCV strains (66). Antigen preparation by this method is slow and labor intensive. However, an advantage of this serological method is that it allows discrimination of false positive staining based on determining the site of intestinal staining (i.e., TCV preferentially infects apical villous epithelium). Alternatively, antigen slides may be prepared using exfoliated epithelial cells collected from bursae of Fabricius of 2-week-old turkeys, 4 days after TCV inoculation (32). Bursae are harvested from infected turkeys, rinsed in cell culture media, and incubated at 4°C for 18–24 hours with gentle stirring to exfoliate epithelial cells. Cells are concentrated by low speed centrifugation and then spotted onto glass slides. TCV-specific antibodies may be detected in experimentally infected turkeys as early as seven days PI using either of the indirect FA methods described above. In a study using indirect FA serology, turkeys infected early in the brooder house remained serologically positive throughout the growout period (40).

Enzyme-linked immunosorbent assays (ELISA) have been described for detection of TCV-specific antibodies in turkeys (1, 27, 38, 49, 52). Turkey coronavirus-specific antibodies may be detected in turkey sera using commercially available, IBV-coated ELISA plates (49). In addition, ELISAs based on recombinant TCV nucleoprotein or spike protein have been developed (1, 27, 38, 52). The IBV ELISA and the recombinant TCV

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nucleoprotein-based ELISAs were shown to be highly sensitive and highly specific compared with the indirect FA procedure (1, 38, 49).

The TCV nucleoprotein-based ELISAs failed to discriminate between IBV- and TCV-specific antibodies (38, 49, 52). However, this is unlikely to be an impediment to specific detection of TCV infection in turkeys, as turkeys are not believed to be susceptible to IBV, and experimental attempts to infect turkeys with IBV have not been successful (33).

A TCV spike protein-specific ELISA was developed based on a recombinant TCV spike protein (27). This ELISA was highly sensitive and highly specific for TCV, and cross-reactivity with IBV-specific antibodies was not observed.

Differential Diagnosis

Enteric disease caused by TCV must be distinguished from other enteric diseases of turkeys, particularly those caused by other viruses, bacteria, and protozoa.

Intervention Strategies

Management Procedures

Prevention is the preferred method for controlling TCV. Turkeys infected with TCV have been shown to shed virus in feces for prolonged periods of time after recovery (10, 66); these turkeys, their feces, and the materials that their feces contact, are potential sources of infection for other susceptible turkeys. Feces from TCV-infected turkeys can be carried on a variety of fomites including clothing, boots, equipment, feathers, and trucks. Other potential vectors such as wild birds, rodents, dogs, and flies also may be involved in transmission from infected to susceptible flocks.

Elimination of TCV from contaminated premises may be accomplished by depopulation followed by thorough cleaning and disinfection of houses and equipment (68). Following cleaning and disinfection procedures, premises should remain free of birds for a minimum of 3–4 weeks.

Vaccination

No licensed vaccine is available.

Treatment

At present, there is no specific treatment for TCV enteritis. Antibiotic treatment has been shown to reduce mortality, most likely by controlling secondary bacterial infections (52, 61, 70). No beneficial effect was observed when glucose, electrolytes, or calf milk replacer was added to drinking water (21). Management procedures that have been effective in reducing mortality include raising brooder house temperatures and avoiding crowded conditions.

Rotavirus Infections

J. Michael Day

Summary

Agent, Infection, and Disease. Avian rotavirus infection is frequently associated with outbreaks of diarrhea and general flock depression; other disease signs attributed to avian rotavirus are consistent with viral enteritis: dehydration, stunting of growth, pasted vents, inflamed vents, anemia due to vent pecking, and litter ingestion.

Diagnosis. Multiplex or individual reverse transcriptase-polymerase chain reaction (RT-PCR) assays targeting specific rotavirus genome segments have implicated rotavirus in outbreaks of enteritis, and rotavirus infection can be visualized via immunohistochemistry. Avian rotavirus can be directly identified in feces or intestinal contents by direct electron microscopy, and electropherotyping using agarose or polyacrylamide can be an effective technique for diagnosis and initial genotyping.

Intervention. Management interventions such as the addition of fresh litter or house cleanout, along with increased general biosecurity measures can be used to prevent and control viral enteritis.

Introduction

In addition to their numerous mammalian hosts, rotaviruses infect many species of birds, including many species of domesticated birds (31, 33, 34, 38, 76, 86, 87, 110, 114, 120, 124). Rotavirus infection in avian species is frequently associated with outbreaks of diarrhea and general flock depression, and is often found in association with a recognized enteric syndrome in poultry (9, 46, 48, 83, 84). The economic impact of rotavirus-associated enteric disease to the poultry industry is not clear, but nonspecific enteric disease with a probable viral etiology is an ongoing industry

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burden (9, 48). There is evidence that certain avian rotaviruses are not strictly species-specific, as rotaviruses from turkeys and pheasants can infect older specific pathogen free (SPF) chickens (125). Although rotaviruses do cause enteric disease in mammals and birds, rotaviruses are often detected in otherwise healthy flocks, particularly when sensitive molecular diagnostic assays are utilized.

Etiology

Classification

The avian rotaviruses are members of the *Reoviridae* family, which is characterized by virions that contain 10–12 linear double-stranded RNA (dsRNA) segments. The rotavirus genome consists of 11 segments of dsRNA with conserved 5' and 3' ends, and each genome segment codes for a single protein with the exception of segment 11, which codes for two (20, 44). The genome is enclosed by a triple-layered capsid (virion) with a characteristic wheel-like appearance (rota = wheel) when viewed via electron microscopy (Figure 12.5). The coinfection of cells with two different rotavirus strains can result in genetic reassortants containing a mixture of genome segments from each parent virus (20, 55).

Morphology

Mature prototypical mammalian rotavirus virions are nonenveloped icosahedrons and have an overall diameter of approximately 1000 Å (100 nm), including the VP4 cell attachment protein "spikes"(44, 90, 92), while the diameter of the outer shell of the rotavirus virion excluding the protruding spikes is approximately 750 Å (55, 91, 93, 130). The smooth appearance of the outer protein shell distinguishes the rotaviruses from other members of the Reoviridae when viewed via electron microscopy (Figure 12.5). Turkey rotavirus virions have a buoyant density in CsCl of 1.34 g/cm^3 (52), while group D rotaviruses isolated from the intestinal contents of pheasant chicks had a density in CsCl of 1.35 g/cm^3. In contrast to mammalian rotaviruses, the pheasant group D virion had a diameter of 800 Å, which is larger than the reported diameter of 730 Å from group A virions of turkeys (21).

Chemical Composition

The rotavirus dsRNA genome is contained within the capsid core and consists of 11 linear segments; avian rotaviruses have genome segments ranging from approximately 3.3 kb to 700 bp, with a total genome size of approximately 19 kb (42, 117, 118). Turkey rotavirus propagated in rhesus monkey kidney cells (MA104 cell line) expressed ten major polypeptides starting at six hours postinfection (PI). (51).

Virus Replication

Rotavirus infected cells display characteristic electron-dense cytoplasmic inclusion bodies called viroplasms, which are viral factories in which genome replication and initial packaging of the viral genome segments occur. Viroplasm formation is directed by the affinity of certain rotavirus structural proteins for newly translated positive-sense RNA [(+)RNA] (89). The replication of the dsRNA genome takes place fully within the newly formed viral cores; the dsRNA genome is thus never exposed to the interior of the cell (44, 89). The association of the rotavirus transcriptase complex with the intact viral core also allows the transcription of each genome segment to occur without complete uncoating of the dsRNA genome.

A group D rotavirus produced electron-dense viroplasms and mature viral particles in pheasant chick duodenal enterocytes by seven days postinfection (DPI), while viroplasms and virions in rough ER were observed by 8 DPI in chicken villous epithelial cells infected with an antigenically novel rotavirus (37). Similarly, PO-13 rotavirus produced virions in the rough ER of MA-104 cells (76).

Susceptibility to Chemical and Physical Agents

Two different turkey rotavirus isolates (AvRV-1 and AvRV-3) propagated in MA-104 cells had no change in viral titers after a 30 minute treatment with chloroform; treatment at pH 3.0 reduced the titer of both isolates approximately 100-fold by 8 hours of treatment. Titers were reduced by about 100-fold after treatment at 56 °C for 30 minutes, but neither virus was inactivated following 8 hours at 56 °C (52). A turkey rotavirus strain Ro/1145/08 was inactivated after incubation at 82.2 °C for 140 seconds in DMEM, but was initially resistant to heating in a thermal cycler gradient from 25 °C to 50 °C. Turkey rotavirus strain Ro/996/07 was similarly inactivated after a 6-hour, 82.2 °C incubation in an effluent decontamination system (EDS) tank designed to treat effluent from a BSL-2/BSL-3 laboratory facility (17). Duck rotavirus strain F-29 was also resistant to chloroform treatment and treatment at pH 3.0 had no effect on viral titers. Heating to 50 °C reduced the titer of F-29 10 times and heating to 50 °C in the presence of 1 M MgCl2 further reduced titers 1000 times (108). The infectivity in cell culture of pigeon rotavirus (PO-13) was resistant to 20% ether, 10% chloroform, and 0.1% sodium deoxycholate (76). Laboratory studies with group A porcine rotavirus identified a phenolic-based disinfectant effectively reduced the titer of rotavirus, even in the presence of a significant amount of organic matter, while glutaraldehyde- and peroxygen-based disinfectants only showed effective reductions in titer in the absence of organic material (16).

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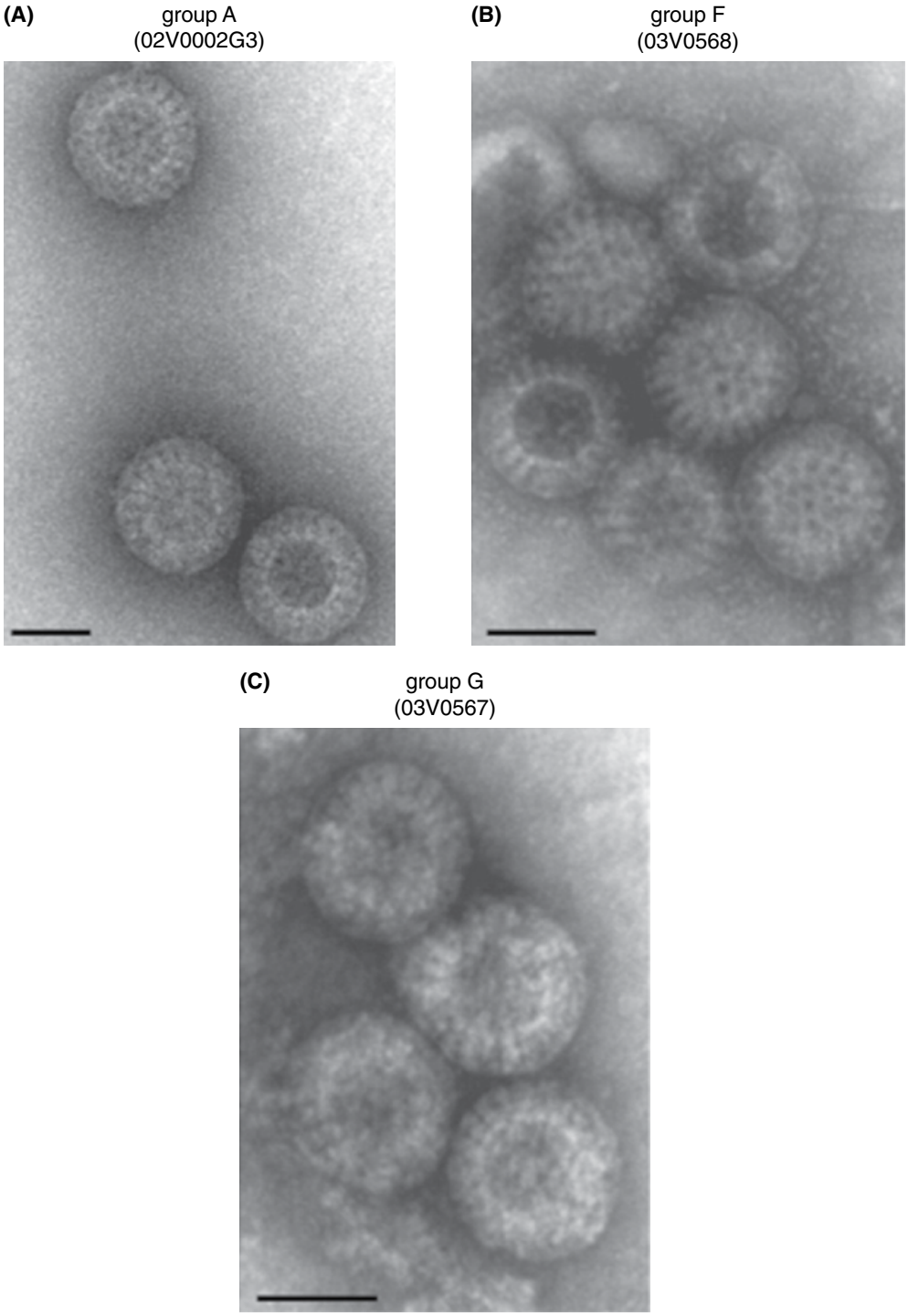


Figure 12.5 Identification of avian rotaviruses in intestinal samples by negative staining electron microscopy. (A) group A rotavirus 02V0002G3, (B) group F rotavirus 03V0568, and (C) group G rotavirus 03V0567. The bar corresponds to 100 nm (55).

Strain Classification

Antigenicity

The antigenicity of rotaviruses is determined by three major structural proteins that comprise the middle and outer layers of the virion: VP4, VP6, and VP7, with multiple serotypes recognized within each serogroup. Seven recognized serogroups comprise the rotaviruses (groups A to G), with groups A–E each formally recognized as a species within the genus *Rotavirus*. Groups F and G do not have formal species recognition, nor does

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an additional group, novel adult diarrhea rotavirus (NADRV or ADRV-N), which has been tentatively placed in a new group H (4, 26, 43, 62). Groups B, C, and E have only been described in mammals, group A has been described in both mammals and birds, and groups D, F, and G have only been described in birds (4). Rotavirus subgroup specificities also reside on the VP6 structural protein. Subgrouping has allowed a classification system for rotaviruses that share the common group A antigen, and places all rotaviruses into subgroups I or II, both I and II, or neither I nor II (4, 40).

Prototypical members of the group D, F, and G rotaviruses have been described in chickens, and include the fully-sequenced rotavirus strain 05V0049 ("Ch-49", group D), rotavirus A4 (group F), and rotavirus 555 (group G) (4, 99, 118). The group D rotaviruses are the most commonly detected rotaviruses in turkey poults with diarrhea (95, 96, 100, 111, 112). Molecular- and cell culture-based evidence exists that group A rotaviruses circulate in turkeys in Germany and the United States (103, 106).

VP4 and VP7 are both part of the rotavirus outer capsid and are antigens that elicit neutralizing antibody, which led to a dual classification system based upon the antigenic properties of each protein (4, 20). VP4 serotypes are P serotypes (derived from VP4's *P*rotease sensitivity) and VP7 G serotypes (VP7 is a *G*lycoprotein). At least 15 G serotypes and 14 P serotypes have been identified based upon virus neutralization assays with hyperimmune sera and monoclonal antibody reactivities, but numerous additional P genotypes exist based upon sequence data (4, 26, 60). Some data does exist regarding avian rotavirus G serotypes in turkeys and chickens (69). Serotype G7 contains group A rotaviruses of chickens, turkeys, and pigeons (13, 14, 26, 41, 98). Sequence data for avian VP7 also suggests novel G serotypes circulate in poultry (56, 60, 97). Sequence data for avian VP4 and VP8* (VP8* contains the antigenic sites that define the P serotypes) has revealed several P genotypes in poultry (98, 103, 117).

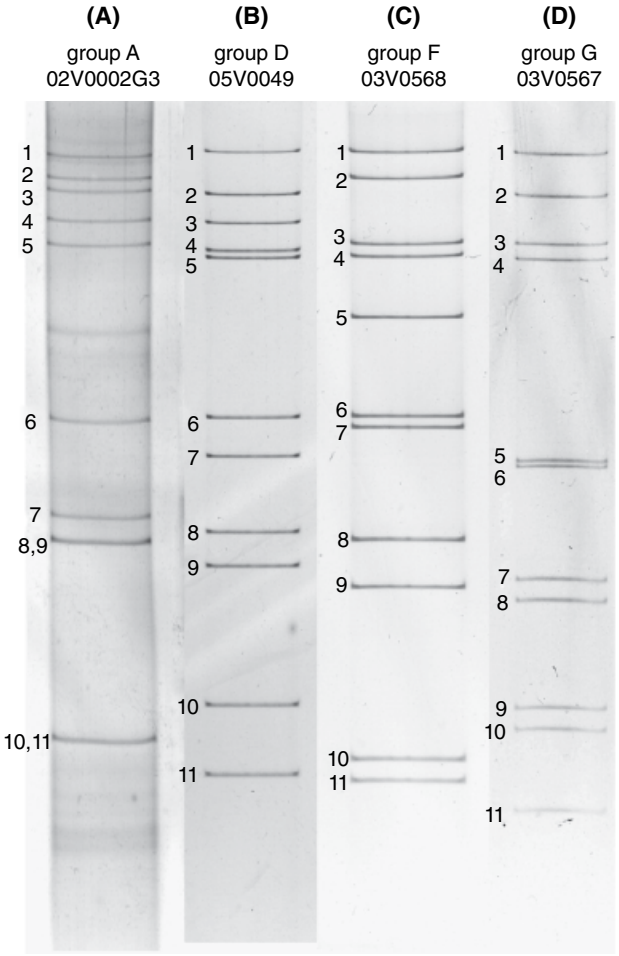


Figure 12.6 Analysis of the banding patterns of avian rotavirus dsRNA genomes after polyacrylamide gel electrophoresis followed by silver staining. RNA segments are numbered according to their mobility. (A) group A rotavirus 02V0002G3, (B) group D rotavirus 05V0049, (C) group F rotavirus 03V0568, and (D) group G rotavirus 03V0567 (55).

Genetic

Analysis of the rotavirus segmented genome via polyacrylamide gel electrophoresis (PAGE)—electropherogrouping—remains a robust method to distinguish rotaviruses in the laboratory (26, 83, 103, 116), and can be utilized tentatively to type newly characterized rotaviruses (Figure 12.6) (49). Five electropherogroups have been established in turkeys and chickens (115), with group D chicken rotaviruses having dsRNA migration patterns similar to turkey and pheasant group D rotaviruses (21, 68, 72, 111, 115).

A uniform classification system for the group A rotaviruses based upon the nucleotide sequences of all 11 genome segments has been proposed by the rotavirus classification working group (RCWG) (59–61). Using this approach, an individual group A rotavirus strain is classified with the notation Gx-P[x}-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx referring to the rotavirus genes VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6, respectively, with each genome segment receiving a numbered genotype. Using this approach, the fully sequenced chicken group A rotavirus Ch-2G3 possesses the genotype constellation G19-P[30]-I11-R6-C6-M7-A16-N6-T8-E10-H8. This uniform classification system will help to identify interspecies transmission and reassortment among the group A rotaviruses (103). To facilitate the implementation of this classification system, a web-based sequence analysis tool has been developed (http://rotac.regatools.be/) (58). In addition to the classification system for the group A rotaviruses, a VP6-based sequence approach has been used to demarcate rotavirus species based upon phylogenetic analysis of

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novel avian rotavirus sequences. This approach determined that the group F rotaviruses are most closely related to the avian group A and D rotaviruses, while the group G rotaviruses are closely related to mammalian group B. A cutoff value of less than 60% nucleotide identity in the VP6 genome segment was used for molecular typing (49, 62).

Laboratory Host Systems

Certain avian rotaviruses can be isolated directly from feces or intestinal contents of birds using primary cell culture (chick embryo liver cells and chick kidney cells) (33, 67–69, 108, 126). Avian rotaviruses from chickens, turkeys, pheasants (group A), and pigeons have also been isolated using the fetal rhesus monkey kidney continuous cell line MA104 (3, 53, 57, 76, 81, 106, 112). Group D pheasant rotavirus could not be propagated in MA104 cells, even after multiple blind passages (21). Pigeon rotavirus has been propagated successfully in MDBK cells (76). Group A avian rotaviruses have been cultivated in normal chicken spleen cells as well as virus-transformed avian lymphoblastoid cell lines (both B and T cell lines) (102). The proteolytic cleavage of rotavirus VP4 into VP8* and VP5* by trypsin primes the virion for efficient infectivity (22, 23), therefore the addition of trypsin to virus inocula and cell culture media generally facilitates avian rotaviruses cell culture (63, 76). Interestingly, the majority of avian rotaviruses that have been successfully isolated and/or propagated in cell culture have been group A avian rotaviruses, with the exception of strain 132 (group D chicken) and duck rotavirus F29, both of which lacked a common rotavirus group antigen at the time of isolation (68, 108).

Certain rotaviruses have been isolated and propagated in embryonating chicken eggs. A rotavirus from a lovebird (*Agapornis*) propagated in chick embryos via the yolk sac resulted in death 4–6 days after inoculation. Group A turkey rotaviruses inoculated into the yolk sac of embryonating chicken eggs also resulted in embryo death (15, 31). Many avian rotavirus strains can also be propagated successfully in their natural hosts (21, 37, 64, 125, 127–129), and in avian species other than their natural hosts (125–127, 129).

Pathogenicity

A survey of turkey flocks revealed group A rotaviruses slightly more often in healthy flocks than in diseased flocks. (94). A retrospective analysis of poult enteritis cases in California from 1993–2003 revealed that RVLVs and "small round viruses" were the most common viruses detected via electron microscopy (124). In a similar analysis in Minnesota, turkey flocks diagnosed with poult enteritis syndrome (PES) were determined via electron microscopy to be infected with rotavirus 48% of the time ("small round viruses" were detected in 17% of these flocks) (48). Another molecular diagnostic study of PES in Minnesota turkeys revealed that 93% of PES cases studied (n = 43) were positive for rotavirus via RT-PCR; electron microscopy detected rotavirus particles in 25 of the 43 PES cases (46).

Pathobiology and Epidemiology

Incidence and Distribution

Rotaviruses have been detected in or isolated from chickens in the United States (86, 126), Italy (27), Argentina (10), Belgium (74), Brazil (2, 12), China (123), Cuba (29), Germany (25, 84), India (75), Bangladesh (85), the United Kingdom (66, 68, 69), and the former Soviet Union (5). Rotavirus has been detected in guinea fowl in Italy via electron microscopy and in the United States via RT-PCR (88, 114). Rotavirus has been detected in or isolated from pheasants in Italy (28, 57), the United Kingdom (30, 34, 35), Hungary (119), and the United States (96, 126). Other reports of rotavirus in avian species include: clinically normal ducks in Japan (108), and the United Kingdom. (121); apparently normal feral pigeons in Japan (76), and diseased racing pigeons in the United Kingdom (33); diseased partridges in the United Kingdom (30) and diseased partridges and Japanese quail in Italy (88). Rotaviruses have been detected in and isolated from wild birds, including a velvet scoter (*Melanitta fusca*) in Japan (110); a lovebird (*Agapornis* species) in the United Kingdom (31); ratites in South Africa (ostrich) and the United States (emu chick) (24, 38); and healthy wild pheasant (*Phasianus colchicus*) and reed bunting (*Emberiza schoeniclus*) in Hungary (120). Antibody to rotavirus has been detected in chickens in Japan (101, 109), ducks in the United Kingdom (67), and pigeons in Belgium (122).

Natural and Experimental Hosts

Naturally occurring rotavirus infections in poultry generally occur in birds that are young, less than about six weeks old (8, 9, 30, 86, 87, 96). This contrasts with experimental rotavirus infection in SPF chickens and turkeys where there is evidence that older birds (56–119 days for chickens, 112 days for turkeys) are more susceptible to enteric disease signs than younger birds (125, 129); further, an outbreak of rotavirus-associated diarrhea has been reported in commercial laying hens between 32 and 92 weeks of age (50). A rotavirus of apparent bovine origin was isolated from 90–150 day old turkeys presenting with diarrhea and low overall performance (3). Natural infections with avian rotaviruses are

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often accompanied by simultaneous or sequential infections with other rotavirus electropherogroups and with other avian enteric viruses such as astrovirus and reovirus (73, 84, 86, 87, 94, 113, 115).

Transmission, Carriers, and Vectors

Rotaviruses are excreted in avian feces (129), and can be readily detected in cloacal swabs using molecular diagnostics (106). Horizontal transmission occurs readily between birds, and rotavirus was the only enteric virus detected via RT-PCR in poults prior to placement on farms during a longitudinal field survey (87). Egg transmission of rotaviruses has not been demonstrated, but rotavirus detection in 3-day-old turkey poults prompted speculation that transmission occurs either in or on the egg (113). Further, rotavirus has been detected in otherwise healthy breeder turkeys up to nine weeks of age (45). Evidence demonstrates that larvae of the darkling beetle can act as a mechanical vector for turkey rotaviruses (19).

Incubation Period, Clinical Signs, Morbidity, and Mortality

In experimentally infected 3-day-old turkeys, watery-to-soft droppings with orange-tinged mucus were passed 2–5 days postinfection (DPI), and impairment of D-xylose absorption from the intestinal tract occurred at 2 and DPI. Inoculated turkeys were depressed with loss of appetite and pasting of the vents between 1 and DPI. When inoculated orally into 3-day-old poults alone or in combination with turkey astrovirus and/or turkey reovirus, rotavirus could be detected via RT-PCR in cloacal swabs from 100% of poults by 2 DPI (36, 106, 127, 128). Similar clinical findings were reported in poults inoculated with crude intestinal homogenates that contained rotavirus, among other agents (47). In the majority of studies, no mortality occurred in experimentally infected turkeys or chickens (105, 106). Mild (64) or no clinical signs (74, 125) were observed following experimental infection of chickens. Chicks had mild diarrhea (68) or passed increased quantities of cecal droppings (64). In a separate experiment, laying hens experimentally infected with rotavirus showed a drop in egg production 4–9 DPI (128). Rotavirus was detected in feces of experimentally infected chickens and turkeys from 24 hours postinfection, and in some birds, shedding continued for more than 16 days (47, 64, 106, 125, 127, 128).

Under field conditions, clinical signs associated with rotavirus infection in broilers vary from subclinical infections to outbreaks of severe diarrhea with associated dehydration, poor weight gain, and increased mortality (2, 10, 67, 69). Similarly, in poults, clinical signs include: (1) very mild diarrhea in the first week of life (39); (2) a more severe disease in 12–21-day-old poults characterized by restlessness, litter eating, and watery droppings with mortality between 4 and 7% (11); and (3) diarrhea in 2–5-week-old poults, with increased mortality and stunting of survivors (66).

Diarrhea and increased mortality has also been reported in 2–3-week-old pheasant chicks in the United States (96). In the United Kingdom, rotavirus infection was associated with stunting and increased mortality in pheasant chicks in the first week of life (34, 35). Six of twenty 2-day-old pheasants inoculated with intestinal contents containing rotaviruses from naturally occurring cases died 5–6 DPI (35); a high mortality rate was also observed in pheasant chicks inoculated with a group D rotavirus (37). In Italy, infected pheasants between 6 and 40 days of age showed depression, drooping wings, yellowish watery diarrhea, and dehydration; mortality was 20–30% (28). Diarrhea, lethargy, and loss of appetite were associated with rotavirus infection in 3–4-month-old racing pigeons in the United Kingdom (33).

Pathology

Gross

The most common finding at necropsy is the presence of abnormal amounts of fluid and gas in the intestinal tract and ceca. Pallor of the intestinal tract accompanied by loss of tonicity may be evident. Secondary findings include dehydration, stunting of growth, pasted vents, inflamed vents, anemia due to vent pecking, litter in the gizzard, and inflammation of the feet (11, 36, 37, 39, 64, 69, 105, 106, 129). Hemorrhages were observed in the cecal walls of some experimentally infected pheasant chicks (35), and discrete, multifocal, superficial, brownish-red erosions were found in the duodenum and jejunum of turkeys experimentally infected at 84 and 112 days of age (129).

Microscopic

Immunofluorescence (IF) studies using chickens and turkeys experimentally infected with rotavirus have demonstrated virus replication in the cytoplasm of mature villous absorptive epithelial cells in the small intestine. Infected cells were most numerous in the distal third of villi. Small numbers of infected cells were detected in colon epithelium, cecal tonsils, and lamina propria of some villi. No IF was observed in proventriculus, gizzard, spleen, liver, or kidney (64, 74, 125, 127, 129). One group A rotavirus grew best in the duodenum of experimentally infected chickens, and a group D rotavirus favored the jejunum and ileum (64).

Microscopic lesions in the small intestines of turkeys experimentally infected with group A rotaviruses consisted of basal vacuolation of enterocytes, separation of enterocytes from the lamina propria with subsequent

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desquamation, villous atrophy accompanied by widening of the lamina propria, scalloping of the villus surface, fusion of villi, and leukocytic infiltration of the lamina propria (105, 129). In general, mean villous lengths were decreased and crypt depths were increased following experimental infection; morphometric changes were more pronounced in the duodenum and jejunum than in the ileum (36, 105, 129). There was infiltration of polymorphonuclear and mononuclear cells into the lamina propria of the cecum and colon in some birds. Scanning electron microscopy demonstrated roughened villous surfaces, irregularly shaped and sized villi, and loss of microvilli in enterocytes located at the tips of villi (36, 129). A group A rotavirus was detected via immunohistochemistry (IHC) in the distal portion of the villi in the jejunum of experimentally infected poults, and separation of enterocytes from the lamina propria was evident at 4 DPI (106) (Figures 12.7 and 12.8). In experimentally infected chickens, minimal leukocytic infiltration of the lamina propria, with minimal loss of microvilli on cells at villus tips was observed (129). Moderate villous atrophy, mainly in the ileum has also been described by other workers in experimentally infected chickens (74). Similar lesions to those found in turkeys were reported in pheasant chicks experimentally infected with a group D rotavirus (37).

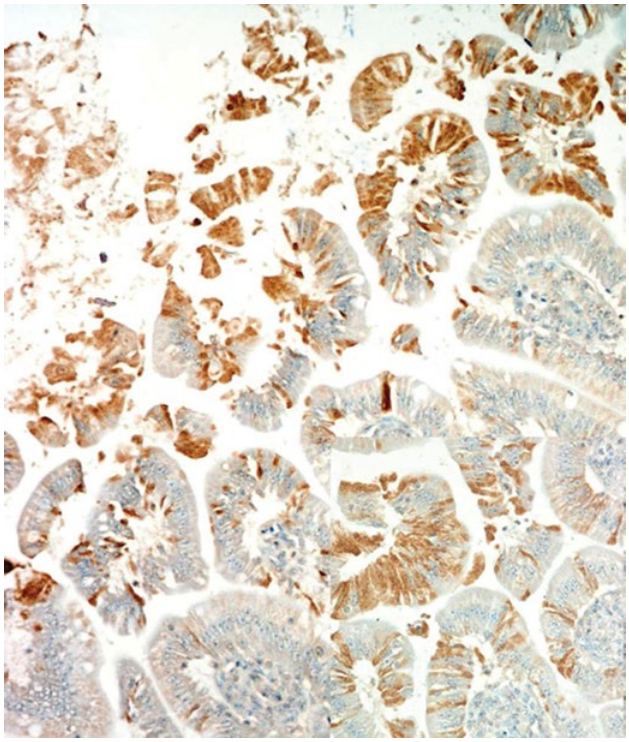


Figure 12.7 Photomicrograph of a section of the jejunum from a poult infected with rotavirus, 4 days postinfection (DPI). (1). Viral antigen staining present in the cytoplasm of the enterocytes at the distal section of the affected villi (brown staining). Primary antibody (hyperimmune serum) prepared from chickens inoculated with oil-emulsion rotavirus vaccine. Immunoperoxidase staining with hematoxylin counterstain, ×400 magnification (122).

Pathogenesis of the Infectious Process

With both avian and mammalian rotaviruses, the target cells are mature columnar absorptive cells that are located in the villous epithelium. Studies with whole virus and with purified VP8 (VP8* is the natural cleavage product of VP4), the cell attachment protein, indicate that when initiating infection avian rotaviruses utilize sialic acid-containing molecules as receptors on the surface of MA104 cells (107). It is not known if a similar mechanism operates *in vivo*.

There is histological evidence that rotavirus infection of enterocytes in the turkey jejunum leads to destruction of the distal portion of the villi (106) (Figures 12.7 and 12.8); structural damage to the villi ("scalloping") is also observed in the turkey duodenum during rotavirus infection (Figure 12.9). The frothy fluids found in the ceca of infected birds may result from impaired absorption of carbohydrates that leads to their fermentation by cecal bacteria, producing metabolites that draw water into the ceca by osmosis (127).

However, malabsorption may not be the only explanation for rotavirus-induced diarrhea. NSP4 proteins of mammalian rotaviruses are enterotoxins, causing diarrhea in suckling mice (6, 7, 26). Avian rotavirus NSP4 glycoproteins have similar biological activity, and there are conserved structural regions in the NSP4 enterotoxin domain between avian and mammalian rotaviruses (77–79).

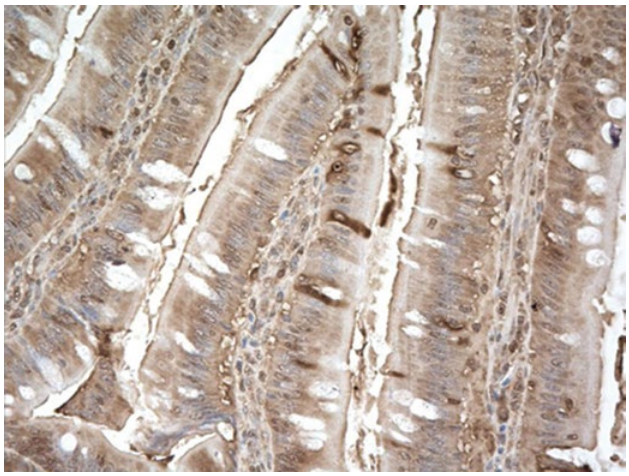


Figure 12.8 Photomicrograph of section of the jejunum from a poult infected with rotavirus, 6 days postinfection (DPI). Viral antigen staining present in the cytoplasm of the enterocytes (brown staining). Primary antibody (hyperimmune serum) prepared from chickens inoculated with VP6 rotavirus vaccine. Immunoperoxidase staining with hematoxylin counterstain. (M. Pantin-Jackwood).

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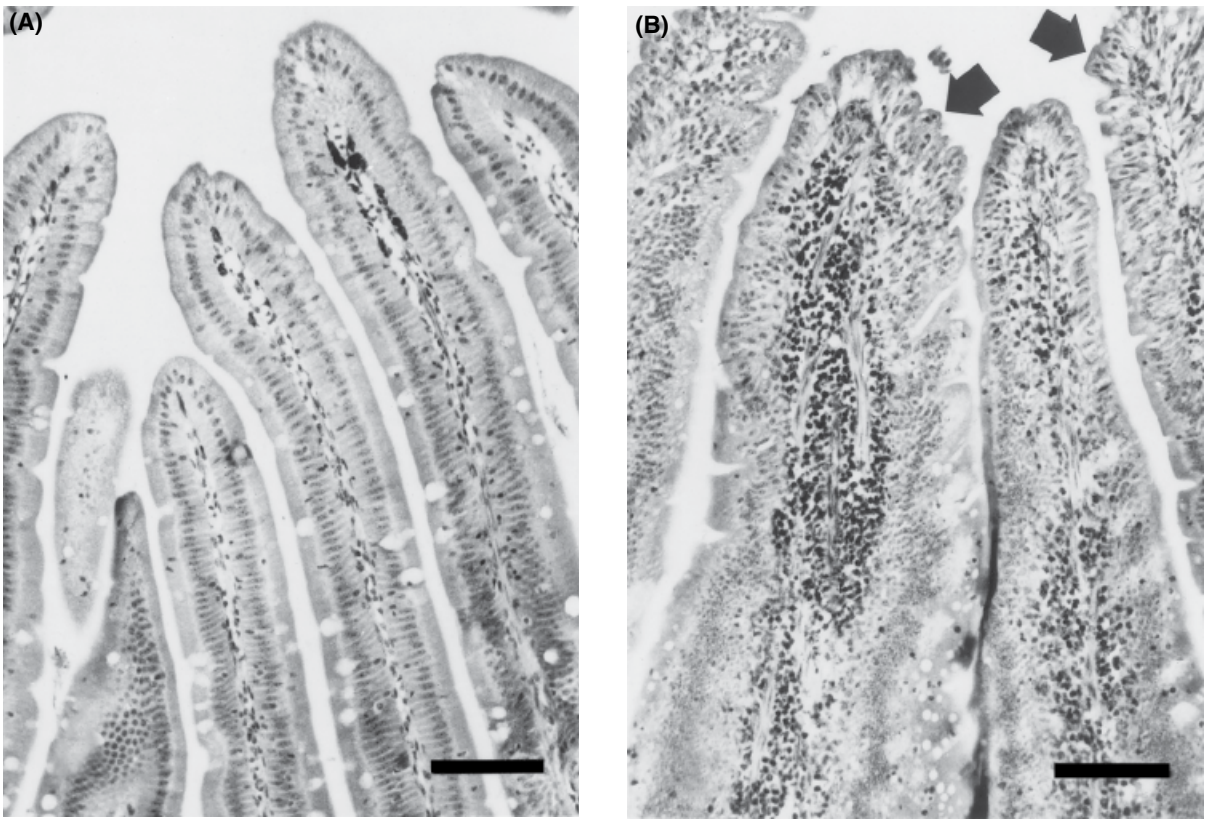


Figure 12.9 Duodenum of SPF turkey poults. (A) Normal villi of an uninfected control poult at 10 days of age. (B) Villi of a 10-day-old poult infected with Tu-2 rotavirus at 7 days of age. Note the remarkable hypercellularity in the lamina propria, scalloping of the villous surface, and basal vacuolation of the epithelial cells at the tips (arrows). H&E stain, bar = 0.1 mm (127).

Immunity

Active

Chickens and turkeys inoculated orally with rotaviruses showed serum antibody responses as early as 4–6 DPI measured by indirect IF. In general, older birds developed higher antibody titers and responded more quickly than younger birds (125, 127, 128). Using immunoglobulin class-specific enzyme-linked immunosorbent assays (ELISAs) (82) to follow antibody responses in chickens experimentally infected with a group A rotavirus, rotavirus-specific IgM, IgG, and IgA were detected in serum, while the intestinal antibody response consisted almost entirely of IgA. Natural killer cell-like activity has been demonstrated in chick intraepithelial leukocytes against rotavirus-infected target cells, and this may be an important *in vivo* immune response (80).

Passive

Maternal antibodies to rotavirus are passively transferred to the avian embryo through the egg yolk and are detectable until about 3–4 weeks of age (127). Progeny of hyperimmunized turkey hens were more resistant to experimental infection with rotavirus at 2 or 5 days of age, but not at 12 days of age. Circulating maternally-derived IgG may protect the intestinal mucosa against rotavirus infection in 1–7 day old poults (74, 105, 127). During the first week of life, maternally-derived anti-rotavirus IgG titers in intestinal washings of poults derived from hyperimmunized (vaccinated) hens were 200–500-fold less than titers in serum. The presence of maternal antibody in the serum in two other studies had no apparent effect on susceptibility of chickens and turkeys to experimental group A rotavirus infection (74, 127). Maternally derived IgG could not be detected in intestinal washings of progeny derived from naturally infected hens (104).

Similarly, an increase in serum neutralizing antibodies was observed in pheasant hens vaccinated with an inactivated group A pheasant rotavirus vaccine (32). These results and those cited previously for the progeny of vaccinated turkeys suggest: (1) that maternally derived antibodies in the progeny of unvaccinated turkeys and pheasants are unlikely to provide significant protection against a field challenge with rotavirus; and (2) that much higher titers of antibody would need to be produced by vaccination to completely protect young birds even for the first week of life.

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Diagnosis

Isolation and Identification of Causative Agent

The classic way to diagnose avian rotavirus infections in the laboratory is to identify the virus in feces or intestinal contents by direct electron microscopy (70, 71). Immune electron microscopy allows rotaviruses of different serogroups to be distinguished, although the technique requires availability of specific reference antisera (100, 111).

Using fecal samples collected from turkeys experimentally infected with a group A rotavirus, negative contrast electron microscopy was more sensitive than a staphylococcal protein-A coagglutination test and a commercial ELISA developed for mammalian group A rotavirus detection (54). Commercially available ELISAs are commonly used to detect group A rotaviruses in mammalian and avian feces. No ELISAs are available to detect rotaviruses of groups D, F, and G.

Diagnosis of rotavirus infection by virus isolation in cell cultures is useful only for group A avian rotaviruses. It has proven extremely difficult to isolate other rotavirus serogroups in cell culture (21, 53, 73, 112). The details of avian rotavirus isolation and propagation in continuous mammalian cell lines and in primary avian cells have been described in detail (53, 63, 71, 76, 81, 108, 112, 126).

A multiplex RT-PCR assay was designed and validated for the simultaneous identification of enteric viruses that are often found concomitantly in turkeys and chickens: avian astroviruses and avian rotaviruses (18). The assay has been used successfully for enteric virus surveys in both turkeys and chickens in different geographical regions of the United States (86, 87), and the NSP4 RT-PCR assay alone has been used for surveys of turkeys affected by PES (45, 46). A one-step RT-qPCR has been developed for the detection of turkey rotavirus NSP4 in fecal samples (1). A conventional RT-PCR assay targeting the rotavirus VP6 gene has been used successfully to amplify rotavirus RNA in field samples from pheasants and wild birds (119, 120). Individual RT-PCR assays targeting specific rotavirus genome segments have allowed in-depth sequence and phylogenetic based analyses of the avian rotaviruses (49, 62), and recent diagnostic RT-PCR assays have been used to detect and differentiate group A and D avian rotaviruses in field samples (83).

Serology

Serologic diagnosis of rotavirus infections is problematic, since the high prevalence of antibody (65, 76) makes results difficult to interpret. Furthermore, the inability to adapt some avian serogroups to cell culture has resulted in gaps in the available battery of antigens. Serologic screening using indirect immunofluorescence (65) or ELISA (82) is useful for establishing and monitoring the status of specific pathogen free flocks.

Differential Diagnosis

Rotavirus infection must be differentiated from other conditions causing diarrhea. Because the clinical signs and pathology of rotavirus infection are not pathognomonic, laboratory diagnosis is necessary. It is not unusual to find other potential viral enteropathogens in flocks with enteric disease and performance problems (30, 38, 86–88, 95, 100, 131).

Intervention Strategies

Management Procedures

No specific treatment or means of control exists for the avian rotaviruses. The effect of diarrhea on the litter can be minimized by increasing ventilation rate and temperature and by adding fresh litter. Where litter is reused several times, infectious agents can build up, and problems are likely to be more severe than in situations in which houses are cleaned and fumigated and fresh litter is used for each batch of birds.

Vaccination

Commercially available vaccines have not yet been developed. Given the extent of antigenic diversity that exists in avian rotaviruses and the difficulty in growing non-group A rotaviruses in cell culture, obvious problems exist in vaccine development. Preliminary work on experimental group A rotavirus vaccines in turkeys (104) and pheasants (32) indicates that inactivated vaccines administered to the breeders are unlikely to protect the progeny against challenge for more than the first week of life.

Astrovirus Infections

Giovanni Cattoli

Summary

Agent, Infection, and Disease. Astrovirus infections are caused by small (25–30 nm in diameter), positive-sense RNA viruses that are spread via the fecal–oral route. Astroviruses (AstVs) are detected in several poultry species. In turkeys, AstVs are among the most common viruses associated with poult enteritis. In chickens,

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AstVs are associated with growth retardation, nephritis, white chicks hatchery disease, and runting stunting syndrome.

Diagnosis. The most popular diagnostic methods are based on reverse transcription-polymerase chain reaction (RT-PCR). Given the variability of AstVs genomes, attention should be given to the proper selection of the RT-PCR primers.

Intervention. Vaccines and chemotherapeutics are not available to date for the control and/or prevention of AstV infections. Strict biosecurity and use of effective disinfectants could reduce their incidence in poultry.

Introduction

Astroviruses (AstVs) have been associated with acute gastroenteritis in a variety of mammals and birds including turkeys, chickens, and guinea fowl, as well as fatal hepatitis in ducks and nephritis in chickens (reviewed in [8]). The identification of AstVs in poultry has greatly increased in recent years, likely due to enhanced surveillance and better diagnostic assays. To date, six different AstVs have been identified in poultry based on species of origin and the characteristics of the viral genome including two chicken-origin AstVs (avian nephritis virus [ANV] and chicken astrovirus [CAstV]), two turkey-origin AstVs (TAstV-1 and TAstV-2), and two duck-origin AstVs (DAstV-1 and DAstV-2) (Figure 12.10A). One must be careful when classifying newly identified AstVs from poultry by species as ANV has been detected in numerous

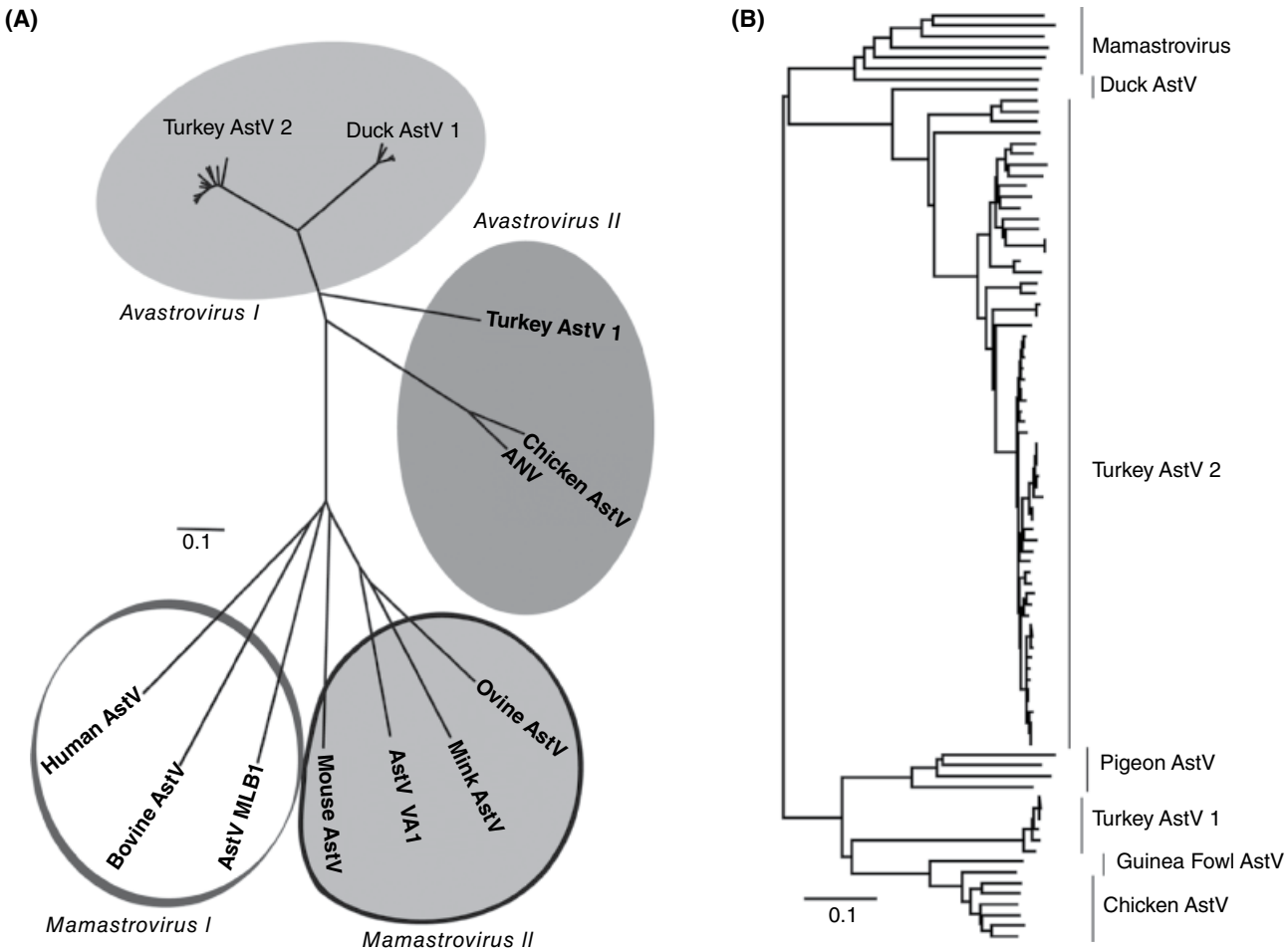


Figure 12.10 Phylogenetic trees of (A) whole genome and (B) capsid sequences of avian astroviruses. The complete nucleotide sequence of the astrovirus genome or an 800-nucleotide segment of the ORF2 capsid gene were aligned using MEGA4 software using the Neighbor-Joining method and a Kimura 2-parameter model with pair-wise deletion (Stacey L. Schukltz-Cherry).

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species including turkeys (10), ducks, and geese (2, 3). Ducks and guinea fowl have also been shown to be infected with TAstV-1 and TAstV-2, respectively (3, 5, 6). These data suggest that AstVs may be able to cross species barriers. Furthermore, it has been recently demonstrated in the United States the presence of TAstV-2 antibodies in people exposed to turkey, although no evidence for avian AstV replication or associated clinical disease has been provided in humans to date (17). Thus, the public health significance of this finding deserves further studies.

Astroviruses are among the most common viruses associated with poult enteritis complex (PEC), poult enteritis syndrome (PES), and poult enteritis and mortality syndrome (PEMS) in turkeys (13, 18, 24, 26). In chickens, AstVs have been associated with growth retardation, nephritis, white chicks hatchery disease, and runting stunting syndrome (RSS) in broilers (1, 9, 33, 41). However, they also can be isolated from clinically healthy birds leading to questions about the role of particular AstV strains in disease pathogenicity (11, 18).

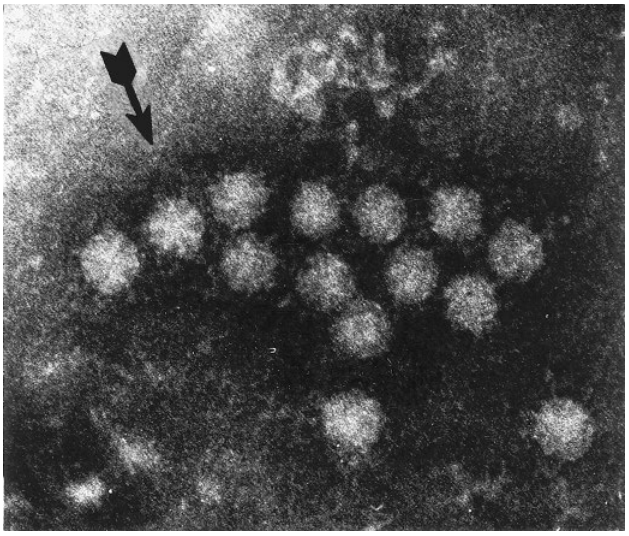


Figure 12.11 A star-shaped astrovirus particle (arrow) among an aggregate of astroviruses from intestinal samples of experimentally infected diarrheic poults, detected by immune electron microscopy. Average particle size is 29.6 nm (26).

Incidence and Distribution

Astroviruses have been detected worldwide and are one of the most prevalent viral infections in poults 1–5 weeks old with enteric disease (26–28). Several studies demonstrated that 86–100% of chicken flocks (23, 41) and up to 100% of turkey flocks (22) are infected with AstVs, frequently in association with other enteric viruses (23, 28). These infections typically occur within the first four weeks of life (27). When flocks were continuously monitored for enteric viral infections from one day of age until market, the first samples positive for viruses always contained AstVs, either alone or with other viruses (27).

Etiology

Astroviruses are small, round viruses typically 25–30 nm in diameter that are naturally spread via the fecal–oral route. The name *Astrovirus* arises from the five- or six-pointed star-like surface projections observed using transmission electron microscopy (TEM) (Figure 12.11) (29). However, only 10% of AstVs in a population may exhibit this morphology, and visualization depends on sample preparation leading to cautions about the use of TEM for diagnosis (8). Astroviruses are nonenveloped and composed of a positive-sense RNA genome 6.5–7.5 kilobase (kb) in length containing three open reading frames (ORF). These reading frames code for nonstructural proteins (ORF1a), a viral RNA-dependent RNA polymerase (ORF1b), and a capsid protein (ORF2). Their replication strategy is quite distinct from other enteric virus families in that they synthesize a subgenomic message during replication, contain a retrovirus-like frame shift signal sequence between ORF1a and ORF1b, and have a serine protease (16). Recently, several avian AstV genomes have been fully sequenced, supporting enhanced phylogenetic analysis. These analyses show that avian AstVs are molecularly distinct from mammalian AstVs and share little sequence similarity in the different gene segments (Figure 12.10A). For example, comparing the most conserved region of the genome, the RNA-dependent RNA polymerase (RDRP) from the human astrovirus type 1 (HAstV-1), to any of the avian viruses show s approximately 40% sequence identity. Among the different avian viruses there is great diversity ranging from only approximately 40% similarity (duck astrovirus-1 to other avian viruses) up to 94–98.9% (within TAstV-2 genomes).

The prototype TAstV-1 strain was originally identified in the United States in 1985 and is genetically and antigenically distinct from TAstV-2 isolates. Even within the more conserved polymerase gene, there is at least 52–60.5% nucleotide divergence among the TAstVs supporting the separation of isolates into two distinct groups (21). The diversity within the TAstV-2 capsid gene is staggering (8, 11, 23, 24) (Figure 12.10B) and there is increasing evidence that AstVs can undergo recombination (8, 21, 37). Genetic analysis suggests that TAstV-1 and TAstV-2 should be reconsidered as subtypes instead of serotypes and that multiple serotypes of TAstV-2 exist (24, 37). Based on the ORF2 sequence, the recently emerged CAstV can also be separated into two groups, namely A and B, which support the previous evidence of two serogroups with low degree of cross-reactivity. Chicken astrovirus groups A and B share low level of amino acid identity, estimated at 38–40% in ORF2 (33).

Given this diversity, a classification based on the genetic criteria is more appropriate than classifying the

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viruses into species within the genus *Avastrovirus* based only on the host of origin as done previously. According to the last classification of the International Committee on Taxonomy of Viruses (ICTV), three new species within the genus *Avastrovirus* are included, namely *Avastrovirus* 1 (TAstV1 and 2), *Avastrovirus* 2 (ANV, CAstV), and *Avastrovirus* 3 (DAstV-1 and 2).

Susceptibility to Chemical and Physical Agents

Astroviruses are extremely stable and resistant to inactivation by phenolics, acidic pH, chloroform, a variety of detergents, heat, ambient temperatures, quaternary ammonia, most alcohols, and lipid solvents (29).

Laboratory Host Systems

Virus isolation in embryonating eggs has been successful for most of the avian AstVs including TAstV and CAstV. Turkey-origin astroviruses can be propagated serially in the yolk sac of 20-day-old turkey embryos or by inoculating 24- to 25-day-old turkey embryos by the amniotic route (29). Turkey-origin astrovirus infection does not result in embryo mortality. Chicken astroviruses can be propagated in 7- or 14-day-old specific pathogen free (SPF) chick embryos following yolk sac inoculation. Infection can result with no lesions for the 14-day-old embryos or a variety of effects in 7-day-old embryos, including early embryo death, dwarfing, and edema (20). Virus isolation in cell culture has been unsuccessful for TAstVs, but successful isolation and propagation of CAstVs in primary chick embryo liver and LMH (chicken hepatocellular carcinoma cell line) cells producing a marked cytopathic effect (CPE) after 4–5 passages has been reported (1). Chicken astroviruses replicate poorly in chick embryo fibroblasts and chick kidney cultures initially, but after several passages will replicate in chick kidney cells inducing CPE.

Pathogenesis and Epidemiology

Turkey-origin astroviruses are commonly detected in 1- to 5-week-old commercial poults suffering from diarrhea (11, 18, 26–29) and have been associated with several enteric disease syndromes (13, 18, 24, 26). Clinical signs of disease usually develop between one and three weeks of age and generally last up to two weeks (29). Severity is usually mild to moderate and is characterized by diarrhea, listlessness, litter eating, and nervousness. Although the mortality rate is low, morbidity occurring as decreased growth (stunting) is of greatest concern. Turkey-origin astroviruses also can be detected in apparently healthy birds but at a lower frequency (11, 29).

Experimentally, TAstV-2-infected turkeys develop a profuse, watery diarrhea by 2 days postinfection (DPI), which continued through 12 DPI. Morphologically, intestines of infected poults are 3–5 times larger than their control counterparts and appeared dilated, distended, and fluid filled by 3 DPI. There is an overall growth depression in infected birds, possibly as a consequence of malabsorption (29). The effect of TAstV-2, turkey rotavirus, and turkey reovirus coinfections in decreased weight gain in broad-breasted white turkey poults showed that poults exposed to all three viruses administered in combination had the lowest body weight, although the weights were not significantly different from control birds (36).

Chicken astrovirus is usually detected in chickens within the first days or week of life. It can be transmitted horizontally and probably vertically, although this has not yet been experimentally documented. Historically, CAstV has been associated with RSS, enteritis, and growth problems especially in broilers. Group B CAstV is generally associated with more severe infections and lesions, even outside the intestinal tract. In India and the Middle East, group B was associated with kidney disease and visceral gout leading up to 40% mortality in broiler chicks (4, 33). Group B viruses sharing 86–90% ORF2 amino acid similarity with those described in India were detected in Europe, North and South America and associated with the so-called white chick hatchery disease. It is characterized by reduction in hatchability (29–68%), chicks with pale plumage, weak, and increased chick mortality.

Histological and Gross Changes

At necropsy, the characteristic pathologic changes associated with TAstV infection are dilated ceca containing yellow, frothy contents and gaseous fluid, loss of tone (gut thinness), and hyperemia of the intestinal tract. TAstV-1-infected poults exhibited histopathologic lesions of the small intestine characterized by mild crypt hyperplasia resulting in increased crypt depth and area beginning in the proximal jejunum as early as 1 DPI, with all portions of the small intestines affected by 5 DPI (29). Unlike some other intestinal viral infections, TAstV infections are not associated with villous atrophy.

A more extensive *in situ* hybridization study with TAstV-2 demonstrated that replication was detected in the upper regions of the small intestine by 24 hours postinfection. Individual degenerating enterocytes were apparent along the basal edge of villi by 2 DPI and continued through day 4. By day 5, mild shortening of the villi was observed as well as occasional clusters of necrotic enterocytes along the villous base, correlating with infection (29). TAstV-2 was localized primarily to the large intestine by day 7 PI. Replication peaked at 3–5 DPI and TAstV-2 was infrequently observed at later time points. There was little evidence of cell death. This lack of

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cellular damage is accompanied by a notable absence of an inflammatory response (12, 29). Several studies suggest that the combination of malabsorption and changes in paracellular permeability may be the cause of the diarrhea (19, 29). Although viral replication is likely an important player, it appears that the viral capsid protein alone can induce acute diarrhea in turkey poults, acting as a novel enterotoxin leading to increased barrier permeability as demonstrated *in vitro* and *in vivo*. It may also explain the systemic spread in TAstV-2-infected poults (17, 29).

Experimental infection of SPF layer chicks with CAstV resulted in a mild diarrhea and limited damage at the base of the small intestinal villi in some birds, and no histological changes outside the gut (1). However, group B CAstV infections have been associated with extraintestinal infections with liver necrosis and pale, swollen kidneys, pancreas, and spleen (20).

Immune Response

The immune response to AstVs is not well characterized. A study with CAstV suggests that vaccination with a purified form of recombinant CAstV capsid protein leads to a partial protection from subsequent RSS challenge (36). There is no such information for TAstVs and neutralizing antibodies against TAstVs have not been isolated. TAstV-2 infection in poults is considered a poor inducer of adaptive immune responses (12). The reason for this could be the reported dysregulation of the immune response by TAstV. Specifically, the responsiveness of peripheral blood lymphocytes (PBLs), thymocytes, and splenocytes isolated from TAstV-2-infected poults was significantly reduced as compared to control birds (29). Functionally, macrophages and heterophils from TAstV-2-infected poults had reduced expression of several key proinflammatory cytokines (25) and upregulation of the immunosuppressive cytokine, TGF-beta (12, 29), further supporting the hypothesis of an impaired innate response during infection.

Despite this impaired immune response, the virus is cleared and clinical disease resolved within 12–14 DPI. Although the mechanism is still unknown, several studies suggest that the induction of nitric oxide (NO) during TAstV-2 infection may be important in halting viral replication in infected poults (12, 25, 29). However, much more work needs to be done to define the immune response to AstV infection, especially if vaccines are considered for control in commercial poultry.

Diagnosis

Commercial poultry appear to be endemically infected with a variety of divergent CAstV and TAstV strains. Thus, diagnostic assays need to be able to not only detect AstVs but also differentiate among different genotypes. Several groups have developed ELISA-based methodologies to detect either CAstV-specific antibodies using recombinant capsid protein (30) or the presence of TAstV-2 antigen in feces or intestinal homogenates (38, 39). One specific group B CAstV antibody ELISA was recently developed and it can be used to screen breeder flocks for seroconversion (31).

There has been increasing use of molecular diagnostic tests for detecting avian AstVs. Several reports described conventional and/or real-time RT-PCR tests for detecting TAstV (14, 32, 35, 40) and CAstV (21, 34). This procedure is completed by pooling the feces or lower intestines from 3–5 birds/flock and either isolating RNA directly or passaging filtered fluids once through embryonating eggs. The RNA from the field sample or from the isolated embryo intestines then is subjected to RT-PCR using oligonucleotide primers specific to two different genes of the viral genome, a conserved region and a diverse region. However, given the diversity displayed by avian AstVs, it can be difficult to select primers that will detect all variants within one type. A recently identified set of degenerate primers within the highly conserved region of the ORF1a that has successfully detected AstVs from multiple mammalian and avian species (7). This reaction yields an approximately 400-nucleotide product that can be sequenced to initially identify the AstV genogroup. A positive finding needs to be followed with the use of genotype-specific primers to the capsid gene to identify the exact strain within the genogroup. However, because the majority of commercial poultry appear to become infected with avian AstVs at some point in their lifetimes, knowing that a flock is AstV positive may be of questionable importance. However, it is possible that future field studies using assays that differentiate among the many strains may point to a role for specific genotypes in disease, necessitating the need for specific testing.

Treatment, Prevention, and Control

No vaccines, chemotherapeutics, or other measures are reported to be fully efficacious for control and/or prevention of avian AstV infections. Vaccines containing CAstV B recombinant capsid protein from different strains were able to elicit specific antibody responses and provided partial protection against RSS challenge (reviewed in [33]). This approach may be worthy of further investigation given the rather clear association between white chick disease, kidney lesions, and the infection with some strains of CAstV. The widespread occurrence of AstVs in commercial poultry coupled with their environmental stability and resistance to inactivation by most widely used disinfectants makes elimination from affected areas difficult (29). It is likely that strict biosecurity, increased downtime between flocks, and use of effective disinfectants could reduce the likelihood of AstV infections; however, this approach requires further investigation.

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Avian Enterovirus-Like Virus Infections

Christopher S. Hayhow

Summary

Agent, Infection, and Disease. Enterovirus-like viruses (ELVs) are picornaviruses that cause disease in a number of avian species, and likely have a worldwide distribution. Most infections occur in young birds during the first few weeks of life with transmission most likely through ingestion of infected feces. The main clinical signs are diarrhea, decreased feed efficiency, uneven growth, and increased mortality.

Diagnosis. Diagnosis of ELV infections in avian species most commonly is accomplished by transmission electron microscopy (TEM) examination of droppings or intestinal samples. Enterovirus-like viruses have been identified using both direct and immune TEM procedures.

Intervention. The role of ELVs as avian pathogens has not yet been fully defined. Consequently, no specific therapeutic or prophylactic measures are available.

Introduction

A number of enterovirus-like viruses (ELVs) have been identified in avian species. The term enterovirus-like is applied to these viruses because they have not been fully characterized; definitive classification awaits further biologic, physicochemical, and molecular characterization. This section addresses ELVs identified in domestic poultry other than duck hepatitis virus types 1 and 3 (Chapter 13) and turkey viral hepatitis virus (Chapter 14).

The economic significance of avian ELVs is not yet known. No evidence suggests that they are transmissible from avian species to humans or other mammals. The extent, if any, to which they spread among different species of domesticated poultry is unknown.

Etiology

Classification

Enteroviruses are 1 of 12 within the family Picornaviridae (22). Members of Picornaviridae contain a single molecule of infectious, positive-sense, single-stranded RNA, 7–8.8 kb in size. Genera within the family Picornaviridae are distinguished *inter alia* by their sensitivity to acid, buoyant density of the virion in CsCl, and clinical manifestations in the affected host. Members of the genus *Enterovirus* are stable at acid pH, have a density of 1.30–1.34 g/mL in CsCl, and replicate primarily in the intestinal tract (21, 22). Most avian ELVs have been classified on the basis of size, morphology, cytoplasmic replication in enterocytes, and resistance to acid pH. However, it is emphasized that these biological criteria are insufficient for definitive classification. This is borne out by nucleotide sequence analyses of genomic RNA, and antigenic analyses, of some avian viruses that possess these biological criteria. Avian encephalomyelitis virus, a virus that initially was considered to be an enterovirus, has been shown to share a high level of deduced amino acid sequence identity with hepatitis A virus (25, 43). Based on the available information, avian encephalomyelitis virus has (Chapter 14) been classified as a species in the genus *Tremovirus* in the family Picornaviridae (22). Similarly, nucleic acid sequence analyses of other viruses— avian nephritis virus and two viruses initially thought to be turkey ELVs—have been identified as members of the family Astroviridae (14, 15, 20, 22, 23, 33, 45, 46). Based on antigenic analyses, it is likely that several viruses initially identified as chicken ELVs will be reclassified in the future as astroviruses, because these viruses have been shown to share antigenic relationships with avian nephritis virus (4, 10, 30, 42).

Morphology

Picornavirus virions are icosahedral (T = 1), nonenveloped, and 22–30 nm in diameter. The virion lacks obvious surface structure, and no surface projections exist (21, 22) (Figure 12.12). The sizes described for most avian ELVs fall within a 22–30 nm range, although a range of 18–24 nm was described for a US turkey ELV (41).

A turkey ELV isolate was determined to have a buoyant density of 1.33 g/mL in CsCl (18).

Chemical Composition

Only limited information is available regarding the chemical composition of avian ELVs. Information on the genome structure of ELVs is available only for a single US isolate from turkeys (18). This virus was shown to possess a single-stranded RNA genome of approximately 7.5 kb. No information is available regarding avian ELV proteins.

Virus Replication

Replication of turkey ELVs has been investigated by both immunohistochemistry and transmission electron microscopy (TEM) (19). Virus replication was shown to

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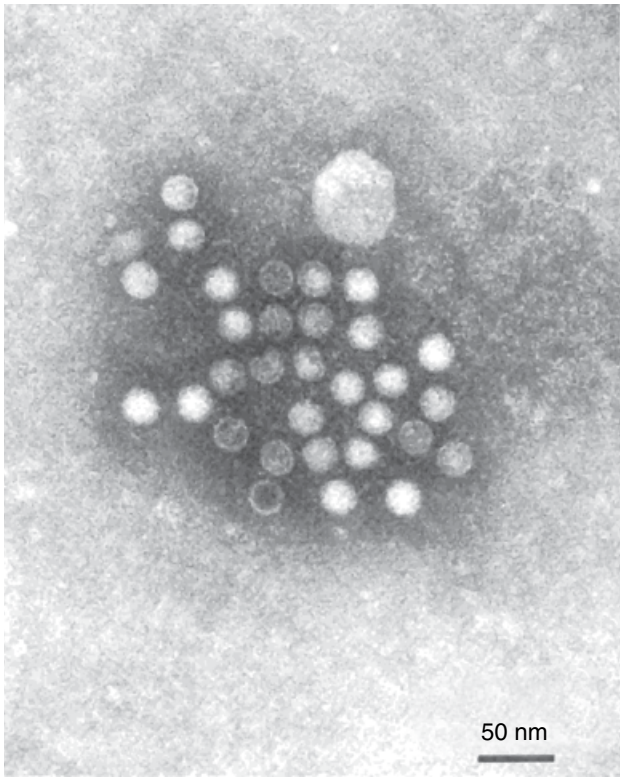


Figure 12.12 Spherical, 18–27 nm enterovirus-like viruses detected in feces of young turkeys with enteric disease. Sodium phosphotungstate.

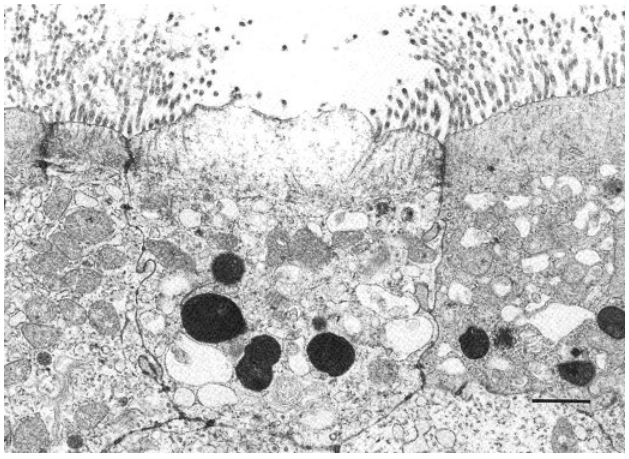


Figure 12.13 Degenerating enterocyte containing cytoplasmic crystalline arrays of enterovirus-like viruses (ELVs).

occur in the cytoplasm of intestinal enterocytes. Crystalline arrays composed of small, round, virus-like particles approximately 23 nm in diameter were observed (Figure 12.13) (19); an earlier study (41) described particles of 17.1 nm. Similar findings to the former were reported for chicken ELVs (6, 9, 26, 28). However, in one detailed study (9), membrane-bound cytoplasmic inclusions containing virus-like particles were detected more frequently in mesenchymal cells and macrophages in the lamina propria than in enterocytes. Some chicken ELVs also replicate in the kidney, and have been implicated in the etiology of baby chick nephropathy (38).

A US turkey ELV was shown by immunofluorescence and immunoperoxidase staining procedures to replicate primarily in the jejunum and ileum of experimentally infected poults. The virus replicated preferentially in those enterocytes located halfway between the tip and base of the villus. Viral antigen was found most abundantly in enterocytes situated immediately above crypt openings (17); similarly, antigens of chicken ELVs were found mostly in cells at the base of the villi (6).

No information is available concerning transcription and translation of the RNA of avian ELVs.

Susceptibility to Chemical and Physical Agents

Avian ELVs that have been tested have been found to be stable at pH 3 and unaffected by solvents such as chloroform and ether (26, 28, 29, 40, 42). No information exists about their sensitivity to disinfectants.

Strain Classification

Because of the difficulties associated with growing avian ELVs in cell culture and other laboratory host systems, little information is available concerning their antigenic relationships. Using cross immunofluorescence, three ELVs isolated from chickens, designated EF84/700 (29), FP3 (40), and 612 (26), were found to be antigenically distinct from each other and also from avian encephalomyelitis virus, avian nephritis virus, duck hepatitis virus type 1, and duck hepatitis virus type 3 (26, 30). Several ELVs isolated in Japan from chicks with baby chick nephropathy (38) and from broilers with a stunting syndrome (42) had biologic and physical properties similar to the G-4260 strain of avian nephritis virus but were antigenically distinct from avian nephritis virus (37, 38). Nucleotide sequence analyses of the genomes of these viruses are needed to determine whether these viruses are enteroviruses or a third serotype of avian nephritis virus (37, 38).

Two strains of turkey ELVs isolated in France were shown to be antigenically unrelated to avian encephalomyelitis and duck hepatitis viruses using cross-neutralization tests (1).

Laboratory Host Systems

Enterovirus-like viruses can be propagated in the laboratory by oral inoculation of neonatal birds of the same species from which they originally were recognized or

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isolated. Depending on the virus, inoculated birds may develop enteric disease and depressed growth rates. Intestinal contents examined by negative contrast TEM 1–3 days postinfection (DPI) normally contain the inoculated virus. Additionally, immune TEM can be used to assist identification of ELVs in intestinal contents of inoculated birds (34, 35, 41). However, caution must be exercised in propagating ELVs in this manner because even specific pathogen free (SPF) birds may be infected with ELVs.

Most chicken ELVs will grow in the yolk sac of 6-day-old embryonating chicken eggs, with approximately 50% of embryos dying within 3–7 DPI. Dwarfing of embryos also may be observed (38). Some of these viruses also can be propagated in the chorioallantoic membrane of embryonating eggs. Immunofluorescent staining of impression smears of yolk sac membranes or cryostat sections of chorioallantoic membrane can be used to confirm virus growth. In addition, some ELVs, for example FP3 and 612, show limited growth in primary cultures of chicken embryo liver or chicken kidney cells. Growth of virus in cell cultures is best detected by immunofluorescent staining (Figure 12.14) because many of these viruses cause little, if any, cytopathology (4, 26, 30).

Samples of feces or intestinal contents that contain chicken or turkey ELVs also may contain reovirus. Reoviruses normally outgrow ELVs in embryos and cell cultures; thus, their presence interferes with attempts to isolate ELVs.

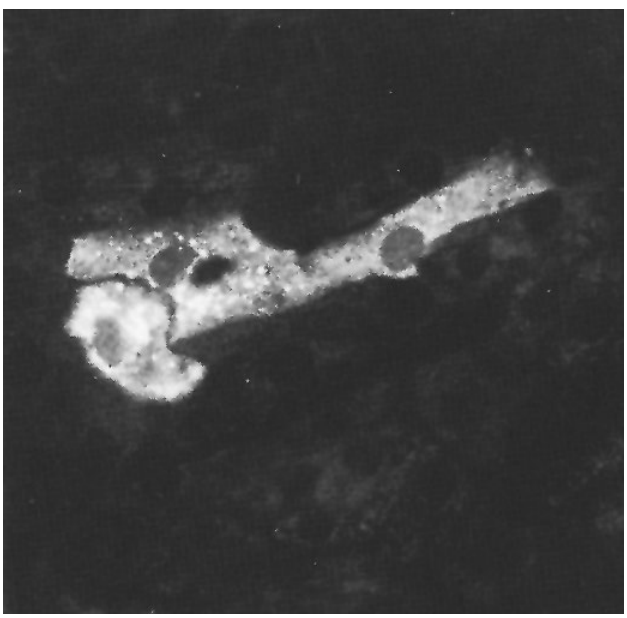


Figure 12.14 Immunofluorescent staining of chicken embryo liver cell culture infected with enterovirus-like virus (ELV) (612 isolate). ×450.

None of four turkey ELVs and two pheasant ELVs detected by negative contrast TEM in the feces and intestinal contents of birds with enteric disease in the United Kingdom produced a cytopathic effect in primary chicken embryo liver cell cultures. However, one turkey virus grew to low titers in embryonating chicken eggs inoculated via the yolk sac (12). Similarly, two isolates of ELVs were made in France from turkey intestinal contents following yolk sac inoculation of chicken embryos (1).

An ELV from guinea fowl with transmissible enteritis was propagated successfully following inoculation of 7-day-old guinea fowl embryos via the yolk sac; however, embryo mortality and lesions were inconsistent. This virus also grew in primary cultures of guinea fowl embryo brain cells; no cytopathic effect was evident, but the presence of virus was demonstrated by inoculation of guinea fowl embryos and 1-day-old guinea fowl (32).

Pathogenicity

The pathogenic role of avian ELVs requires further clarification. Although field and experimental evidence suggests that they may cause enteric disease in young turkeys, chickens, and guinea fowl, and nephropathy in baby chicks, additional studies are needed to define their importance.

Pathobiology and Epizootiology

Incidence and Distribution

Examination of feces using negative contrast TEM has led to the discovery of ELVs in a number of avian species. The presence of ELVs in intestinal contents of young turkeys and chickens was described in the United Kingdom in 1979 (27). Subsequently, ELVs were identified in the feces of turkey poults in the United States (34, 35, 36), Italy (32), and France (1); in chickens in Belgium (5), the United States (11), Malaysia (3), South Africa (26), Italy (32), Holland (39), and Germany (39); in guinea fowl with transmissible enteritis in Italy (24, 32) and France (2); and in partridges (13) and pheasants (12) in the United Kingdom. In addition, ELVs have been found in feces and enterocytes of cockatoos and galahs with enteric disease in Australia (31, 44) and in the gut contents of ostriches showing signs of enteritis in South Africa (8). Based on these findings, it is likely that avian ELVs have a worldwide distribution.

Natural and Experimental Hosts

Infections with ELVs have been described in turkeys, chickens, guinea fowl, partridges, pheasants, ostriches,

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and psittacine species. The majority of naturally occurring infections in domestic poultry have been identified in young birds during the first few weeks of life. However, a chicken ELV was isolated from the meconium of a dead-in-shell chicken embryo (40), indicating that infection with these viruses may occur in adulthood.

Transmission, Carriers, and Vectors

The principal site of replication of ELVs is the small intestinal epithelium (Figure 12.15); some chicken ELVs also replicate in the kidney (38). Thus, infection most likely is spread horizontally through ingestion of infected feces, but other routes of spread cannot be ruled out. Isolation of a chicken ELV from meconium of a dead-in-shell chicken embryo suggests that this virus is vertically transmitted (40); it is likely that other ELVs also are transmitted in this manner. Additionally, evidence indicates darkling beetle larvae may act as mechanical vectors for turkey ELVs (7).

Clinical Signs

The main clinical signs associated with naturally occurring ELV infections in domestic poultry are diarrhea, decreased feed efficiency, and uneven growth. Increased mortality also may occur. Disease is most frequently seen in birds in the first few weeks of life. Enteric disease has been reproduced experimentally in neonatal birds inoculated orally with ELVs.

In 3- or 4-day-old SPF turkeys experimentally infected with a US turkey ELV, depression, watery droppings, and pasted vents were observed. Signs were first evident 3–4 DPI. Similar signs occurred in poults inoculated at 2, 3, and 4 weeks of age. Decreases in body weight gain also were observed. Enterovirus-like viruses were detected in greatest numbers in the intestinal contents of inoculated turkeys at 3 and 4 DPI but were present in some birds up to 14 DPI (17, 19, 41).

Abnormal feces and transient stunting of growth of variable severity were observed in broiler chickens dosed orally with ELVs (5, 26, 30). Specific pathogen free chicks inoculated orally with Japanese ELVs showed diarrhea and variable mortality (up to 53.3%), dying between 2 and 6 DPI (38).

An ELV isolated from guinea fowl with transmissible enteritis in Italy suppressed weight gains of commercial guinea fowl when they were inoculated orally at one day of age (32).

A naturally occurring syndrome associated with ELV infection in young galahs and sulfur-crested cockatoos was characterized by intractable profuse diarrhea, wasting, and death (31, 44).

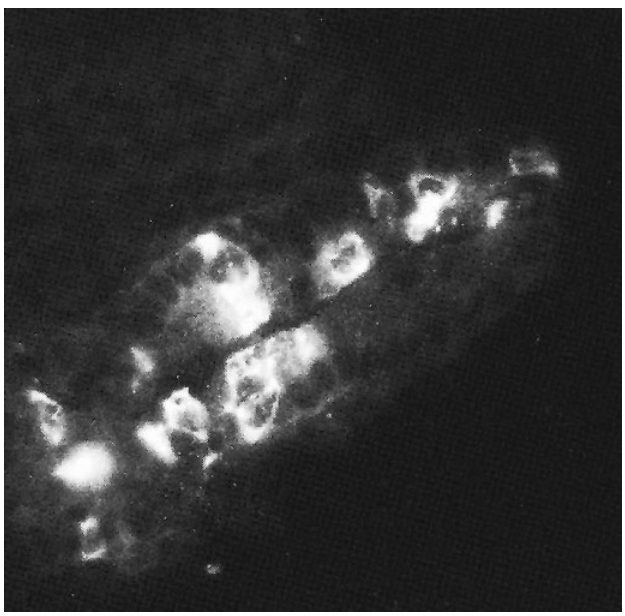


Figure 12.15 Specific immunofluorescence in epithelium of jejunal villus of chicken infected with enterovirus-like virus (ELV) (612 isolate). ×450.

Pathology

Gross lesions in turkeys experimentally infected with a US turkey ELV consisted of thin-walled, dilated ceca filled with yellow, foamy fluid and extreme paleness of the serosa of the gastrointestinal tract; catarrhal secretions were detected in the small intestines. Morphometric studies indicated varying degrees of shortening of the villi and elongation of crypts along the length of the small intestine (17, 19, 41). In naturally occurring infections in turkeys, ELVs usually occur as a component of mixed infections. Interestingly, poults experimentally infected with a combined turkey ELV/group A rotavirus inoculum were more severely affected in terms of clinical signs, body weight gain, and lesion severity than poults that received either inoculum alone (17).

Chicks experimentally infected with Japanese ELVs and that died 2–6 days after inoculation showed microscopic changes characteristic of baby chick nephropathy (i.e., nephrosis and visceral urate deposition) (38). Chicks experimentally infected with ELVs from broilers in Belgium with a runting syndrome had pale small intestines with watery and sometimes filamentous contents of the small intestine and ceca (5).

In a naturally occurring syndrome associated with ELV infection in young galahs and sulfur crested cockatoos, the intestine was dilated with mucoid fluid and gas, and the walls appeared thickened. Microscopic

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lesions in the intestine consisted of villus atrophy and fusion, elongation of the crypts of Lieberkühn, marked epithelial cell proliferation in the crypts and shortened villi, with inflammation of varying severity (31, 44).

Thin section TEM has revealed the presence of intracytoplasmic crystalline arrays of particles resembling enteroviruses in the enterocytes of infected chickens (6, 28) and cockatoos (31, 44).

Pathogenesis of the Infectious Process

The nature of the microscopic lesions in the small intestine of affected birds suggests that infections with ELVs produce malabsorption and diarrhea due to destruction of small intestinal villus epithelial cells. Measurement of absorption of D-xylose from the intestines of experimentally infected turkey poults confirmed that a transient malabsorption was present in poults inoculated at three days of age, but not in poults inoculated at two weeks of age (17). However, it also has been suggested that the turkey ELV exerts its effects by altering the cellular physiology of the villus epithelial cells, altering the normal intestinal flora, or through a systemic mechanism (as evidenced by a transient lymphopenia) (41).

Replication in other organs, such as the kidneys, also may contribute to the pathogenesis of these viruses.

Immunity

The development of active immunity to ELV infections has not been investigated. Similarly, the extent to which passively acquired maternal antibodies provide protection from these infections is unknown.

Diagnosis

Isolation and Identification of Causative Agent

Diagnosis of ELV infections in avian species most commonly is accomplished by TEM examination of droppings or intestinal samples. ELVs have been identified using both direct and immune TEM procedures. For direct TEM, droppings or intestinal contents are prepared as suspensions (10–20%) in phosphate-buffered saline and centrifuged at 800 × g for 20 minutes to remove large particulate material. The supernatant fluid subsequently is centrifuged at 15,000 × g for 20 minutes in a benchtop centrifuge, and the resultant pellet is resuspended in approximately 500 µL of distilled water and 100 µL of 2% phosphotungstic acid. After mixing, the material either is sprayed onto formvar-filmed copper grids, or a drop of the material is placed on the grid for 1–3 minutes and removed by blotting on bibulous paper. Enterovirus-like viruses also may be detected in droppings or intestinal contents using immune TEM (35); however, this procedure requires availability of specific antisera.

Confirmation that particles observed by TEM are animal viruses is achieved by isolating the viruses in turkey or chicken embryos or in cell cultures as described previously. Antigenic characterization of the isolate depends on the availability of serogroup-specific antisera. Yolk sac membranes or chorioallantoic membranes from inoculated embryos may be prepared as impression smears or cryostat sections and examined by immunofluorescent staining using serogroup-specific antisera. This serologic procedure distinguishes between isolates of known serogroups and aids in the identification of new serogroups.

An antigen-capture ELISA was described for detection of turkey ELV in turkey intestinal contents (16). The procedure was shown to be a rapid, highly sensitive, and specific method for diagnosis of the virus.

Serology

Antibodies to ELVs have been detected by serum neutralization and indirect immunofluorescence tests (6, 30, 38); however, because virus isolates and reference antisera are not widely available, routine serologic diagnosis is not recommended. Serology is, however, useful to determine the status of SPF birds with respect to ELV infections.

Differential Diagnosis

Enteric disease associated with ELVs needs to be distinguished from similar conditions caused by other enteric viruses, such as rotavirus, astrovirus, parvovirus, and coronavirus; clinical signs and lesions are not pathognomonic. However, mixed infections of ELVs and other enteropathogens occur commonly, and it may be difficult to identify the relative importance of each constituent of mixed infections.

Intervention Strategies

The role of ELVs as avian pathogens has not yet been fully defined. Consequently, no specific therapeutic or prophylactic measures are available. Given the importance of some of the conditions with which avian ELVs have been associated, it would be prudent to develop better diagnostic methods for these viruses to investigate their epizootiology and pathogenicity more fully.

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Enteric Parvovirus Infections of Chickens and Turkeys

J. Michael Day

Summary

Agent, Infection, and Disease. Infection with the turkey and chicken parvoviruses may be associated with runting-stunting syndrome or poult enteritis complex. Signs include decreased weight gain, poor feathering, diarrhea, and food malabsorption.

Diagnosis. Conventional or real-time polymerase chain reaction (PCR) assays targeting specific chicken or turkey parvovirus genes have implicated parvovirus in outbreaks of enteritis, and are a useful tool for experimental diagnosis. The parvoviruses can be directly identified in feces or intestinal contents by electron microscopy and visualized via immunohistochemistry.

Intervention. Management interventions such as the addition of fresh litter or house cleanout, along with increased general biosecurity measures can be used to prevent and control viral enteritis.

Introduction

Chicken and turkey parvoviruses are members of the Parvovirus family, subfamily Parvovirinae (8). Chicken and turkey parvoviruses were first identified by transmission electron microscopy (TEM) (21, 42) during the early 1980s. Parvoviruses have been detected in cases of enteric diseases of poultry (2, 31, 32, 39, 48), in association with cerebellar hypoplasia in day-old broilers (27), and in cases of beak atrophy and dwarfism in ducklings (6, 43). The complete genome sequences of selected avian parvoviruses have been determined (5, 8, 14, 26, 44), and diagnostic assays have been developed and used to determine the prevalence of parvoviruses in the poultry industry domestically and abroad (11, 22, 28–30, 37, 47). Parvoviruses are frequently detected in chicken and turkey flocks affected by enteric disease and non-specific enteritis (2, 10, 11, 13, 30–32, 39, 47). Pathogenicity studies indicate that chicken- and turkey-origin parvoviruses induce typical enteric disease signs in susceptible young birds (9, 17, 45). Although the exact economic significance of parvovirus infection has not been determined, data suggest that they play a significant role in the etiology of the recognized enteric disease syndromes of poultry, including runting-stunting syndrome (RSS) of broilers, and poult enteritis complex (PEC) and poult enteritis syndrome (PES) of turkey poults (9, 10, 13, 35). Chicken and turkey parvoviruses have no known public health significance.

Etiology

Classification

Classification of parvoviruses was initially based on biological characteristics and structural properties, including whether an isolate could replicate autonomously, and whether the virus contained multiple transcriptional promoters (40). Sequence-based phylogenetic analysis has become the dominant method to classify parvoviruses, with genera identified as a group of species that represents a single branch of a phylogenetic tree (22, 35, 46).

The use of a particle-associated nucleic acid sequencing technique led to the identification and sequencing of the chicken parvovirus (ChPV) genome and the full-length coding region of turkey parvovirus (TuPV)(8). While similar to each other, ChPV and TuPV differ significantly from other members of the Parvovirus subfamily within the family Parvoviridae and group together phylogenetically (Figure 12.16). The high amino acid identity observed between the ChPV and TuPV suggests that they recently diverged from a common ancestor. The ChPV *Galliform aveparvovirus 1* is recognized as the prototypical member of the genus *Aveparvovirus* within the *Parvovirinae* subfamily (8, 25). Both ChPV and TuPV are genetically distinct from the duck and goose parvoviruses, which are in a different genus (25, 41).

Morphology

Parvoviruses are small, isometric (icosahedral), nonenveloped DNA viruses approximately 25 nm in diameter that contain linear, single-stranded genomes (40, 41) (Figure 12.17). The virions contain a nonsegmented genomic ssDNA molecule between 4 and approximately 6.3 kb in length, which terminates in short palindromic sequences that can create duplex hairpin telomeres at both ends of the genome. The capsid is comprised of 60 copies of the 60–70 kDa structural virus protein VP2, which lacks the N-terminus of the structural protein VP1; both VP1 and VP2 are encoded by the same capsid-specific gene cassette (1, 41).

ChPV was first detected via electron microscopy in samples taken from the gut of 10-day-old broilers affected by stunting syndrome (21). Typical ChPV particles measured 19–24 nm in diameter (Figure 12.17), and their buoyant density in CsCl ranged between 1.42 and 1.44 g/mL. TuPV was first visualized in intestinal intranuclear inclusions of turkeys suffering from enteric disease (42).

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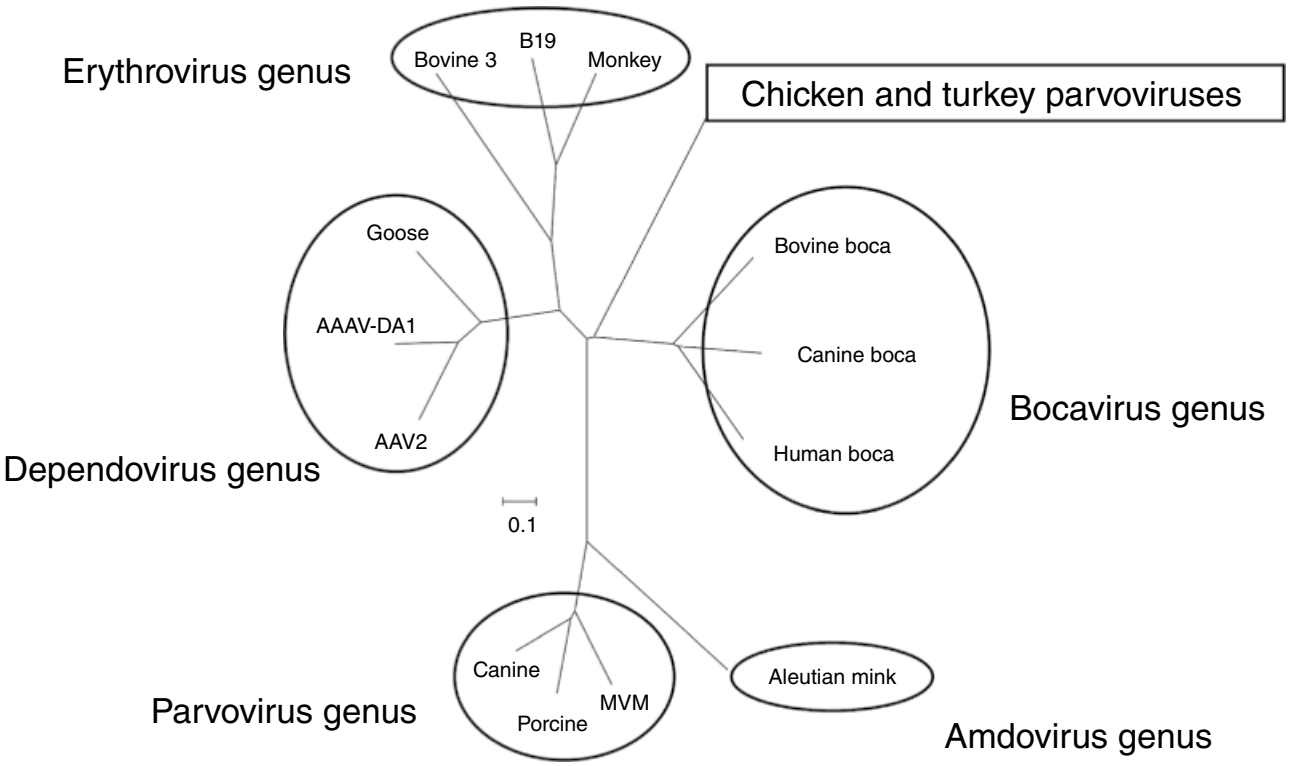


Figure 12.16 A phylogenetic tree prepared using the full coding sequence of the indicated parvoviruses. Representative genera within the *Parvovirinae* are indicated. The evolutionary relationships were inferred using the Neighbor-Joining method.

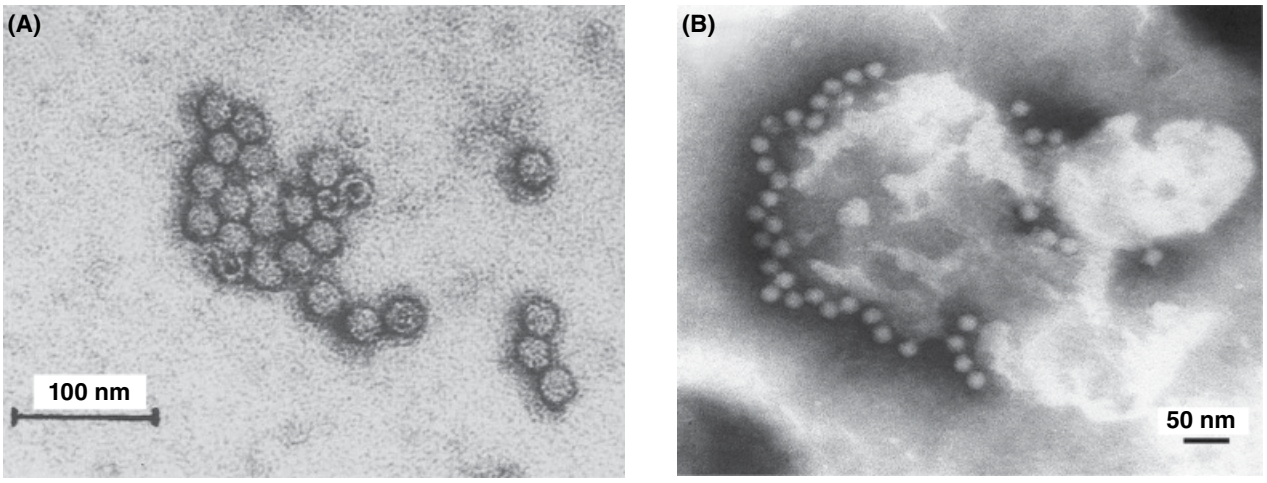


Figure 12.17 Electron micrograph of (A) purified chicken parvovirus (Janos Kisary) and (B) parvovirus from wild turkey fecal sample (Dallas Ingram).

The individual TuPV particles had hexagonal profiles and were approximately 15–20 nm in diameter.

Chemical Composition

Studies on the genome structure of ChPV revealed that the virions contained a single genome which served as a template *in vitro* for second-strand synthesis in the presence of DNA polymerase and appropriate precursors (19). The 5,275 nt ChPV genome is flanked on the 5' and 3' ends by 206 nt direct repeat sequences, each of which contains 39 nt inverted repeats presumed to form a hairpin structure similar to the structures common in other parvoviruses (1, 8, 12, 38). The genomes of ChPV and TuPV are similar to those of other parvoviruses, with two major predicted open reading frames (ORFs)

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encoding non-structural (left ORF, 2095 nt) and structural (right ORF, 2028 nt) proteins. The three structural proteins encoded by the parvovirus genome (VP1, VP2, VP3) together comprise the parvovirus virion and are responsible for the induction of neutralizing antibodies during an infection (34).

Virus Replication

Chicken parvovirus and TuPV, like other autonomous parvoviruses, are able to replicate without helper viruses (20). Transmission electron microscope (TEM) studies and immunohistochemistry (IHC) showed that the primary target cells for replication *in vivo* are located in the small intestine (18, 21, 32, 42, 45). Negative contrast TEM revealed numerous parvovirus-like particles in the intestinal suspension taken from affected birds. In subsequent studies, specific nuclear fluorescence was seen in the epithelial cells in the small intestine of chickens infected experimentally with ChPV ABU strain at one day of age (18, 21). A similar finding was reported of the presence of turkey parvovirus-like particles in the epithelial cells of the small intestine (42). Due to the inability to propagate ChPV and TuPV in cell culture or avian embryos, our knowledge about replication mechanisms in infected cells is limited.

Susceptibility to Chemical and Physical Agents

Chicken parvovirus has been tested for stability in an effluent decontamination system (7). Although the nonenveloped parvoviruses are resistant to inactivation, the study demonstrated that a standard treatment cycle of 82.2 °C for 6 hours totally inactivated 6 $\log_{10}$/mL chicken infectious dose 50 of the chicken parvovirus ChPV-P1 strain. Parvoviruses can be inactivated by formalin treatment, beta-propiolactone, hydroxylamine, sodium hypochlorite, ultraviolet light, and with certain photocatalysts (1, 15, 41).

Strain Classification

There is little information available about the antigenic relationships of ChPV and TuPV. Enzyme-linked immunosorbent assay (ELISA) using chicken parvovirus VP2 as antigen could successfully detect antibodies raised against various chicken and turkey isolates, indicating that these parvoviruses are related antigenically (37, 45).

Although it is less reliable when applied at the species level, phylogenetic analysis is becoming the most frequently used technique for strain classification of parvoviruses (40). Comparative sequence analysis of full genome and partial genome sequences of ChPV and TuPV strains in the United States, Hungary, and Croatia indicated that they are closely related, yet they represent different species within the *Aveparvovirus* genus (2, 8, 28, 31, 32, 47). Currently, strain classification is based on the phylogenetic analysis of a 561 bp PCR amplicon from the highly conserved NS1 nonstructural viral gene. The phylogenetic tree constructed based on the nucleotide sequences of this NS1 gene segment revealed a dominant clustering of the virus strains of different species origin; however, occasionally parvoviruses from chickens are grouped with turkey strains and vice versa (28, 47).

Laboratory Host Systems

To date, ChPV and TuPV have not been isolated or propagated in embryonating chicken eggs or in cell culture. Although chicken parvovirus infection has been demonstrated in primary chicken fibroblast cell culture by indirect immunofluorescence (IF) staining of the nucleus with antiserum to the ChPV ABU strain, subsequent efforts to establish productive infection in cell cultures or in embryos were unsuccessful (20).

Pathogenicity

The pathogenic role of chicken and turkey parvoviruses in enteric disease syndromes of poultry requires further investigation. Although field and experimental data strongly suggest that they play a significant role in the etiology of enteric diseases, additional studies will be necessary to determine their exact pathogenic properties.

Pathobiology and Epizootiology

Incidence and Distribution

Recent surveys indicated that ChPV and TuPV are widely prevalent in poultry farms in the United States, Brazil, and several European countries, including Hungary, Poland, and Croatia (2, 11, 28, 30–32, 35, 39, 47). Chicken parvovirus has also been described in chicken flocks in Korea (22, 23). Like other parvovirus infections, ChPV and TuPV were present in birds as early as four days of age.

Natural and Experimental Hosts

Chickens and turkeys are the only natural hosts in which ChPV and TuPV infections have been detected. Fast-growing broilers appear the most susceptible to virus infection and clinical disease; however, productive virus replication has been documented in white leghorn chickens (17). Clinical disease could only be induced in broilers but not in white leghorns following experimental infection with the ChPV ABU strain. The susceptibility of birds to ChPV- and TuPV-induced enteric diseases is strongly

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age related (21, 32, 45). The most frequent infections occur within the first week of age and clinical signs appear between 7 and 28 days of life. Older birds do not show clinical signs but react to viral infection immunologically by producing virus-specific serum antibodies (45).

Transmission, Carriers, Vectors

Experimentally infected birds shed ChPV in their feces as early as four days of age and this results in fast and efficient horizontal bird to bird transmission of the infection (45). Because parvoviruses are stable in the environment, infectious viruses can be easily detected in the litter, providing an additional source of infection to newly placed chicks. With the advent of high-throughput sequencing technology, parvoviruses are commonly described in wild birds, including in enteric samples. A novel avian parvovirus initially described in a viral community analysis of wild pigeon droppings was most closely related to previously described turkey- and chicken-origin parvoviruses (3, 4, 16, 33).

Clinical Signs

The main clinical signs associated with naturally occurring ChPV and TuPV infections are similar to the disease signs observed during poultry enteric disease syndromes such as RSS and PEC (9). The commonly observed signs are: (1) impaired growth and poor feathering ("helicoptering") (17, 21, 32); (2) intestinal malabsorption of nutrients such as carotenoids which results in the production of mucoid yellowish droppings and paleness of the birds, particularly in the shanks in affected broilers (17, 42); (3) watery diarrhea and mustard-yellow feces that can be observed as early as 4–7 days of age (45); and (4) osteoporosis that leads to bone deformation of the tibiae at the age of 2, 3, and 4 weeks (42). Case histories of parvovirus-associated enteropathy in turkeys included listlessness, depression, and stunting, and affected birds were reported to suffer from splayed legs. The economic significance of the enteric syndromes is primarily associated with poor production, failure of affected birds to grow, increased cost of therapy, poor feed conversion efficiency, and in severe forms immune dysfunction and increased mortality (9, 13, 32).

Following experimental infection of young broiler chickens with SPF chick-passaged ChPV, enteric disease signs were observed, characterized by serious growth retardation, poor feathering, and bone disorders (17, 45). Importantly, by the fourth week of life the body weight gain of broiler chicks infected with the ChPV was nearly 40% lower than that of the controls. Similar results were obtained with commercial and SPF broilers and turkey poults which were experimentally infected with ChPV ABU-P1 strain (a derivative of the ABU strain) and TuPV1078, respectively (45). Following infection, 2-day-old SPF broiler chickens and turkey poults showed characteristic signs of enteric disease (watery diarrhea and growth retardation). Viral growth in the gut and viral shedding was detected for 4–7 days PI (DPI), which was followed by viremia and generalization of infection.

Poults showing parvovirus-associated enteropathy were known to have originated from eggs characterized by poor hatchability (42). Experimental infection of chicken embryos with the ABU ChPV resulted in 15% death rate, the surviving embryos began to break through the eggshell 24–48 hours later than the uninfected controls, and on average, only 30–40% of the embryos hatched (17). The chicks were poorly developed and 80–90% of them died within one week post hatching.

Pathology

The intestines are the major target organs in naturally occurring parvovirus-associated RSS and PEC cases (17, 29, 45). In a recent study, naturally occurring parvovirus infections were described in Hungarian broiler flocks experiencing RSS and in young turkeys with PEC (31, 32). Examined birds from all of the flocks with clinical signs of enteric disease presented higher than normal daily mortality, stunted growth, and diarrhea. At necropsy, segments of the small intestine contained large amounts of gas and mucus. Histological examination revealed moderate to severe distension of the intestinal crypts and acute catarrhal enteritis in the jejunum and duodenum (Figure 12.18A). Nodular lymphohistiocytic pancreatitis also was observed (Figure 12.18C). Transmission electron microscopy studies revealed the presence of parvovirus-like particles in the intestinal contents and positive nuclear staining was detected by indirect IHC in the epithelial cells and inflammatory cells from the lamina propria of the duodenum and jejunum in both chicken and turkey samples (Figure 12.18B). While the parvovirus specific staining was restricted to the small intestine in chickens, positive reactions were observed in the follicles of the cloacal bursa, liver, and exocrine pancreas in turkeys (Figure 12.18D).

Lesions observed at necropsy of turkeys affected with parvovirus-induced enteropathy also were restricted to the gastrointestinal tract (42). Gizzards contained large quantities of litter and grit but little feed. The small intestines, and occasionally the ceca, were distended by mucus, gas, and fluid feces. Electron micrographs of ileal mucosa revealed that intranuclear inclusions were completely filled with loosely packed hexagonal virus particles of 15–20 nm in diameter.

In experimental infections, parvovirus replication was detected by IHC in the small intestine of infected broilers and turkey poults (45). Virus replication was most prevalent in the epithelial cells of the duodenum; however, PCR assays detected ChPV nucleic acid in different

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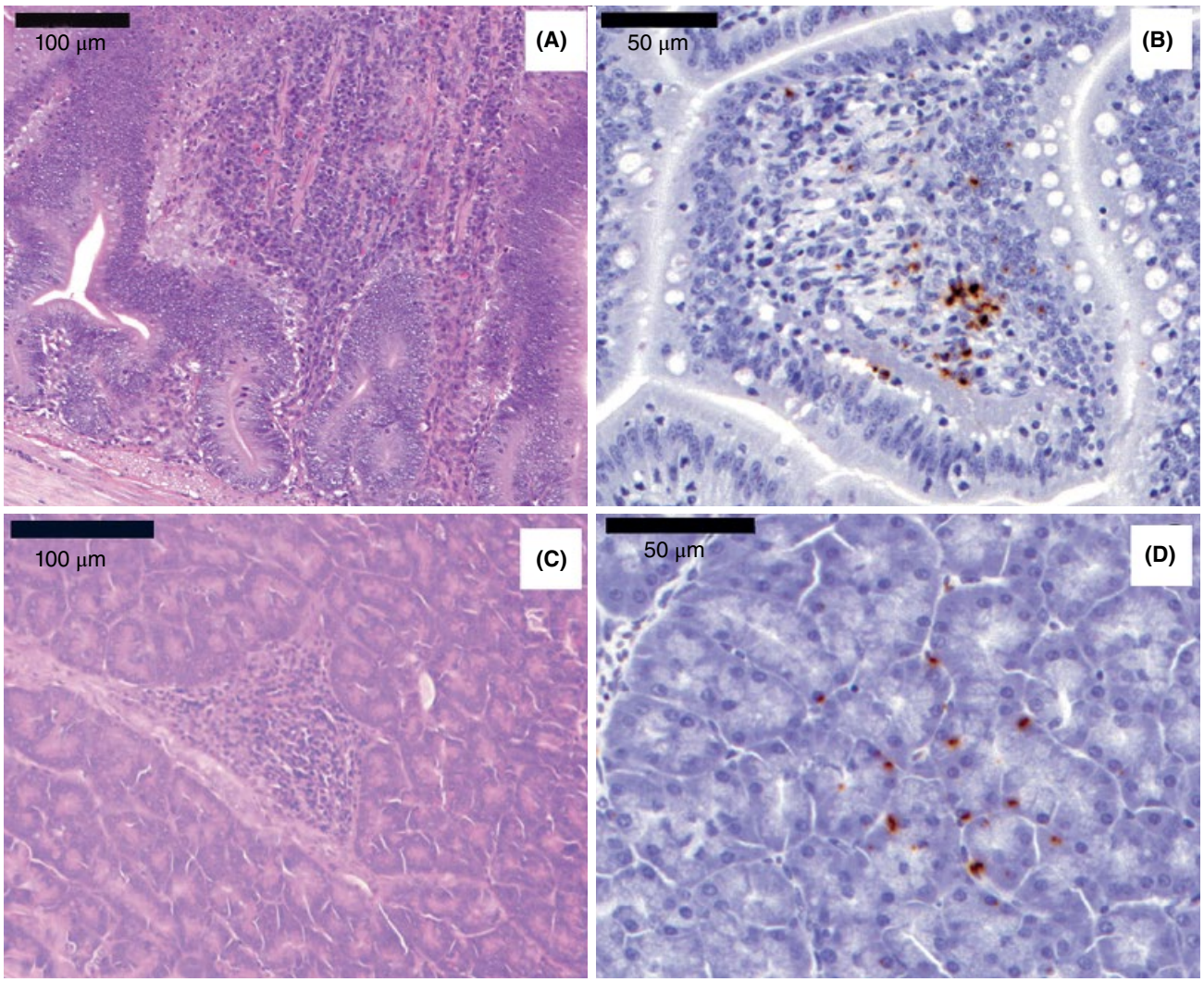


Figure 12.18 Distension of crypts with increased number of mononuclear leukocytes in the (A) jejunum and (B) nodular pancreatitis, with (C) lymphocytes and histiocytes in parvovirus-infected chickens. Indirect IHC staining shows parvovirus-positive nuclear staining in the inflammatory cells from the lamina propria of (B) jejunum and staining in the (D) exocrine pancreas. (Elena Alina Palade)

sections of the small intestine between 4 and 35 DPI. Generalization of infection occurred between 7 and 14 DPI and presence of parvovirus in liver, spleen, and thymus could be detected by PCR through the end of the study at 5 weeks of life.

Pathogenesis of the Infectious Process

As is the case with many poultry enteric viruses, ChPV and TuPV can be frequently detected via molecular assays or EM from both healthy and diseased individuals, especially at an early age (10, 11, 31, 32, 47). Maternally acquired virus-specific antibodies play a significant role in the epidemiology of clinical disease, and the level of passive immunity determines the susceptibility of the progeny following virus infection (45).

Immunity

Passive Immunity

Adult breeding chickens and turkeys that have been naturally infected with parvoviruses transfer maternal IgG type antibodies through the egg yolk to their progenies (37, 45). This passively acquired immunity progressively declines, halves at about 14 days of age, and cannot be detected after three weeks of life. The presence of maternally derived antibody has a significant correlation with protection against chicken parvovirus-induced enteric disease.

Active Immunity

Chickens and turkeys inoculated with parvoviruses developed antibody responses as early as 4–7 DPI (37, 45). Using an immunoglobulin class-specific ELISA,

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the primary serum antibody response was IgM type that subsequently converted into IgG type (37). These antibodies could be detected for at least 35 DPI in experimental conditions. Similar serum antibody response was demonstrated in chickens following inoculation with a baculovirus vector that expressed chicken parvovirus VP2 protein (36, 37, 45). The level and duration of antibody response, however, was somewhat lower than those observed following live virus infections.

Diagnosis

Electron microscopy was the main technique to identify parvovirus infections in chicken and turkey flocks during the early 1980s (21, 42). An indirect IF assay was developed in 1985 for direct detection of viral antigens to monitor parvovirus infections in broiler flocks (18). Positive cells were detected in the intestinal tract of ChPV-infected birds and no specific fluorescence was observed when anti-goose parvovirus antibody was used. These data agreed with previous observations, which indicated that goose parvovirus was not neutralized by serum against chicken parvovirus.

Comparative genome sequence analysis has indicated a high level of homology of ChPV and TuPV NS1 nonstructural genes, and a diagnostic PCR test targeting those regions was developed (47, 48). This conventional PCR assay proved to be highly sensitive and specific for detecting ChPV and TuPV in clinical samples from experimentally infected birds and field samples (2, 28, 31, 32). For quick detection of ChPV in field samples a real time PCR also was developed and found to be more sensitive and less laborious than the previously described PCR assay (39).

Serologic assays are experimentally used as diagnostic tools to confirm the presence of parvoviral infections and to evaluate the immune status of parents and their progeny. To detect parvovirus-specific antibodies in chicken serum samples a capture ELISA was developed (37). A previously described approach was used to clone and express viral structural proteins in insect cells from recombinant baculovirus vectors (24). This ELISA demonstrated the presence of maternally derived parvovirus-specific antibodies in chicken serum samples and virus-specific antibodies in sera following experimental infection of birds (45).

Intervention Strategies

Management Procedures

The widespread occurrence of parvoviruses in poultry suggests that it is not practical to keep commercial flocks free from infections. Sound biosecurity measures are necessary to prevent accumulation of virus on the premises and control the spread of parvoviruses directly or indirectly by feces, litter, boots, or equipment. Parvoviruses are exceptionally stable and can survive for a long time in the environment (1, 40). If litter is being reused several times combined with a short downtime between placements, parvovirus infection will build up, and infection of newly placed birds will likely result in much more severe diseases compared to those flocks with which fresh litter is used.

Vaccination

At this time there is no commercial vaccine to prevent parvovirus-induced enteric diseases in poultry. Given the difficulties to propagate the viruses *in vitro* there are obvious challenges for vaccine development. It has been shown that maternal antibodies to chicken parvoviruses can reduce the severity of the disease (45), indicating that vaccination of breeder flocks would likely result in protection of the progeny during the most susceptible early days of their lives.

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13

Viral Infections of Waterfowl

Introduction

Simone T. Stoute

Interests in viral infections of waterfowl are more diverse than for poultry and focus on diseases associated with wild waterfowl, problems associated with raising birds commercially and problems created by migratory waterfowl in the transmission of infectious diseases to commercially reared waterfowl and poultry. The objective of this chapter is to bring together all of the viral diseases affecting waterfowl.

This chapter has detailed subchapters on the following diseases:

- Duck hepatitis, a major disease of young ducklings attributed to at least five different viruses. Three genotypes of duck hepatitis A virus (DHAV), duck atrovirus type 1 (DAstV-I), and duck astrovirus type 2 (DAstV-II).
- Duck virus enteritis (DVE), caused by a herpesvirus poses a major threat to both commercially reared and wild waterfowl.
- Parvovirus infections of geese and Muscovy ducks. Although Muscovy duck parvovirus (MDPV) and goose parvovirus (GPV) are closely related, they are genotypically distinct. MDPV has been recognized in the United States.
- Goose hemorrhagic polyomavirus (GHPV) is the cause of hemorrhagic nephritis enteritis of geese (HNEG).

Other virus infections associated with waterfowl will be covered in this introduction.

RNA viruses associated with waterfowl include Picornaviridae (DHA, duck picornavirus [DPV]), Astroviridae (DastV-1 and DAstV-2), Paramyxoviridae (avian metapneumovirus, avian paramyxoviruses), Orthomyxoviridae (avian influenza), Flaviviridae (West Nile virus), and Reoviridae (Duck reovirus, Muscovy duck reovirus, and goose reovirus); DNA viruses include Herpesviridae (DVE, goose herpesvirus), Adenoviridae (duck adenovirus), Circoviridae (circovirus-like infection of ducks and geese), Hepadnaviridae (duck hepatitis B virus), Parvoviridae (MDPV and GPV), and Polyomaviridae (GHPV).

Oncogenic viruses of waterfowl are not included in this chapter.

Avian Paramyxoviruses

Avian paramyxoviruses (APMV) serotypes 1, 4, 6, 8, and 9 have been associated with infections in waterfowl. Infections by these viruses have been demonstrated through virus isolation or serology in various waterfowl species. However, most of these APMV serotypes are generally considered to be apathogenic in waterfowl species.

While waterfowl are considered to be natural reservoirs of APMV-1, there are several reports of APMV-1 strains associated with pathogenicity in waterfowl. Wen et al. reported a virulent APMV-1 genotype III causing respiratory and neurological disease in duck layers in China (54). Zou et al. (56), and Jinding et al. (22) reported an APMV type 1 causing high mortality in geese in China. Shi et al. (42) reported an APMV-I isolated from a Muscovy duck that was pathogenic for Muscovy and Pekin ducks.

APMVs are dealt with in more detail in Chapter 3.

Avian Metapneumovirus

Avian metapneumoviruses (aMPV), members of the family Paramyxoviridae, were first isolated in 1980s. They have been of most concern to the turkey industry and were not detected in the United States until 1996. US turkey isolates of aMPV are designated aMPV type C, which is different from the types A and B found in Europe. Isolates from ducks in France have been assigned to subgroup C but European and American subgroup C viruses belong to different genetic lineages (51). Ducks often play a role as nonclinical carriers of

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aMPV and can serve as a potential source of infection for domestic turkeys (43). Turpin et al. (53) surveyed wild birds for aMPV type C in Georgia, South Carolina, Arkansas, and Ohio. Avian metapneumovirus type C was isolated from oral swabs of American coots and Canada geese, demonstrating that wild waterfowl can serve as aMPV reservoirs.

In 1999, Toquin et al. (51) reported the isolation of a pneumovirus from 42-week-old Muscovy ducks exhibiting coughing and decreased egg production. Mortality was about 2%. Lesions of general congestion, splenomegaly and tracheitis were identified. The isolate was confirmed as a pneumovirus by RT-PCR on the N gene. Using monospecific aMPV antisera, the Muscovy duck virus isolates reacted most strongly with the aMPV Colorado (type C) antiserum when compared with antisera against aMPV A, B, and non-A and non-B types.

Avian metapneumovirus RNA has been isolated from the nasal turbinates or swabs of mallard ducks, wild geese, and sentinels captured in the north central United States. (44). The aMPV M gene from wild birds had more than 96% predicted amino acid identity with the MN/2 A turkey aMPV isolate. McComb et al. reported the detection of aMPV viral RNA in choanal–tracheal swabs collected from 8-week-old sentinel mallard ducks allowed to mingle with wild waterfowl in central Minnesota (32). The authors also detected aMPV viral RNA in choanal swabs from Canada geese, blue winged teal, and snow geese (32).

In Minnesota, United States, aMPV-negative mallard ducks placed next to a turkey farm experiencing a severe outbreak of aPMV infection did not develop clinical disease but infectious aMPV was recovered from choanal swabs after two weeks and anti-aMPV antibodies were detected after four weeks. (43).

Bennett et al. demonstrated aMPV in wild Canada geese (*Branta canadensis*) and blue winged teal (*Anas discors*) by reverse transcriptase-polymerase chain reaction (RT-PCR) (5). Using Canada geese isolates, Bennett et al. (5) investigated the genomic structure of the virus. All but one of the eight genes were similar to those of a turkey aMPV isolate in terms of size, sequence identity, and genome organization. This virus replicated in the upper respiratory tract of experimentally challenged domestic turkeys without eliciting clinical signs and could be horizontally transmitted to naïve turkeys and elicit aMPV specific antibody production. The authors suggested that this virus may be a safe and effective vaccine for commercial turkeys (5).

In 2014 Sun et al. reported a subgroup C aMPV circulating in Muscovy duck breeders in South China (49). Upper respiratory disease, decreased egg production, thin-shelled eggs and mortality exceeding 10% was noted in some of the affected Muscovy flocks.

Avian Influenza Virus

Until relatively recently waterfowl typically do not experience significant disease problems due to avian influenza viruses (AIVs), but infections in these birds are widespread. Avian influenza viruses can be recovered from migratory waterfowl, particularly ducks; at least 30 of 149 species of ducks, geese, and swans have yielded virus but natural infections are usually considered asymptomatic (50).

The picture changed dramatically in late 2002 when HPAI (H5N1) occurred in geese, ducks, and swans, among other avian species, at two waterfowl parks in Hong Kong (13). The range of pathological changes present in the various waterfowl examined in this outbreak resembled those reported generally for HPAI viruses in chickens (50). High mortality in ducks and geese was also reported in H5N1 outbreaks in India in 2011, Indonesia in 2012 and Bangladesh in 2013 (12, 16, 43).

Since the outbreak in 2002, H5N1 HPAI has spread through Asia, Europe, and parts of Africa. The role of migratory waterfowl in the spread of HPAI H5N1 has been investigated. Chen et al. (9) described an outbreak in bar-headed geese (*Anser indicus*) at Qinghai Lake in Western China in May 2005; more than 1,500 birds died. Hulse-Post et al. (20) reported that the H5N1 HPAI can revert to non-pathogenic forms in ducks. Thus, wild waterfowl may appear uninfected by the H5N1, but still may continue to circulate the virus. Wild waterfowl migration was also implicated in the rapid global expansion of HPAI intercontinental group A (icA) H5N8 clade 2.3.4.4 in poultry in Asia in 2014 with subsequent spread through Europe, Asia, and North America by 2015 (27).

There are multiple reports regarding the role of waterfowl in avian influenza virus transmission and additional details of this virus are covered in Chapter 6.

West Nile Virus

West Nile virus (WNV) is a member of the Japanese encephalitis virus antigenic complex of arthropod-borne flaviviruses (Flaviviridae) that are transmitted through mosquitoes to a variety of mammals and birds (25). The notoriety of this virus has increased since 1999 when an epizootic causing death in wild American crows (*Corvus brachyrhynchus*) began in New York (36). This was the first time that WNV had been detected in North America. At the same time, WNV-positive cases occurred in a number of wild bird species, humans, and horses, and at zoological collections of mammals and birds in New York (25, 26, 48).

Outbreaks of WNV involving ducks and geese have been reported from Israel and Romania (26, 35, 40). In the New York outbreak, Steele et al. (48) examined birds from two wildlife facilities, which had either died or were euthanized after suspected of being infected with WNV. Lesions in emaciated Anseriformes included cerebral

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hemorrhages, massive necrotizing splenitis, splenomegaly, nephritis, and congestion in the kidneys. One mallard and two bronze-winged ducks were involved with this study. In the mallard, abundant antigen was demonstrated by immunohistochemistry in the brain, heart, liver, kidney, and pancreas, and to a lesser extent in the adrenal gland and intestine. Virus in excess of 10^2 pfu/0.2 ml was isolated from brain, heart, spleen, liver, and kidney; virus also was demonstrated by RT-PCR in these same tissues. Only immunohistochemistry was performed on the tissues from the bronze-winged ducks, but results obtained were similar to those in the mallard.

In the 1999 outbreak in New York, an Abyssinian blue-winged goose (*Cyanochen cyanopterus*), a Rosybill duck (*Netta peposcaca*), and a domestic goose (*Anser anser*) showed asymptomatic seroconversion to WNV. In the domestic goose and trumpeter swan (*Cygnus cygnus buccinator*) morbidity and recovery were recorded. No deaths were recorded in any of the birds. The New York isolates of WNV yielded an E gene nucleotide sequence that was closely homologous to that from the WNV isolated from a goose in Israel in 1998 and also from a 1996 Romanian isolate.

Bird to bird transmission of West Nile virus in geese has been proposed and some studies have reported direct (non-vector) transmission of WNV in geese (1, 3). Banet-Noach et al. concluded that horizontal transmission can occur in commercial flocks and may be aggravated by cannibalism and feather picking of sick birds (3).

Detection of WNV neutralizing antibodies has been reported in wild migratory ducks in Japan (39) and South Korea (55) and domestic (23) and wild ducks (33) in India.

Duck, Muscovy Duck, and Goose Reoviruses

Muscovy duck reovirus infections have been described. In France, it is considered to be a major virus disease of Muscovy ducks. In 2- to 4-week-old ducks, the disease is acute, morbidity is high, and mortality can reach 10%. Clinical signs include apathy, diarrhea and difficulty moving. Lesions include fibrinous pericarditis, splenic necrosis, and hepatic necrosis and mononuclear hepatitis and exudative synovitis of leg tendons. Palya et al. (37) reported reovirus causing disease in young geese. The disease was characterized by splenitis and hepatitis with miliary necrotic foci during the acute phase, and epicarditis, arthritis, and tenosynovitis during the subacute/chronic phase.

Cross-neutralization tests have demonstrated that Muscovy duck reovirus is antigenically distinct from chicken reovirus S1133. Banyai et al. (4) investigated the genetic variability among goose reoviruses (GRV). The S4 genome segment of 5 GRVs shared substantial structural similarity with Muscovy duck reovirus (DRV). The authors consider GRV and DRV to belong to a species distinct from others established within the subgroup 2 of orthoreoviruses.

Hollmen et al. (17) reported the isolation of a reovirus from common eider ducks (*Somateria mollissima*) in Finland. The virus was isolated from the bursa of Fabricius, inoculated into Muscovy duck embryo fibroblasts. The relationship of this reovirus to DRV and GRV has not been reported.

A reovirus causing duck viral swollen head hemorrhagic disease in Pekin ducks has been reported from China (28), and an indirect immunoperoxidase assay was developed for detection of the virus. Liu et al. (29) report the isolation and characterization of a reovirus causing spleen necrosis in Pekin ducklings. DNA sequencing revealed that the isolate was closely related to Muscovy duck reoviruses.

Goose Herpesvirus (GHV)

In Australia, a herpesvirus has been implicated in a peracute disease of domestic geese that caused 97% mortality over a 24-day period. Clinical signs and gross pathology were similar to those seen with DVE infections. Histologically, button ulcers and large plaques overlying lymphocyte aggregates were present on the small intestinal mucosa. Focal necrosis and hemorrhages were seen in the livers. Numerous intranuclear hepatic inclusion bodies were observed. A herpesvirus was isolated in various primary chicken and duck embryo cell cultures. This virus was not neutralized by DVE antiserum. In experimental transmission studies, the virus caused 100% mortality in adult domestic geese, 50% mortality in 1-day-old commercial ducklings, and 25% mortality in 4- to 6-week-old ducklings (24). However, Pekin ducks are not susceptible to GHV (15).

Using cross-neutralization tests in cell cultures, GHV was compared with five other avian herpesviruses. No significant cross neutralization was reported, confirming that GHV is antigenically distinct from DVE viruses (15).

Adenoviruses

Duck adenovirus 1 (DAdV-1) (previously known as group 3 avian adenovirus, egg drop syndrome-1976 virus [EDS], avian adenovirus EDS, and egg drop syndrome virus) is a member of the genus *Atadenovirus, s*pecies Duck adenovirus A. Ducks and geese are assumed to be the natural hosts of this virus, but there is sparse evidence of respiratory disease in waterfowl associated with this virus. Ivanics et al. (21) reported in 2001 an acute respiratory disease in goslings in Hungary attributable to EDS virus.

Aviadenoviruses have also been isolated from mallards and a Muscovy duck (7). In Muscovy ducks, mortality occurred, and the isolated adenovirus was tentatively named Duck adenovirus 2.

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Unclassified Adenoviruses Hollmen et al. (18) reported the isolation of an adenovirus from long-tailed ducks (*Clangula hyemalis)* collected during a die-off in the Beaufort Sea off the north coast of Alaska. The authors reproduced the disease experimentally in long-tailed ducks; no mortality was recorded but clinical signs included watery feces and blood in the feces. Challenged ducks seroconverted. The virus could not be neutralized by reference antisera to group 1, 2, or 3 avian adenoviruses and may represent a new serotype.

Hollmen et al. (19) also reported on an adenovirus associated with impaction of the posterior small intestine with mucosal necrosis and the cause of death in 10 male common eider ducks (*Somateria mollissima*) in the northern Baltic Sea near Finland. The adenovirus isolated from cloacal swans could not be neutralized with reference antisera to group 1, 2, or 3 avian adenoviruses. The virus caused clinical signs of illness and gastrointestinal pathology in an experimentally infected mallard duckling.

Cheng et al. (10) reported attempts to characterize a virus causing enteritis in goslings in China. They identified an adenovirus and named the virus new gosling viral enteritis virus (NGVEV).

For further references on characterization, see previous editions of *Diseases of Poultry*. For more information on adenoviruses see Chapter 9.

Circovirus-Like Infection of Ducks and Geese Soike et al. (47) first reported in 1999 the presence of circovirus-like particles, approximately 15 nm in diameter, in cloacal bursa, splenic, and thymic tissues from a flock of Czech hybrid geese with a history of increased losses and runting. Since then a virus has been isolated from two female mulards showing characteristic signs of circovirus infection (46). This duck circovirus (DuCV) has been shown to be closely related phylogenetically to goose circovirus (GoCV), but is still distinct.

Avian circovirus infections which occur in the first months of life are characterized by developmental and/or feathering disorders. The virus invades the lymphoid tissues and leads to immunosuppression, growth retardation, and an increased probability of secondary infections. In geese the only apparent gross lesions in 2-week-old and 9-week-old birds were a cloudiness of the air sacs (47). Lesions included lymphocytic depletion and histiocytosis of lymphoreticular tissue; and was most apparent in the cloacal bursa. Basophilic globular intracytoplasmic inclusions were found in the bursa follicular and epithelial cells. In naturally infected commercial Muscovy, mule, Pekin ducks, and White Roman geese in Taiwan, 4- to 6-week-old birds had clinical signs of loss of wing and body feathers, necrosis of feather follicles, and stunted growth. The most common lesion was polyserositis (8).

Smyth et al. (45) investigated a circovirus infection in geese by *in situ* hybridization using a GoCV DNA probe that showed that circovirus DNA could be demonstrated in the cloacal bursa, spleen, thymus, bone marrow, liver, kidney, lung, and heart.

Diagnostic tests for GoCV and DuCV are mostly PCR based (8). Liu et al. (30) have since reported the development of an indirect enzyme-linked immunosorbent assay (ELISA) for the detection of DuCV infection in flocks using recombinant capsid protein as antigen.

Glavits et al. (14) investigated an outbreak of West Nile virus infection in a goose flock in Hungary. Histological lesions suggestive of WNV and circovirus were identified and both virus infections were diagnosed by molecular diagnostic tests.

A duck circovirus (DuCV) was detected by PCR in bursal and thymic samples from Pekin ducks from New York. The birds exhibited bursal and thymic atrophy and arthritis caused by *Staphylococcus aureus* (2). This is the first report involving Pekin ducks. Genetic diversity of DuCV has been reported.

Miscellaneous Viral Infections

Tembusu viral infection is an emerging flavivirus infection reported in ducks and geese in Asia. Infection has been associated with anorexia, diarrhea, decreased egg production, ataxia and paralysis. Tembusu virus infection has been associated with lesions of hepatomegaly, meningeal congestion, myocardial hemorrhage, and pulmonary edema (31).

Tsai et al. (52) reported that 77.3% of 611 ducks and 70.9% of 542 geese in Taiwan were positive for antibodies to Japanese encephalitis virus.

Bidin et al. (6) reported the first evidence of avian nephritis virus (ANV) in ducks. The report from Croatia confirmed the diagnosis by RT-PCR; high nucleotide and amino acid identities were shared with other ANV polymerase (ORF 1b) genes.

Avian bornavirus (ABV) associated with non-suppurative inflammation in the central peripheral and autonomic nervous systems has been detected in wild, free-ranging Canada geese and trumpeter swans (*Cygnus buccinator*) in Ontario, Canada. The virus was detected in brain by immunohistochemistry and RT-PCR (11). ABV also has been detected in healthy Canada geese by RT-PCR; the virus was propagated in duck embryo fibroblast cells and detected by RT-PCR after two passages and indirect fluorescent antibody assay after three passages antibody (38).

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Duck Hepatitis

Hsiang-Jung Tsai

Summary

Agent, Infection, and Disease. Duck hepatitis is an acute, highly fatal, and contagious viral disease of young ducklings. The disease is characterized by a short incubation period, sudden onset, opisthotonos, high mortality, and characteristic liver lesions. Three distinct types of duck hepatitis virus (DHV) have been identified. The most internationally distributed and economically important is DHV type I, a picornavirus. Three genotypes, and probably also serotypes, of DHV type I have been identified and designated as duck hepatitis A virus (DHAV) types 1, 2, and 3. While DHV type 2 and DHV type 3 viruses are two distinct astrovirus.

Diagnosis. A presumptive diagnosis can be made on the basis of the characteristic disease pattern in the flock and gross pathological lesions in the liver, but isolation and identification of the causative agent are necessary to confirm the diagnosis. The disease caused by all DHV types in ducklings is indistinguishable, and molecular diagnostic methods are commonly used for rapid identification and differentiation of the etiological DHV types.

Intervention. Resistance against DHV may be conferred to ducklings by active immunization of ducklings with live vaccine, or by injecting ducklings with immune serum or yolk, or by maternal antibodies derived from breeding stock immunized with live or inactive vaccines.

Introduction

Definition and Synonyms

Duck hepatitis (DH) or duck viral hepatitis (DVH) is a highly fatal, rapidly spreading, viral infection of young ducklings characterized primarily by hepatitis. The disease can be caused by at least five different viruses. Traditionally, viruses causing DH were classified into three serotypes: DHV type 1, DHV type 2, and DHV type 3. The most pathogenic and widespread is DHV type 1 (6, 12, 28, 68, 70). According to the Ninth Report of the International Committee on Taxonomy of Viruses, DHV type 1 has been renamed duck hepatitis A virus type 1 (DHAV-1) and is classified in the newly proposed genus *Avihepatovirus* in the Picornaviridae family (141). Two newly described DHAV genotypes (which may also be serotypes), DHAV-2, isolated in Taiwan (114), and DHAV-3, isolated in South Korea (65) and China (38), also belong to the *Avihepatovirus* genus. Both DHV type 2 and DHV type 3 viruses now belong to the genus *Avastrovirus* in the family Astroviridae, and are referred to as duck astrovirus type 1 (DAstV-1) and type 2 (DAstV-2), respectively (11). For other reviews of DHV types 1 and 3, see Calnek (12), and for DHV type 2, see Gough and Stuart (47).

In addition to the five viruses that are etiologically associated with liver disease in ducks, a member of the hepadnavirus group (duck hepatitis B virus; DHBV) can also be found in wild and domestic ducks. Within the Picornaviridae family, three novel duck picornaviruses have also been found in domestic ducks. One belonged to the genus *Sapelovirus* (115), another to the genus *Megrivirus* (74), and the third a newly proposed genus, *Aalivirus* (120). Also, a novel duck astrovirus has been detected in newly hatched ducklings, and the nucleic acid sequence indicates that it is genetically different from DAstV-1 and -2 viruses (78). All of these novel duck picornaviruses, novel duck astroviruses, and DHBVs are not known to be associated with hepatitis in ducklings.

Economic Significance and Public Health Significance

Duck hepatitis is of economic importance to all duck-growing farms because of the high potential mortality if not controlled. All three DHV types are not known to have any public health significance.

History

Duck Hepatitis Virus Type 1

DHV type 1 was first observed in young White Pekin Ducks on Long Island in 1945 (72). Since then, DHV type 1 has been reported in duck-raising areas worldwide (137). The causative pathogen, DHAV-1, was first isolated in chicken embryos in 1950 (71), and was originally classified as an enterovirus (124) until the complete genome was determined in 2006 (68). Presently, it is classified in the new genus *Avihepatovirus* in the family *Picornaviridae* (113). In 2007, two antigenically and genetically distinct viruses were identified: DHAV-2, isolated in Taiwan (113), and DHAV-3, isolated in South Korea (66). For historical details of the disease, please refer to the previous edition of *Diseases of Poultry* (126).

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Duck Hepatitis Virus Type 2 and Type 3

Duck hepatitis virus types 2 and 3 were recognized as separate entities because they induced hepatitis in DHV type 1-immune ducklings (6, 12, 37, 44, 45, 47, 52, 84, 109, 111). Duck hepatitis virus type 2 was first isolated from an outbreak of hepatitis in ducklings in Norfolk, England in 1965 by Asplin (6). The disease disappeared from commercial flocks by 1969, but reappeared from 1983 to 84 on three farms, again in Norfolk, England (44). DHV-2 was originally regarded as a picornavirus, but later was characterized as an astrovirus by morphology in 1984, and later renamed as duck astrovirus 1 (DAstV-1) in 1984 (42, 45, 84). Since the outbreaks in the mid-1980s, there have been no additional outbreaks of the disease in that area (43). Outside of the UK, outbreaks of DVH caused by DAstV-1 have been reported in China in 2008 and 2012 (17, 37). Duck hepatitis type 3 was first reported in 1969 by Toth (111), and last observed in 1975 (53) on Long Island in the United States. Duck hepatitis type 3 was originally thought to be a picornavirus and later was characterized as an astrovirus in 2009 (109).

Etiology

Classification

Duck Hepatitis Virus Type 1

Duck hepatitis A virus (DHAV) belongs to the genus *Avihepatovirus* in the Picornaviridae family. As the only species in its genus, DHAV consists of three distinct genotypes and probably also serotypes. They are designated DHAV-1, DHAV-2 and DHAV-3 (109).

Duck Hepatitis Virus Type 2 and 3

Duck hepatitis virus type 2 belongs to the genus *Avastrovirus* in the Astroviridae family, and has been renamed duck astrovirus 1 (DAstV-1) (42, 85, 109). Duck hepatitis virus type 3 was originally classified as a picornavirus (53), but more recently it was reclassified as also an astrovirus and was named duck astrovirus type 2 (DAstV-2) (109).

Morphology

Duck hepatitis virus type 1 viruses are of typical picornavirus morphology; naked with an icosahedral capsid and estimated to be 20–40 nm in size (94, 95, 108). Duck hepatitis virus type 2 viruses have an astrovirus-like morphology, and a diameter of 28–30 nm, as seen by electron microscopy (EM), has been reported (46). Duck hepatitis virus type 3 viruses have a cubic symmetry, and are about 30 nm in diameter as observed in cytoplasmic crystalline arrays of infected cultured duck kidney (DK) cells by EM (52).

Chemical Composition

Duck Hepatitis Virus Type 1

The complete genome of DHAV-1 isolates from Korea (68), Taiwan (113), and China (28, 75) have been determined and shown to comprise a positive-sense, single-stranded ribonucleic acid (RNA) of about 7,800 nucleotides in length, with a poly (A) tail at the 3′ end. The genomic organization of DHAV-1 is typical of a picornavirus, with a single, long, open reading frame (ORF) flanked by 5′ and 3′ untranslated regions (UTRs). The ORF can be translated into a large polyprotein, about 2,200 amino acids in size, which appears to be cleaved into 12 mature products, in order from the 5′ to 3′ end, forming its structural (VP0, VP3, and VP1) and nonstructural proteins (2A1, 2A2, 2A3, 2B, 2C, 3A, 3B, 3C, and 3D). Three in-tandem 2A genes are unique features of DHAV-1. Among them, 2A1 protein is an aphthovirus-like 2A protein, and 2A3 protein is a human parechovirus-like 2A protein, whereas 2A2 protein is not related to any known picornavirus proteins (113). The 2A2 protein of DHAV-1 was shown to induce apoptosis in primary cell culture (14).

The DHAV genes for VP0, VP1, and VP3 encode capsid proteins, which could be the main viral antigen epitopes that confer specific antigenicity. Among them, the VP1 protein has been shown to probably play a vital role in receptor binding, virulence, immunogenicity, and protection against DHAV (75). Sequence analysis results have shown that mutations were distributed mainly in the gene encoding VP1 (17, 75, 118, 122, 138). Li et al. (72) also showed that VP1 of DHAV-1 is a target of neutralizing antibodies and involved in receptor-binding activity. The gene encoding the 3D RNA-dependent RNA polymerase, which is responsible for the synthesis of the viral RNA, is highly conserved among DHV-1 strains (105). DHAV-1 has no hemagglutination activity (34, 108).

Duck Hepatitis Virus Type 2 and 3

Astroviruses are non-enveloped, single-stranded, positive-sense RNA viruses. The genome arrangement is ORF1a and ORF1b at the 5′ end, encoding the viral protease and the RNA-dependent RNA polymerase, and ORF2 at the 3′ end encoding the precursor capsid protein (11).

The complete genome of DAstV-1 is about 7,752 nucleotides (nt) in length with a 30 nt poly(A) tail, a 7,483 nt coding region, a 22 nt short 5′ UTR, and a 247 nt 3′ UTR. The coding region includes three overlapping ORFs (ORF1a, ORF1b, and ORF2), which encode polypeptides of 1,240; 516; and 731 amino acids, respectively. The ORF2 of DAstV was not found to be in the same reading frame as either ORF1a or ORF1b, which was distinct from all other astroviruses (17, 37).

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Virus Replication

Adamiker (2) reported on the *in vivo* replication of DHAV-1. Six-day-old ducklings were infected intranasally and intramuscularly with DHAV-1 and killed 1–24 hours later; their livers were examined by EM. Virus-like particles were detected at 1 hour and 18–20 hours postinfection (PI) (2). In another study, one-day-old ducklings were subcutaneously injected with a virulent or an attenuated strain of DHAV-1 and killed 36 hours later. The results indicated that both virus strains replicated well in the liver, spleen, and intestine; however, the virulent strain replicated more efficiently than the attenuated strain (104).

Yao et al. (140) studied the replication cycle of DHAV-1 in duck embryonic hepatocytes by monitoring the dynamic changes of the relative DHAV-1 gene expression. The results suggested that the absorption of DHAV-1 progressive increase from 15–90 minutes and reached a peak at 90 minutes PI, and replication of DHAV-1 gradually increases and reached a peak at 11–13 hours PI, and the release of DHAV-1 was in steady state after 32 hours PI.

Susceptibility to Chemical and Physical Agents

Duck Hepatitis Virus Type 1

DHAV-1 is resistant to lipid solvents, such as ether and chloroform; relatively heat stable; and capable of survival for long periods of time under usual environmental conditions. DHAV-1 has been shown to resist treatment with ether or fluorocarbon (88), chloroform, pH 3 and trypsin (108), and 30% methanol or ammonium sulfate (54), 2% lysol or 0.1% formalin (8). Complete inactivation of DHAV-1was reported with 1% formaldehyde or 2% caustic soda within two hours at 15–20°C, and 2% calcium hypochlorite within three hours at 15–20°C (91). It was reported that most of DHVA-1 was inactivated after 30 minutes at 56°C (54) and that heat stability was unaffected by a 1 M solution of divalent cations (Mg^{2+}) (110).

Using cell culture-grown DHAV-1, the half-life of the virus was determined to be 48 minutes at 50°C. However, in the presence of NaCl, Na_2SO_4, $MgCl_2$, or $MgSO_4$, the virus was protected from inactivation at that temperature (24). It has been shown that DHAV-1 can survive for 21 days at 37°C (90). Under more natural environmental conditions, the virus can survive at least 10 weeks in uncleaned, infected brooders, and for longer than 37 days in moist feces stored in a cool shed (8). At 4°C, the virus has been shown to survive more than 2 years (8, 32) and at – – 20°C for as long as 9 years (53).

Tseng and Tsai (114) demonstrated that DHAV-2 is similar to DHAV-1 in several physical and chemical characteristics. Both viruses are resistant to chloroform, formalin, pH 3.0, and 1 M Mg^{2+} treatment at 56°C and 60°C. Also, both viruses were sensitive to these temperatures in the absence of the cation, with DHAV-2 being more resilient.

Duck Hepatitis Virus Type 2 and 3

DAstV-1 is resistant to chloroform, pH 3.0, trypsin treatment, and heating at 50°C for 60 minutes. Formaldehyde fumigation and standard disinfection procedures have eliminated the infection from contaminated premises (47).

DAstV-2, as an RNA virus, is also insensitive to iododeoxyuridine (IUdR). It has been found to be resistant to chloroform and pH 3.0, but sensitive to 50°C irrespective of the presence of 1 M $MgCl_2$ (52).

Strain Classification

Duck Hepatitis Virus Type 1

The diseases caused by all the different DHV types cannot be differentiated based on clinical manifestation and pathology. But DHV type 1, 2, and 3 are genetically and serologically distinct from each other. Among DHV type 1, both DHAV-2 and DHAV-3 have also been shown to be distinct genetically and serologically from DHAV-1 (38, 65, 113). It is unknown whether DHAV-2 is serologically distinct from DHAV-3. However, the nucleic acid sequence differences that have been found in capsid-coding regions (approximately 30% divergence in nucleotides) suggest that they may belong to different serotypes (118).

A variant strain of DHAV-1, named DHAV-1a, has been described (98). Partial cross-protection between types 1 and 1a has been demonstrated *in vitro* and *in vivo* (98, 135). Viruses differing or serologically distinct from DHAV-1 have been recognized as causes of hepatitis in ducklings in India (92) and Egypt (100). The Indian isolate is known to be distinct from DHAV-l, but its relationships to the other DHV types are not known.

Duck Hepatitis Virus Type 2 and 3

DAstV-1 has been compared with astrovirus isolates from chickens and turkeys in cross-protection and transmission studies, and found to be antigenically distinct (47). Phylogenetic analyses have shown that DAstV-2 is more closely related to turkey astrovirus type 2 than DAstV-1 (109).

Laboratory Host Systems

Duck Hepatitis Virus Type 1

DHAV-1 is readily propagated in chicken and duck embryos (70). Levine and Fabricant (70) were the first to propagate the virus in the allantoic sac of 9-day-old chicken embryos. Ten percent to 60% of the embryos

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died by the fifth or sixth day PI and were stunted or edematous (Figure 13.1). Death of the chicken embryos inoculated with the attenuated strain was found to occur as early as the third or fourth day PI (Tseng and Tsai, unpublished data). Hwang and Dougherty (61) passaged a DHAV-1 strain as two lines in 10-day-old chicken embryos. The serially passaged lines became nonpathogenic for newly hatched ducklings after the twentieth and twenty-sixth transfers. The virus titer in chicken embryos was 1–3 $\log_{10}$ lower than when grown in ducklings.

Golubnichi et al. (41) reported successful growth and an extensive cytopathic effect (CPE) in duck embryo fibroblasts inoculated with chick embryo-adapted DHAV-1. Woolcock (127) reported that primary duck embryo liver (DEL) cells are particularly sensitive to DHAV-1, with a CPE characterized by cell rounding and necrosis. A plaque assay has also been developed for DHAV-1 in primary monolayers of duck embryo kidney (DEK) cells (132) and DEL cells (127). Kaleta (62) described a microneutralization assay using attenuated DHAV-1 in primary DEK cells, which was used to monitor immune responses to vaccines (129).

Recently, Wang et al. (119) described the establishment of a duck embryo epithelial cell line and suggested its use for DHAV-1 propagation and vaccine development.



Figure 13.1 Normal 15-day-old chick embryo (right). Fifteen-day-old chicken embryo inoculated six days previously with duck hepatitis A virus (DHAV) type 1 (left). Note small size, hemorrhage, and edema.

A description of other attempts to grow and assay DHAV-1 in other host systems and cell cultures of various origins can be found in the previous edition (136).

Duck Hepatitis Virus Type 2

DAstV-1 has been replicated in embryonating chicken eggs following several blind passages in the amniotic sac, and attenuation of pathogenicity was found to occur after serial passage in chicken embryos (44). DAstV-1 has not been propagated efficiently in various duck and chicken cell cultures (44, 136); therefore, production of enough antigen for diagnostic tests is difficult.

Duck Hepatitis Virus Type 3

DAstV-2 has been grown successfully in embryonating eggs of ducks, but not of chickens (52, 136). During the first passages embryo deaths were erratic and did not occur until the eighth or ninth day PI, but this was reduced with higher passages. In severely affected embryos, the CAMs were discolored and the surface of the affected areas had a dry crusty or cheesy appearance. Underneath, the CAM was edematous and thickened up to 10 times normal. Embryo lesions included stunting, edema, skin hemorrhages, flaccid appearance, gelatinous fluid accumulations and enlargement of liver, kidneys, and spleen. Attenuation of pathogenicity for ducklings, accompanied by increased pathogenicity for duck embryos, was found to occur following serial passage in embryonating duck eggs inoculated by the CAM route (51).

Liver and kidney cell cultures of duck embryo or duckling origin were shown to support replication of the virus (52). Woolcock (127) reported that DAstV-2 failed to produce plaques in primary DEK and DEL cell monolayers.

Pathogenicity

Until recently, DHAV-I infection had only been associated with acute hepatitis in ducklings; however, it has now been reported to cause pancreatitis and encephalitis in Muscovy ducks (49, 50). After 20 or more passages in chicken embryo passages, DHV-1 was found to have lost its pathogenicity for ducklings (5, 56, 58, 93). Hwang and Dougherty (61) reported that chicken embryo-passaged strains, while nonpathogenic for ducklings, multiplied in the tissues, but at lower titers. Field strains were found in high concentrations in duckling brain, while chicken embryo-passaged strains could not be detected or were present in low titers. A similar attenuation of pathogenicity has been reported when DHAV-1 was passaged in duck embryos (10). Embryo passage-attenuated DHAV-1 strains are still capable of causing very mild and transitory histologic changes after inoculation (97, 107), and reversion to virulence occurs after back-passage in young ducklings (133, 134).

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Pathobiology and Epidemiology

Incidence and Distribution

Duck Hepatitis Virus Type 1

DHAV-1 is worldwide in distribution (125, 128). DHAV-2 has been reported in Taiwan (114) and DHAV-3 has been reported in South Korea (65), China (37), and Viet Nam (28). Recent studies indicated a higher prevalence rate of DHAV-3 infection than DHAV-1 infection in Korean, Vietnamese, and Chinese duck farms (29, 80, 103).

Duck Hepatitis Virus Type 2 and 3

Previously, DHV type 2 had only been reported in England. Recently, outbreaks in commercial duck flocks of DH caused by DAstV-1 have also been reported in China (17, 37). Disease caused by DHV type 3 is only known to have occurred in the United States.

Natural and Experimental Hosts

Duck Hepatitis Virus Type 1

Young ducks and geese are susceptible to DHAV-1 infections. Experimental infections in goslings (4) and mallard ducklings (36) have been reported. In experimentally exposed birds, no mortality occurred in chicks, Muscovy ducklings, or pigeon squabs; low mortality occurred in young turkeys and quail, while high mortality occurred in young pheasants, geese, and guinea fowl. All exposed birds became infected with DHAV-1 (60). In another experimentally exposed study, Tsiay duck (*Anas platyrhynchos* var. *domesticus*) and Cherry Valley ducklings were highly sensitive, and Pekin ducklings and hybrid ducklings of Tsiay duck and Pekin duck were relatively sensitive. Muscovy ducklings were least sensitive, whereas mule ducklings showed intermediate sensitivity to infection (Tseng and Tsai, unpublished data).

Field observations have indicated that all duck breeds are susceptible to DHAV-1 infection, while chickens and turkeys were resistant. Asplin (8) reported that young chickens can contract an inapparent infection and pass it on through contact with other chicks. Rahn (91), however, found that day-old and week-old poults exposed to DHAV-1 developed signs, lesions, and neutralizing antibody. Poults, after either oral or intraperitoneal exposure, had mottled livers and enlarged gall bladders and spleens. DHAV-1 was isolated from livers up to 17 days after oral exposure of day-old poults. Schoop et al. (199) and Reuss (94) failed to infect chickens experimentally.

In 2004, DHAV-2 was isolated in Taiwan from 1-week-old goslings in a white Roman goose flock experiencing mortality exceeding 70%. The affected goslings displayed liver hemorrhage typical of DH (114). Recently DHAV-3 has been reported to cause a new disease in overfed geese in China characterized by hemorrhagic hepatitis. The flocks showed about 20–40% morbidity and less than 5% mortality (76).

Duck Hepatitis Virus Type 2 and Type 3

Ducks appear to be the only species affected by DAstV-1, and no wildlife reservoirs or vectors have been detected. All recorded outbreaks have initially involved ducks kept on open fields; therefore, wildfowl, gulls, and other wild birds have been suspected as being vectors (41).

DAstV-2 has been found to have low pathogenicity for ducklings experimentally infected, and only ducklings appear to be affected by the virus. Subcutaneous (SC) or intramuscular (IM) inoculation of liver homogenate from infected ducklings into susceptible day-old ducklings is unreliable. Intravenous inoculation may increase the effectiveness (13).

Age of Host Commonly Affected

In naturally occurring outbreaks, all three DHV types have been found only in young ducklings. The ducklings have been found to gradually become more resistance as they grow older. Adult breeders on infected premises have not been found to become clinically ill and have been found to continue in full production.

Transmission, Carriers, and Vectors

Duck hepatitis A virus is excreted in the feces from infected ducklings and is transmitted horizontally by direct contact between birds or through fomites, such as brooders, water, feed, equipment. Under field conditions, DHAV spreads rapidly to all susceptible ducklings in the flock. Although high mortality and rapid spread of the disease on farms indicate extreme contagiousness, occasional exceptions have been observed. In one pen, 65% of the ducks died, while in an adjoining pen which was separated only by a 14 inch curb, mortality was negligible (135).

Field experience with DHAV-1 has indicated that egg transmission does not take place. Newly hatched ducklings produced by breeders on infected premises have remained well when housed where no ducks were being kept (5). Ducklings may also become infected by respiratory or oral routes (54, 90). The portal of entry may be the pharynx or upper respiratory tract, because the virus administered in a capsule failed to produce infection (112).

Recovered ducks may excrete virus in feces for up to eight weeks after infection (94). Asplin (8) reported that there is strong field evidence to incriminate wild birds as mechanical carriers of the virus over short distances. He also suggested the possibility that an unknown host acting as a healthy carrier might be responsible for new outbreaks at great distances. However, no serologic evidence of DHAV-1 infection was found in 520 wild

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aquatic fowl of 6 species (7), or in 4 species of 36 wild ducks taken from ponds where DHAV type 1 had occurred in domestic ducks (116). In addition, all 153 wild duck embryonating eggs from an infected area were susceptible to experimental infection.

Of possible significance in the epizootiology of the disease is a report indicating that brown rats (*Rattus norvegicus*) could act as a reservoir host of DHAV-1 (27). Ingested virus remained alive up to 35 days and the virus was excreted 18–22 days postinoculation (PI). Serum antibodies were also present 12–24 days PI. However, there has been no evidence of the involvement of any vectors in transmission of DHAV-1.

Incubation Period

The incubation period for DHAV-1 is 18–48 hours (130). For DAstV-1 and DAstV-2 infection, the incubation period is 2–4 days (44, 111).

Clinical Signs

Duck Hepatitis Virus Type 1

Onset and spread of DHAV-1 are very rapid, with practically all mortality occurring within 3–4 days. Affected ducklings at first fail to keep up with the brood. Within a short time, they stop moving and squat down with eyes partially closed. Birds fall on their sides, kick spasmodically with both legs, and die with heads drawn back (Figure 13.2). Death occurs within an hour or so after signs are noted. During the height of severe outbreaks, ducklings can die very rapidly. The clinical signs caused by all three DHAV genotypes are similar.

Duck Hepatitis Virus Type 2

The clinical course of DAstV-1 infection is similar to that of DHAV-1 and can be seen in ducklings immune to DHAV-1 infection. Deaths occur within 1–4 days, usually within 1–2 hours after the appearance of clinical signs, which include polydipsia with loose droppings, excessive urate excretion, and sometimes convulsions and acute opisthotonos (47). Affected ducks usually die in good condition, and the time of death and mortality rate (10–50%) depend on the age of the ducks (44). Survivors excrete virus for at least one week after infection (47) and rear normally, with little evidence of retarded growth (44). Mature ducks are refractory to the disease (44).

Duck Hepatitis Virus Type 3

Ducklings dying from DAstV-2 infection show the typical clinical signs of DHV type 1 infection such as outstretched legs and opisthotonos. Mortality rarely exceeds 30%, but gross pathologic changes are similar to those caused by DHV type 1 (111).



Figure 13.2 Duckling dead from infection with duck hepatitis A virus (DHAV) type 1. Note typical opisthotonos.

Morbidity and Mortality

Duck Hepatitis Virus Type 1

Morbidity is 100% and mortality in young ducklings infected with DHAV-1 varies according to age. In some broods less than one week old, mortality may reach 95%. In 1- to 3-week-old ducklings, mortality may be 50% or less. In 4- to 5-week-old ducklings, morbidity and mortality are low or negligible (136).

Duck Hepatitis Virus Type 2 and Type 3

Mortality due to DHV type 2 varies between 10 and 25% in 3- to 6-week-old birds, and can be up to 50% in 6- to 14-day-old birds (44). DHV type 3 is less severe than DHV type 1 and 2, and mortality rarely exceeds 30% (111).

Pathology

Gross

Duck Hepatitis Virus Type 1. Principal lesions due to DHAV are found in the liver, which is enlarged and displays distinct punctuate or ecchymotic hemorrhagic foci (Figure 13.3). Frequent reddish discoloration or mottling of the liver surface is seen. The spleen is sometimes enlarged and mottled. In numerous cases, the kidneys are swollen and renal blood vessels congested. The lesions caused by all three DHAV genotypes are similar.

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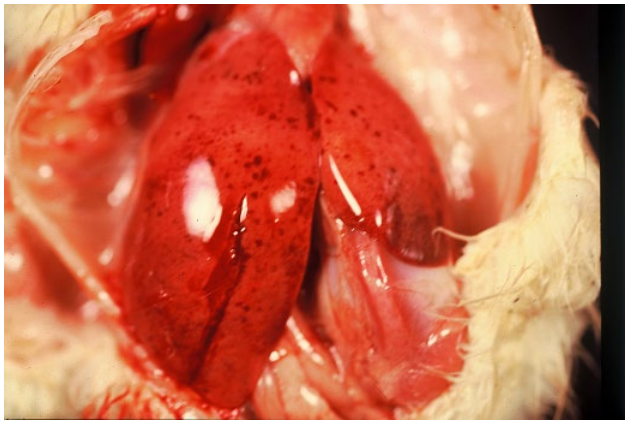


Figure 13.3 Enlarged liver with diffuse petechial and ecchymotic hemorrhages caused by duck hepatitis A virus (DHAV) type 1 infection.

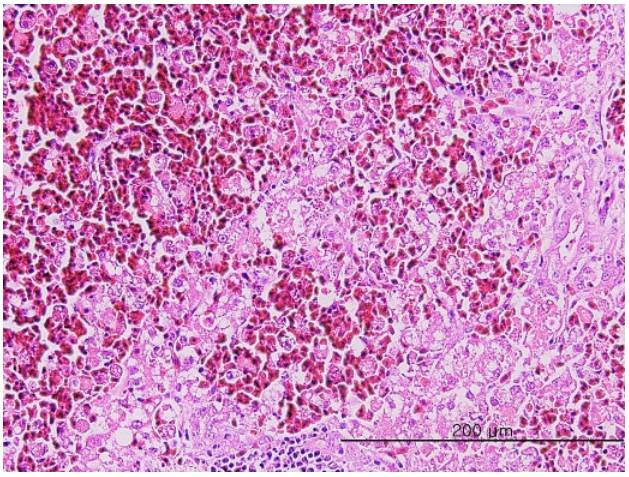


Figure 13.4 Microscopic lesions in liver of duckling dead from infection with duck hepatitis A virus (DHAV) type 1. Note acute lesions show massive hepatocyte necrosis and hemorrhage.

Duck Hepatitis Virus Type 2 and Type 3. Lesions caused by all three types of DHV are similar. Occasionally, small hemorrhages are seen in the intestinal wall and on the heart fat of DAstV-1-infected ducklings (44).

Microscopic and Ultrastructural

Duck Hepatitis Virus Type 1. Microscopic changes in DHAV infection consist of necrosis of hepatic cells and varying degrees of inflammatory cell infiltration and hemorrhage (Figure 13.4); survivors with more chronic lesions have shown regeneration of liver parenchyma and widespread bile duct hyperplasia (Figure 13.5) (33). Peng showed by EM that DHAV invades many tissues in the duckling and causes swelling, hemorrhage, and necrosis of the liver, spleen, kidneys, and pancreas. Pathological changes have also been seen in the central nervous system and the bursa of Fabricius in infected ducklings (87). Adamiker (1) examined spleen and

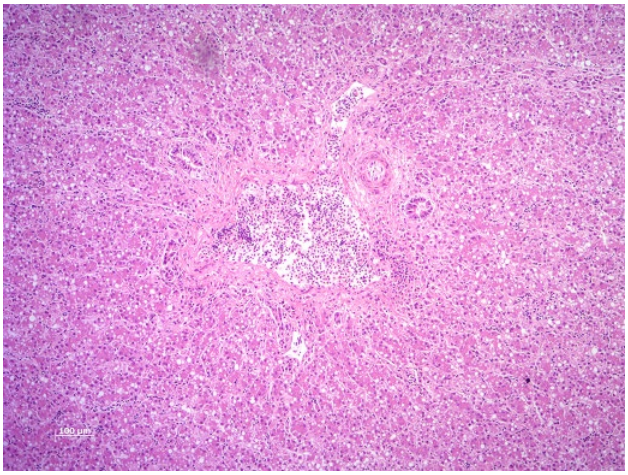


Figure 13.5 Microscopic lesions in liver of duckling dead from infection with duck hepatitis A virus (DHAV) type 1. Note chronic lesions show extensive bile duct proliferation.

muscle of ducks infected with DHAV-1 by EM. The spleen showed regressive changes at 6 hours PI and became necrotic by 24 hours. There were degenerative changes in the nuclei of plasma cells that may have been caused by the virus. Virus particles were not identified. Only slight changes were seen in muscles.

Duck Hepatitis Virus Type 2 and 3. Lesions of DAstV-1 and DAstV-2 infections are similar to those of DHAV infection. Microscopic changes in the acute case are characterized by extensive necrosis of the hepatocyte cytoplasm and bile duct hyperplasia is widespread.

Pathogenesis of the Infectious Process

Duck hepatitis A virus infection causes hepatic necrosis and apoptosis, resulting in liver injury, which plays an important role in disease pathogenesis (102). Ahmed et al. (3) reported that, in clinical cases of DHAV-1 infection, there were lower serum levels of total protein and albumen, and elevated levels of alkaline phosphatase, glutamic pyruvic transaminase (GPT), bilirubin, and creatinine. The serum levels of GPT and glutamic oxaloacetic transaminase were increased in relation to severity of infection (84).

Song et al. (104) reported that the main target organs of both a virulent (SH) and an attenuated (FC64) strain of DHAV-1 were liver, spleen, and intestine. Infection with the SH strain was lethal to the 1-day-old ducklings at 36 hours PI, and apoptosis and visible lesions were demonstrated in the liver. Interferon-gamma (IFN-γ), interleukin 2 (IL-2), inducible nitric oxide synthase (iNOS), and nitric oxide (NO) production were strongly upregulated by SH infection, which may contribute to the pathogenicity of SH. However, the FC64 strain was nonpathogenic. The induction of type I IFNs,

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IFN-stimulated genes, and IL-6 by FC64 infection was several hundred-fold greater than that of SH infection. The intensive induction of cytokines by FC64 may be involved in restriction of virus replication and stimulation of adaptive immune responses.

Immunity (Active, Passive)

Duck Hepatitis Virus Type 1

Recovery from DHAV-1 infection results in solid immunity and neutralizing antibodies in serum. Active immunity can be induced in adult ducks by injection of certain strains of the virus (5). Some strains require repeated injections to obtain high levels of antibody (85). Virus-neutralizing activity was revealed in both immunoglobulin M (IgM) and IgG classes of sera of actively-immunized ducks (112). Davis and Hannant (26) reported that neutralizing antibody was present 4 days post vaccination of 2-day-old ducklings. Song et al. (104) reported that in ducklings inoculated with an attenuated strain of DHAV-1, high levels of neutralizing antibodies were produced and maintained for 45 days.

Passive immunity can be conferred to ducklings by injection of serum from recovered or immunized birds. Passive antibodies may also be transferred through yolk to hatched ducklings to protect them. Toth and Norcross (112) have shown that it is IgG, and not IgM, that is transferred from the dam to the newly hatched ducklings.

Duck Hepatitis Virus Type 2 and 3

Gough and Stuart (47) found that survivors of DAstV-1 infection were immune to further infection. Detectable antibody levels following infection were shown to be low, using a virus-constant serum neutralization test in embryonating chicken eggs (42). An active immune response to DAstV-2 infection can be stimulated in adult ducks by inoculation of attenuated virus. This immunity may be passively transferred via the yolk to progeny.

Diagnosis

Isolation and Identification of the Causative Agent

Although a presumptive diagnosis can be made on the basis of the characteristic disease pattern in the flock and gross pathological lesions in the liver, isolation and identification of the causative agent are necessary to confirm the diagnosis. Liver specimens can be collected at postmortem for virus identification.

Duck Hepatitis Virus Type 1

The presence of DHAV may be confirmed by molecular diagnostic methods, or one or more of the following procedures (125, 128):

- Inoculation, SC or IM, of the isolate into 1- to 7-day-old DHAV susceptible ducklings. The characteristic clinical disease should follow, with deaths often occurring within 24 hours. Ducklings should show the gross pathology attributable to DHAV. The virus should be reisolated from the livers to confirm the diagnosis.
- Inoculation of serial dilutions of the liver homogenate into the allantoic sacs of embryonating duck eggs (aged 10–14 days) from a DHAV-free flock, or chicken eggs (aged 8–10 days). DHAV-infected duck embryos should die within 24–72 hours; chicken embryos are more variable and erratic in their response and usually take 5–8 days to die. The allantoic fluid is opalescent or a pale greenish-yellow. Gross pathological changes in the embryos include stunting and SC hemorrhages over the whole body, with edema, particularly of the abdominal and hind limb regions. The embryo livers may be swollen, red, and yellowish in color, and show necrotic foci. The liver lesions and embryo stunting become more apparent in embryos that take longer to die. Histological changes include: proliferation of granulocytes in various organs, focal necrosis of the liver, bile duct hyperplasia, and SC edema. Inclusion bodies should not be found (35).
- Inoculation of primary cultures of DEL cells (127). Serial dilutions of the liver homogenate containing DHAV-1 should cause a CPE that is characterized by cell rounding and necrosis. When overlaid with a maintenance medium containing 1% agarose (wt/vol), the CPE gives rise to plaques approximately 1 mm in diameter.

A rapid and accurate diagnosis of DHAV-1 can be made using the direct fluorescent antibody (FA) technique on livers of naturally occurring cases or inoculated duck embryos (82, 117). Virus isolation and identification procedures have been reviewed (51, 110).

Duck Hepatitis Virus Type 2

Traditionally the most reliable diagnostic method for DHV type 2 has been EM examination of liver homogenates for the detection of astrovirus-like particles. Now, molecular diagnostic methods are more sensitive and are widely used.

The virus can only be isolated, with difficulty, following repeated passage in the amniotic sac of embryonating chicken or duck eggs. However, these are difficult and expensive processes because the embryos may respond erratically only after four passages and no deaths may be seen during earlier passages. Few embryos have been shown to die in less than seven days, but infected embryos have appeared stunted and had greenish necrotic livers in which astrovirus-like particles could be demonstrated by EM (44). Inoculation of susceptible ducklings with virus gives a variable response; mortality up to 20% may occur within 2–4 days PI. (44, 125, 128).

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Duck Hepatitis Virus Type 3

Duck hepatitis virus type 3 may be tentatively identified by inoculation of liver suspension onto the CAM of 10-day-old embryonating duck eggs if embryo lesions and mortality patterns develop as above-described (125, 128). Alternatively, virus may be isolated and identified in DK or DEK cultures examined by immunofluorescence 48–72 hours PI using DAstV-2-specific antibody. A direct FA test in duckling livers and DEK or DK cells has been described for DAstV-2 (125, 128).

Molecular Diagnostic Methods

Several reverse transcriptase-polymerase chain reactions (RT-PCR) have been developed and are useful for identifying DHAV-1 infections (22, 38, 66). Chen et al. (22) reported the detection limit of RT-PCR DHAV-1 RNA as 3 pg/10 µl. They also demonstrated that RT-PCR was the most sensitive when the detection rates were compared on 185 clinically suspected DHAV-1-infected liver tissues by RT-PCR, enzyme-linked immunosorbent assay (ELISA), and virus isolation methods.

Reverse transcriptase-loop-mediated isothermal amplification (RT-LAMP) assays for the detection of DHAV have also been developed, and it has been shown that the assay is as sensitive as RT-PCR (72, 105). Duplex and multiplex RT-PCR have been developed for the simultaneous detection and differentiation of DHAV types (55, 67, 123).

Yang et al. (139) reported the development of a one-step real-time RT-PCR assay (rRT-PCR) based on primers to a conserved region in the *3D* gene for the detection of DHAV-1. The detection limit of this assay was 10 viral genomic copies per reaction. An astrovirus-specific RT-PCR assay based on the ORF1b region of astroviruses (109) has been reported for use to detect DAstV-1, DAstV-2 and other duck astroviruses. Multiplex RT-PCR has been developed for the simultaneous detection of DHAV-1, DHAV-3, and DAstV-1 (16).

Serology

Duck Hepatitis Virus Type 1

Due to the short course of the disease, serologic tests have not been useful in diagnosing acute outbreaks of DHAV-1. However, the neutralization test is useful for virus identification, titration of serologic response to vaccination, and epidemiologic surveys.

Both *in vivo* and *in vitro* neutralization tests have been developed for DHV type 1. Hwang (57) first described an accurate, reproducible DHAV-1 neutralization test in chicken embryos. Modifications of this procedure have been described (46, 112), including those adapted to duck embryos or ducklings (51) and tissue culture (41). Woolcock et al. (135) first described a plaque-reduction test for neutralization antibody. This assay was considerably more sensitive than assays in eggs. A microneutralization assay in DEK cells has been developed as a more practical, rapid, and economical alternative to other tests for the diagnosis of DHAV-1 (62). This assay has been adapted to monitor the serum neutralization antibody responses of ducks to vaccines in field and laboratory trials, and a titer of less than $4 \log_2$ is considered to be negative (129).

An indirect ELISA using the whole DHAV virus particle as a coating antigen has been shown comparable to the neutralization test (141). Recently, indirect ELISAs using the VP1 or VP3 proteins of DHAV-1 as coating antigens have also been described (77, 101). For reviews of other serologic tests such as the passive hemagglutination (HA) test, indirect HA test, and agar gel diffusion precipitin test, please see the previous edition of this textbook (136).

Duck Hepatitis Virus Type 2

An indirect ELISA has been developed based on the expressed C-terminal ORF2 protein of DAstV-1 (121).

Differential Diagnosis

The sudden onset, rapid spread, and acute course of disease caused by DHAV-1 are characteristic. Hemorrhagic lesions in the liver of ducklings up to three weeks of age are pathognomonic. Since all three DHV types cause similar disease outbreaks, differential diagnosis is needed because immunization is serotype-specific and does not confer protection against infection with heterologous type and serotypes.

Possible synergistic effects of DHAV-1 with *Chlamydophyla psittaci* (15) and influenza virus (48) have been suggested. Other potential causes of acute mortality in ducklings include avian influenza, duck viral enteritis, *Pasteurella anatipestifer* infection, salmonellosis, coccidiosis, and aflatoxicosis. Aflatoxicosis may cause ataxia, convulsions, and opisthotonos as well as microscopic lesions of the bile duct with hyperplasia suggestive of DHV, but does not cause the same characteristic liver hemorrhages.

Intervention Strategies

Management Procedures

Duck hepatitis virus type 1 can be prevented by strict isolation, particularly during the first 4–5 weeks of age. In areas where the disease is prevalent, however, it is very difficult to obtain the necessary degree of isolation. Contact with wild waterfowl should be prevented and, since rats may act as a reservoir host of the virus, pest control is needed. Panikar (86) as well as Kaszanyitzky

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and Tanyi (63) demonstrated the feasibility of eradicating DHAV-1 in selected areas where isolation can be achieved. In both studies, vaccination of breeder ducks was used as part of the program.

Vaccination

Type of Vaccine

Duck Hepatitis Virus Type 1. Resistance against DHAV-1 may be conferred to ducklings by three methods: injection of immune serum or yolk, as described under Treatment (see below); immunization of breeding stock to ensure high levels of passively transferred antibody in the hatched ducklings; and direct active immunization of ducklings with live avirulent strains of DHAV-1.

Attenuated DHAV-1 strains suitable for vaccine use have been produced by passage in chicken embryos (5, 39, 40, 99) or duck embryos (10, 96). Up to this time, various strains of chicken embryo-passaged DHAV-1 have been used most frequently as vaccines. Davis (25) reported that triple plaque-purified strains of DHAV-1 used for vaccines could revert to virulence as readily as non-cloned virus. This finding has been confirmed with various egg-passaged levels of DHAV-1 (131). Davis suggested rapid passage as a method to increase genetic stability.

Recently, live attenuated DHAV-3 vaccine has been developed in South Korea (64). Also, a live attenuated duck enteritis virus vector vaccine, which was designed to express VP1 of both DHAV-1 and DHAV-3, has shown protection to challenge of virulent strains of DHAV-1 and DHAV-3 (142).

Duck Hepatitis Virus Type 2 and Type 3. An experimental DAstV-1 live attenuated virus vaccine has been shown to protect ducklings from challenge with virulent virus, but this vaccine has never been produced commercially (47). Also, a DAstV-2 live attenuated vaccine has been used experimentally in breeder ducks to confer passive immunity to ducklings, but this vaccine has not been available commercially (136).

Field Vaccination Protocols and Regimes

Breeders. It is generally suggested that two or three doses of attenuated virus vaccine be given in order to achieve satisfactory levels of protection of progeny (30, 59, 62, 93). Rispens (96) recommended that two doses of attenuated virus vaccine be administered to breeders at least six weeks apart; passive immunity was transmitted to progeny for about nine months after the second vaccination. Woolcock (130) recommended administering the attenuated virus vaccine subcutaneously at the neck of breeder ducks at 16, 20, and 24 weeks of age and every 12 weeks thereafter, throughout the laying period.

The application of inactivated vaccines has also been investigated. Multiple inoculations of inactivated vaccine are needed to provide passive immunity to progeny for a complete laying cycle. A single inoculation of inactivated vaccine given to breeder ducks, which has been previously primed with live DHAV-1, at the time before the birds come into lay, also provides passive immunity to progeny ducklings for a complete laying cycle (46, 129). For example, Gough and Spackman (46) reported that effective levels of duckling protection can be secured by administering three doses of inactivated, oil-emulsion vaccine. They also reported that live DHAV-1 vaccine administered at 2–3 days of age, followed by inactivated vaccine at 22 weeks, producing significantly higher virus-neutralizing (VN) antibody levels than did three doses of inactivated vaccine. Finally, they reported that inactivated vaccine prepared from virus grown in duck eggs gave a better antibody response than virus grown in chicken eggs. Woolcock (129) also investigated the use of inactivated DHAV-1 vaccines in breeder ducks. He showed that ducks primed with modified live virus at 12 weeks of age, and boosted with inactivated DHAV-1 vaccine at 18 weeks of age, developed VN antibody titers that were 16-fold higher than those in ducks that received only the MLV priming. This level of immunity was sufficient to protect ducklings hatched through a complete laying cycle (eight months), as demonstrated by challenging progeny with virulent DHAV-1. He also showed that only ducks given multiple inoculations of inactivated vaccine developed titers of $6 \log_2$ or greater, which was considered the minimum protective level.

Ducklings. Chicken embryo-attenuated strains of DHAV-1 have been shown to induce a considerable degree of protection in one-day-old ducklings inoculated by the SC, IM, intranasal (IN), or foot-web route (5, 23, 40, 93, 143). Effective mass vaccination of ducklings by aerosol and drinking water routes have also been reported (54, 69, 85, 86). However, Sung et al. (106) reported that IM administration of attenuated DHAV-1 vaccine showed more efficient protection than by the oral or eye drop route of administration. Newly hatched ducklings injected intramuscularly with an attenuated DHAV-1 rapidly developed resistance in 3 days (59). Oral exposure required up to six days for protection to occur. There was evidence that vaccination would be of benefit even at the start of an outbreak.

Results from studies on the effects of maternal antibodies on vaccination of ducklings with live attenuated DHAV-1 have not been consistent (9, 79). However, field experience has indicated that successful practical duckling vaccination depends on the absence of maternal antibodies, and is influenced by time and severity of exposure to virulent virus. Vaccination is also less effective when ducklings are exposed to virulent virus early in

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life, especially in endemic areas and on heavily infected premises. Judicious application of good hygiene and sanitation methods can help with this problem (136).

Kim et al. (64) reported on the development of a chicken embryo-attenuated strain of DHAV-3. One-day-old ducklings vaccinated intramuscularly with this virus were fully protected from challenge with pathogenic virus at 2–3 days post vaccination.

Treatment

Duck Hepatitis Virus Type 1

As soon as the cause and nature of DHAV-1 infection were recognized (70), it became apparent that ducklings might be protected by administration of serum from immune ducks. This procedure proved to be highly successful in laboratory experiments and in the field. For many years, the Duck Research Laboratory (DRL) at Cornell University at Eastport, Long Island, New York, kept a bank of antiserum processed from blood collected at the time of slaughter from recovered birds. Intramuscular injection of 0.5 ml DHAV-1 antiserum into each duckling of a brood at the time of the first deaths in an outbreak was an effective control method.

Rispens (96) has suggested passive immunization by injection of yolk from eggs produced by hyperimmune breeder ducks. At the DRL, Long Island, New York, this procedure has been modified by substituting yolk from eggs produced by specific pathogen free chicken hyperimmunized with DHAV-1. For immunization, yolk antibody preparations with a minimum neutralizing index of 10^3, as determined by the constant-yolk/varying–virus method, would be considered to be satisfactory (125).

Recently, Chinese herb medicine has been studied with respect to treatment and effect on DHAV-1. Bush Sophora Root polysaccharide and its sulfate (19–21), astragalus polysaccharide and its sulfate (18), icariin and its phosphorylated structural modification (137), and a flavone/polysaccharide-containing prescription drug (31) have shown some antiviral activities against DHAV. Also, phosphorylated *Codonopsis pilosula* polysaccharide has been shown to reduce the replication of DHAV-1 in duck embryonic hepatocytes, probably through a reduction in expression of IFN-β (83).

Duck Hepatitis Virus Type 2 and Type 3

Inoculation of susceptible ducklings with convalescent serum obtained from DHV type 2-infected ducks has been used successfully to control the disease in the field (47). Also, convalescent sera obtained from DHV type 3-infected ducks have been used effectively in the field to control outbreaks.

Duck Virus Enteritis (Duck Plague)

Samia A. Metwally and Anchun Cheng

Summary

Agent, Infection, and Disease. Duck virus enteritis (DVE), also called duck plague, is one of the major contagious and fatal diseases of ducks, geese, and swans. The agent can spread via horizontal and/or vertical transmission and causes death, vascular damage, and subsequent internal hemorrhage, lesions in lymphoid organs, digestive mucosal eruptions, severe diarrhea and degenerative lesions in parenchymatous organs of infected birds. Huge economic losses are caused by high morbidity and mortality, decreased egg production, and hatchability. Duck virus enteritis is worldwide in distribution.

Diagnosis. The diagnosis of DVE includes virus isolation, serological, and molecular tests in combination with clinical manifestations and histopathology.

Intervention. Vaccination of live-attenuated or inactivated vaccines can prevent disease in broiler and breeder ducks.

Introduction

Definition and Synonyms

Duck virus enteritis (DVE) is an acute, contagious herpesvirus infection of ducks, geese, and swans, characterized by vascular damage, tissue hemorrhages, digestive mucosal eruptions, lesions of lymphoid organs, and degenerative changes in parenchymatous organs. Synonyms for the disease are duck plague, eendenpest (Dutch), peste du canard (French), Entenpest (German), and duck virus enteritis. Although Bos (5) first used the term duck plague, it was proposed as the official name by Jansen and Kunst in 1949 (29). Subsequently, DVE, based on principal features of the disease and to distinguish it from fowl plague, has become the preferred term.

Economic Significance

In duck-producing areas of the world where the disease has been reported, DVE has produced significant economic losses in domestic and wild waterfowl due to mortality, condemnations, and decreased egg production.

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The first outbreak in the United States was in 1967 and caused losses in excess of $1 million during a one-year period for the small, but concentrated, duck industry of Long Island, New York (42).

Public Health Significance

Duck virus enteritis is primarily a disease of waterfowl. No known risk to human health has been reported.

History

In 1923, Baudet (4) reported an outbreak of an acute, hemorrhagic disease of domestic ducks in the Netherlands. Bos (5) suggested that the disease was caused by a new distinct viral disease of ducks, which he termed "duck plague." These observations were further supported by more detailed studies on virus propagation, incidence and distribution, pathology, and immunity (28, 67)

After that, serious outbreaks in migratory waterfowl, zoos, and game farm flocks have been reported (17). The latest outbreak was reported in Germany (2007) in ducks and geese kept in captivity to prevent spread of highly pathogenic avian influenza (H5N1) (34). Duck virus enteritis was delisted from the OIE Terrestrial Animal Health Code in 2010 due to its limited spread across country borders.

For detailed historical details of DVE, see prior editions of this book.

Etiology

Classification

The causative agent of DVE is a herpesvirus (Anatid herpesvirus 1), belonging to the *Mardivirus* genus of Alphaherpesvirinae subfamily (55). Duck enteritis virus is non-hemagglutinating and non-hemadsorbing (13).

Morphology

Ultrastructure, Size, and Density

Ultrathin sectioning and transmission electron microscopy (TEM) of infected duck embryo fibroblasts (DEF) were employed to investigate the morphology of duck enteritis virus (DEV) (23). The nucleic acid of DEV was round in shape with diameter of 35-45 nm and was often in a cluster in the nucleus of DEF. The nucleocapsid of DEV was round in shape with diameter of 90–100 nm and could be observed both in nucleus and cytoplasm of DEF. The mature DEV which had the structures of envelop and tegument was spherical in shape with diameter of 150–300 nm and located in cytoplasmic vacuoles (Figure 13.6).

Study on the morphogenesis of DEV in infected DEF cells demonstrated that the attached DEV probably penetrated the cell membrane by direct fusion between the viral envelop and the plasma membrane. Progeny nucleocapsids assembled in the nucleus and exited from this compartment into the cytoplasm by budding through the inner nuclear leaflet into the interconnected perinuclear cisterna and cisternae of endoplasmic reticulum. DEV tegumentation occurred in cisternae of endoplasmic reticulum and the tegumented progeny nucleocapsids obtained their final envelop by budding into cytoplasmic vesicles. Intravesicular mature DEV particles were discharged from the cell through exocytosis of the cytoplasmic vesicles by cells, or through rupture of the virus-containing vesicles (20). Intracytoplasmic inclusion bodies and intranuclear inclusion bodies could be observed respectively in the cytoplasm and nucleus of infected DEF. With the appearance of progeny DEV in DEF, some densely electron-stained, virus-related structures which were rod-shaped, U-shaped, or of circle, semicircle, or concentric circle in appearance, could be observed in the cytoplasm of DEF.

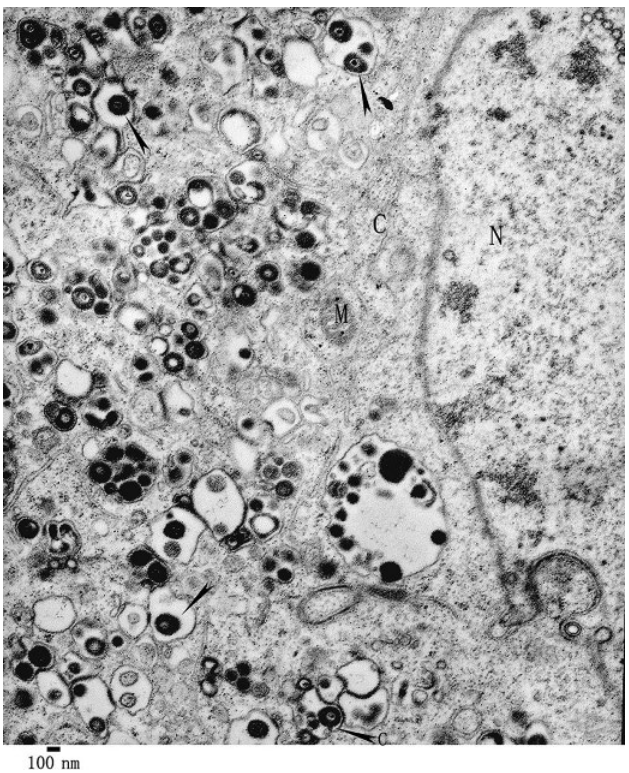


Figure 13.6 The morphology and distribution of mature duck enteritis virus (DEV) particles in host cells. Microscopically, liver shows focal areas of necrosis filled with fibrin from duck virus enteritis (DVE). Intranuclear inclusion bodies can be seen in hepatocytes near areas of necrosis. Arrow: The mature particles located in the cytoplasmic vacuoles. N: nucleus, C: cytoplasm.

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Chemical Composition

The virion contains DNA (33). RNase treatment on thin sections had no effect on ultrastructural morphology of the virus, and exposure to DNase led to the removal of the central core without affecting the envelope. Fluorescence of intranuclear inclusion bodies in cell cultures stained with acridine orange also was consistent with the presence of DNA. Inactivation by pancreatic lipase indicates that the virions contain an essential lipid.

Virus Replication

Development of the virus in cell cultures was studied by electron microscopy (EM) and growth curves of intracellular and extracellular virus (21, 22, 70). Examination of thin sections revealed development forms only in the nucleus 12 hours postinoculation (PI). By 24 hours, in addition to viral forms in the nucleus, larger particles with an envelope were observed in the cytoplasm. Virus titrations of similar cell cultures demonstrated new cell-associated virus 4 hours PI, with maximum titer at 48 hours. Extracellular virus was first detected 6–8 hours PI and reached maximum titer at 60 hours (33). Increased incubation temperatures of tissue cultures (39.5–41.5°C) favored viral replication, especially of less virulent strains (7). Viral glycoprotein C plays an active role in the adsorption of DEV over DEF to enhance the infectivity and hence blocking the gC can be a critical strategy in preventing the viral establishment in the host cells (25, 32).

In a susceptible host, virus replicates primarily in the mucosa of the digestive tract, especially in the esophagus, and then spreads to the cloacal bursa, thymus, spleen, and liver. The epithelial cells, lymphocytes, and macrophages of these organs are the principal sites of viral replication (60, 86). Since DEV can replicate quickly in many cell types and tissues, it is considered as a pantropic virus that leads to pathological lesions in many different organs (54).

Susceptibility to Chemical and Physical Agents

The virus was found to be sensitive to ether and chloroform. Exposing virus for 18 hours at 37°C to trypsin, chymotrypsin, and pancreatic lipase markedly reduced or inactivated it, and papain, lysozyme, cellulase, DNase, and RNase had no effect. Besides, heating, calcium chloride drying and exposure to extreme pH resulted in rapid inactivation (50).

For susceptibility details of DEV to chemical and physical agents, see prior editions of this book.

Strain Classification

Antigenicity, Immunogenicity, or Protective Characteristics

Although differences in virulence among DEV strains have been noted, all appear to be immunologically identical and antigenically related (66). The virus is immunologically distinct from other avian viruses, including fowl plague, Newcastle disease, duck hepatitis (13), and other herpesviruses (59).

A herpesvirus was isolated from domestic geese in Australia showing gross pathological and histopathological changes similar to those seen in DVE (36). The virus isolate was antigenically and genomically distinct from DEV as shown by protection, serological analysis, and genetic characterization by restriction endonuclease digestion and polymerase chain reaction (PCR) assays (19).

Genetic or Molecular Characteristics

Duck enteritis virus is currently grouped into the *Mardivirus* genus of the Alphaherpesvirinae subfamily. Complete genomic analysis of the CHv strain of DEV demonstrated that the genome comprises of 162,175 nucleotides encoding 78 putative proteins (81). Further analysis showed DEVs have a typical type D herpesvirus genome arrangement pattern (UL-IRS-US-TRS) which are consistent with genomic organization of the members of Alphaherpesvirinae. Like most members of them, the genes in the UL region are well conserved, while the genomic arrangement of IRS-US is similar to that of Marek's disease virus and equine herpesvirus 1. Moreover, comparative genomic analysis of DEVs demonstrated that, although similar to other herpes viruses, the DEV genome also show variation (83). For example, the LORF3 segment of the European strain (2085) was 33 bp shorter than that of Asian DEV strains (CHv, VAC). A 181 amino acid domain present in virulent strains was absent in attenuated strain.

Laboratory Host Systems

Duck enteritis virus can be propagated in duck embryo fibroblasts, duck embryo liver or kidney primary cells, and the chorioallantoic membrane (CAM) of 9- to 14-day-old embryonating duck eggs. The virus can be adapted to grow in embryonating chicken eggs and chicken embryo cell cultures (13). Moreover, continuous cell line CCL-141 and Vero cell were also demonstrated suitable for DEV growth in recent years (2, 53). However, they are unsatisfactory for primary isolation. The virus produces a cytopathogenic effect in inoculated cell cultures (13), and intranuclear inclusions have been observed in infected chicken and duck embryo cell cultures (24) (Figure 13.7). Plaque assays have been used to measure virus and neutralizing antibody titers (13). In the presence of complement, antibodies to DEV are capable of lysing infected duck embryo fibroblasts (38).

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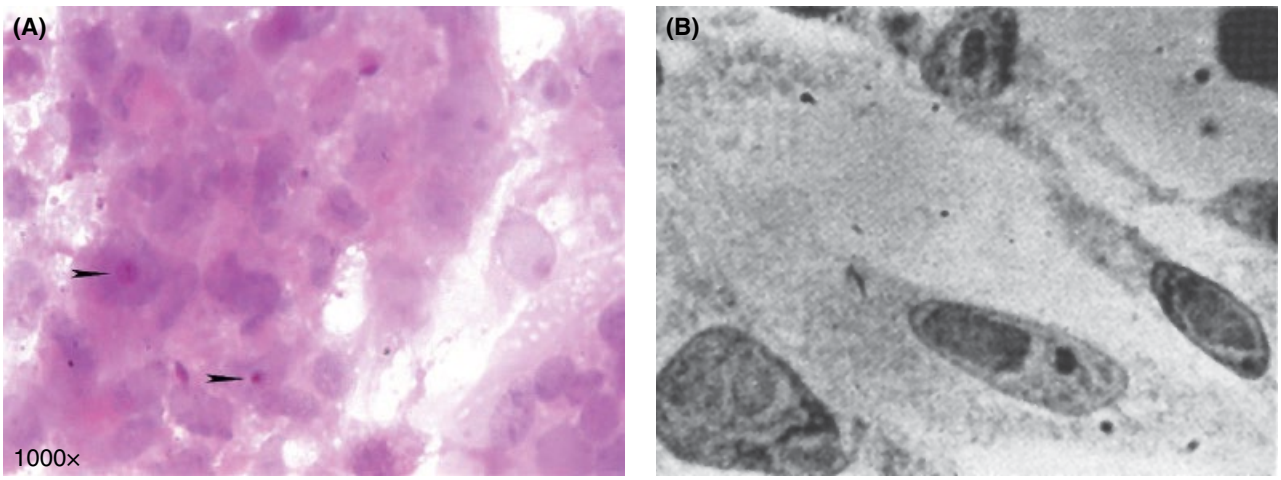


Figure 13.7 Inclusion bodies in duck embryo fibroblasts infected with duck virus enteritis (DVE). (A) ×1000. (B) ×310,000.

Pathobiology and Epizootiology

Incidence and Distribution

In addition to the Netherlands, DVE has been reported in China and confirmed in France, Belgium, India, Thailand, England, Canada, Hungary, Denmark, Austria, Viet Nam, Germany (34), Egypt (16), Poland (80), and Bangladesh (1). Epidemiological investigations suggested an association between the incidence of DVE in wild birds and farmed poultry as samples from wild birds and poultry were found to be positive/carrier, by various virological and serological investigations. Higher flock density is usually the main contributing factor for initiation of DVE outbreaks. Reports demonstrated that DVE had a worldwide distribution that regularly occurred throughout the year except in August and September, and approximately 86% of these outbreaks have been reported from March to June due to the physiological changes and breeding during spring season (15).

Natural and Experimental Hosts

Although the virus can be adapted by serial passage to grow in embryonating chicken eggs and chickens up to two weeks of age (41), natural susceptibility to DVE has been limited to members of the family Anatidae (ducks, geese, and swans) of the order Anseriformes. Naturally occurring outbreaks have occurred in a variety of domestic ducks (*Anas platyrhynchos*) including white Pekin, Khaki Campbell, Muscovy ducks (*Cairina moschata*) (43), Indian runner, hybrids, and native ducks of mixed breeding, domestic geese (*Anser anser*), and mute swans (37) ranging from seven days of age to mature breeders. Gray call ducks have been found to be resistant to lethal infections. Outbreaks of DVE in domestic ducks are frequently associated with aquatic environments cohabited by wild waterfowl (43).

In addition to domesticated species, mallards (*A. platyrhynchos*), Garganey teal (*A. quer quedula*), gadwall (*A. strepera*), European widgeon (*A. penelope*), wood ducks (*Aix sponsa*), shovelers (*Spatula clypeata*), common pochards (*Aythya ferina*), common eiders (*Somateria mollissima*), white-fronted geese (*Anser albifrons*), bean geese (*A. fabalis*), and mute swans (*Cygnus olor*) were susceptible to lethal infection. The first reported outbreaks of spontaneous DVE in wild waterfowl on Long Island, New York (42) confirmed DEV present in mallards, black ducks (*A. rubripes*), a Canada goose (*Branta canadensis*), a bufflehead (*Bucephala albeola*), a greater scaup (*Aythya marila*), and a mute swan. Mallards were more resistant to lethal infections and were considered a possible natural reservoir of infection (79).

European teal (*A. crecca*) and pintails (*A. acuta*) were resistant but produced antibodies against DEV as a result of experimental exposure, but herring gulls (*Larus argentatus*) and black-headed gulls (*L. ridibundus*) of the order Charadriiformes were not susceptible to experimental infection and failed to produce antibodies against DVE (74).An experimental study (78) showed blue-winged teal (*A. discors*) and Canada geese were extremely susceptible to DEV and experienced high mortalities. Blue-winged teal had few gross lesions at necropsy.

A study on susceptibility of waterfowl to Lake Andes strain of DEV showed that blue-winged teals, wood ducks, and redheads were highly susceptible; Muscovy ducks and gadwalls were moderately susceptible; mallards and Canada geese were less susceptible; and pintails were the least susceptible (17, 66). An outbreak in captive ducks and geese reported in Germany in 2005 identified 14 additional susceptible Anseriform species that were collected by a hobbyist from different continents (34).

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Transmission, Carriers, and Vectors

Duck virus enteritis can be transmitted by direct contact between infected and susceptible birds or indirectly by contact with a contaminated environment. As the waterfowl is dependent on an aquatic environment, transmission through water seems to be a prime source. New outbreaks of DVE can be prevented after limiting the access of domestic ducks to open water bodies that are cohabited by free-flying waterfowl. However, once the infection has been established, they may become carriers and shed DEV into the environment, causing new foci of infection by movement into susceptible flocks or onto bodies of water previously free of virus contamination. Experimentally, DVE can be transmitted via oral, intranasal, intravenous, intraperitoneal, intramuscular, and cloacal routes. Horizontal spread is the principal mode of transmission, while vertical transmission has been reported in persistently infected waterfowl (15).

The course and direction of the infection are dependent on population density as well as rate of transmission between infected and susceptible birds. Recovered birds become carriers and shed the virus periodically (9). Like other herpesviruses, DEV latency and reactivation have been blamed for precipitating outbreaks in domestic and migrating waterfowl populations. The trigeminal ganglion, lymphoid tissues, and peripheral blood lymphocytes have been shown to be the latency site for the virus postinfection with DEV (62). According to the reports, the US2 protein of DEV plays an active role in penetrating the susceptible host cell and subsequent spread of virus from one cell to another cell in the host and facilitates the establishment of DEV infection in susceptible birds (76).

Incubation Period

In domestic ducks, the incubation period ranges from 3–7 days. After overt signs appear, death usually follows within 1–5 days.

Clinical Signs

In domestic breeder ducks, sudden, high, persistent flock mortality is often the first observation. Mature ducks die in good flesh. Prolapse of the penis may be evident in dead mature males. In laying flocks, a marked drop in egg production may be noted during the period of highest mortality.

As infection progresses within a flock, more signs are observed. Photophobia, associated with half-closed pasted eyelids, loss of appetite, extreme thirst, droopiness, ataxia, ruffled feathers, nasal discharge, soiled vents, and watery diarrhea appear. Affected ducks are unable to stand; they maintain a posture with drooping outstretched wings and head down suggesting weakness and depression. Sick ducks forced to move may show tremors of head, neck, and body. Young ducklings 2–7 weeks of age show dehydration, loss of weight, blue beaks, conjunctivitis, lacrimation, nasal exudate, and often a blood-stained vent.

Morbidity and Mortality

Total mortality in domestic ducks may range from 5%–100%. Because the birds showing clinical signs usually die, morbidity closely approaches mortality. Adult breeder ducks tend to experience higher mortality than young ducks. No differences in mortality rates are found in mallard and white Pekin ducks experimentally infected with DVE and *Riemerella anatipestifer*, indicating that these pathogens do not act synergistically (52). However, mallards immunosuppressed with cyclophosphamide and challenged with a sublethal dose of DVE may increase mortality rate (18). Secondary bacterial infections with *Pasteurella multocida*, *R. anatipestifer*, and *E. coli* are often seen in a natural outbreak of a low virulent strain in young ducklings as a result of the immunosuppressive effect of the virus (61).

Pathology

The specific pathologic response to DVE depends on species affected (41), age, sex, stage of infection, and virulence and intensity of virus exposure (43).

Gross

Lesions of DVE are associated with disseminated intravascular coagulopathy and necrotic degenerative changes in mucosa and submucosa of gastrointestinal tract in lymphoid and parenchymatous organs. These collective lesions, when present, are diagnostic of DVE.

Petechial, ecchymotic, or larger extravasations of blood may be found on or in the myocardium and other visceral organs and their supporting structures, including the mesentery and serous membranes. On the epicardium, especially within coronary grooves, closely packed petechiae give the surface a red "paintbrush" appearance (Figure 13.8). The latter lesion is observed more frequently in mature breeder ducks than in young ducklings. When heart chambers are exposed, endocardial mural and valvular hemorrhages also may be observed. Surfaces of liver, pancreas, intestine, lungs, and kidney may be covered with petechiae. In mature laying females, hemorrhages may be observed in deformed, discolored ovarian follicles, and massive hemorrhage from the ovary may fill the abdominal cavity. Lumina of intestines and gizzard are often filled with blood. The esophageal–proventricular sphincter appears as a hemorrhagic ring.

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Figure 13.8 Petechial hemorrhages in the epicardium and coronary heart fat of duck infected with duck virus enteritis (DVE) virus.



Figure 13.9 Petechial hemorrhages and ulceration of esophageal mucosa from duck infected with duck enteritis virus (DEV) virus. Note the lack of inflammatory response and presence of intranuclear inclusion bodies.

Specific digestive mucosal lesions are found in the oral cavity, esophagus, ceca, rectum, and cloaca. Each of these lesions undergoes progressive alterations during the course of the disease. Initially, macular surface hemorrhages appear, which are later covered by elevated, yellow-white crusty plaques. Subsequently, the lesion becomes organized into a green superficial scab devoid of its former hemorrhagic base. Lesions range in size from approximately 1–10 mm in length. In the esophagus and cloaca, lesions may become confluent; however, close inspection will often reveal their composite structure. In the esophagus, macules occur parallel to longitudinal folds. When macular concentrations are numerous, small lesions may merge to form larger ones covered with a patchy diphtheritic membrane (Figure 13.9). In young ducklings, individual lesions in the esophagus are less frequent; sloughing of the entire mucosa is more common, and the lumen becomes lined with a thick yellow-white membrane. Oral erosions can be found at

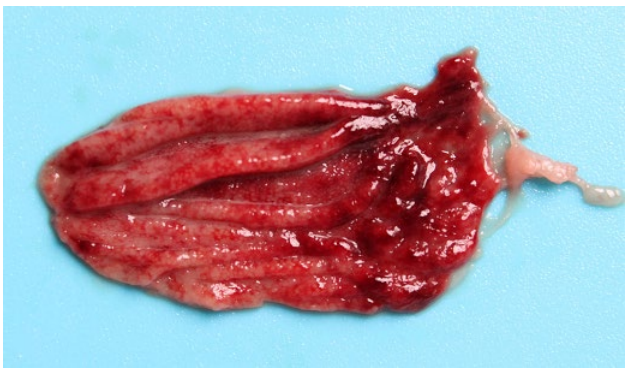


Figure 13.10 Extensive hemorrhages lesions of bursa of Fabricius with duck enteritis virus (DEV).

openings of sublingual salivary gland ducts in chronically infected waterfowl (6). Meckel's diverticulum may be hemorrhagic and contain a fibrinous core (57).

In ceca, macular lesions are singular, separated, and well-defined between mucosal folds. The external surface of affected ceca often presents a barred, congested appearance. Rectal lesions are usually few in number with greatest concentration at the posterior portion of the rectum, adjacent to the cloaca. In the cloaca, macular lesions are densely packed; initially, the entire mucosa appears reddened. Later, individual plaque-like elevations become green and form a continuous scale-like band lining the lumen of the organ.

All lymphoid organs are affected. The spleen tends to be normal or smaller in size, dark, and mottled. The bursa of Fabricius is intensely reddened during early infection (Figure 13.10). The exterior becomes surrounded by clear, yellow fluid that discolors adjacent tissue of the pelvic cavity. When the lumen of the bursa is opened, pinpoint yellow areas are found in an intensely hemorrhagic surface. Later, walls of the bursa become thin and dark, and the bursal lumen is filled with white coagulated exudates. Intestinal annular bands appear as intensely reddened rings visible from external and internal surfaces. Yellow pinpoint areas can be observed on the mucosal surface. Later, the entire band becomes dark brown and tends to separate at its margins from the mucosal surface. The multifocal necrosis of gut-associated lymphoid tissue causes ulceration covered by fibrinous pseudomembranes (Figure 13.11). The thymus is atrophied and has multiple petechiae (Figure 13.12) and necrotic focal areas on the surface and cut section and is surrounded by clear, yellow fluid that infiltrates and discolors subcutaneous tissues of the adjacent cervical region from the thoracic inlet to the upper third of the neck. The latter lesion is of importance in meat inspection and is easily detected when the opened neck of the carcass is observed on the processing line.

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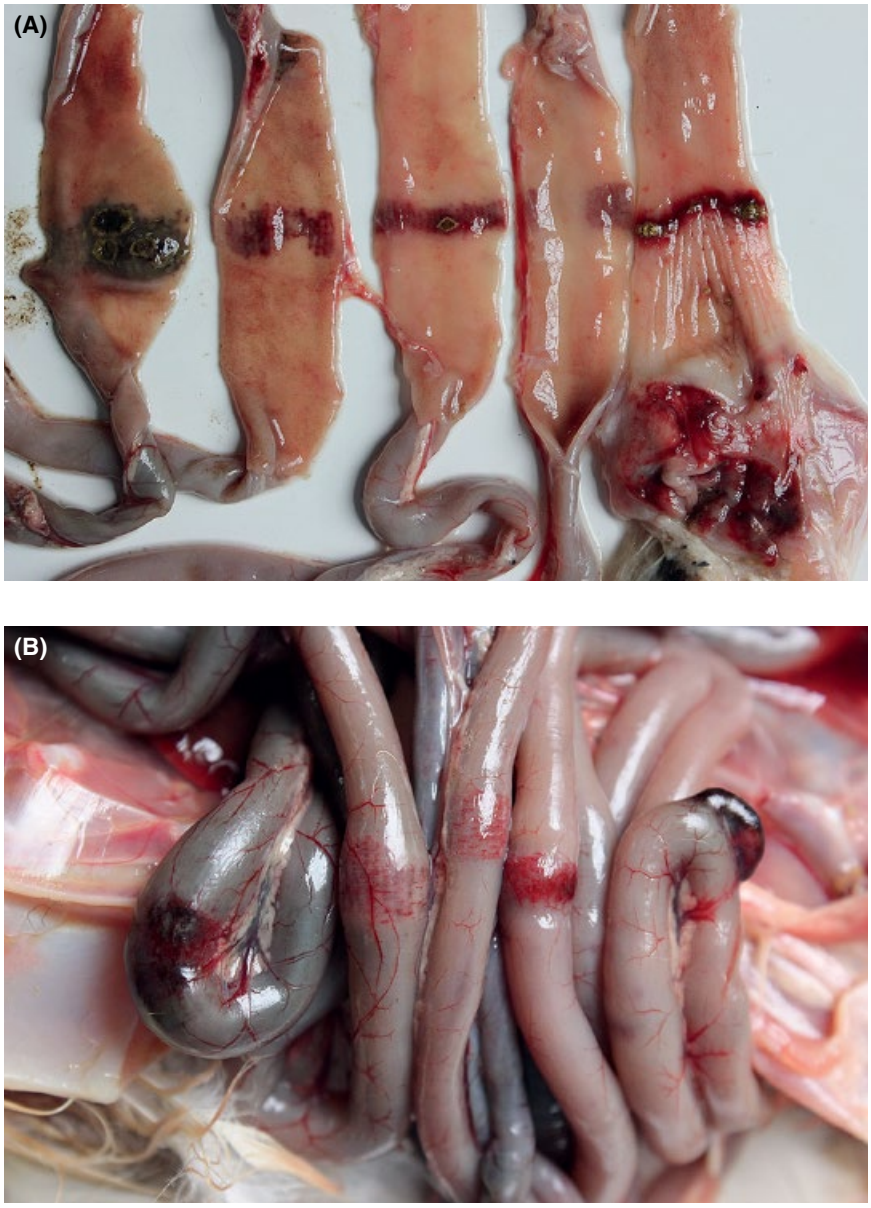


Figure 13.11 (A) Multifocal necrosis of gut-associated lymphoid tissue resulting in ulceration covered by fibrinous pseudomembranes of duck enteritis virus (DEV). (B) Also note the reddened ring visible on the external surface of the intestine.

During early stages of infection, the entire liver surface has a pale copper color with an admixture of irregularly distributed pinpoint hemorrhages and white foci (Figure 13.13), giving it a heterogeneous, speckled appearance. Late stages of infection are characterized by dark bronze or bile-stained livers without hemorrhages; the white foci are larger and appear more distinct on the darker background.

Although these lesions are consistent with DVE infection, each age group responds distinctively. In ducklings, tissue hemorrhages are less pronounced and lymphoid lesions are more prominent. In mature domestic ducks with naturally regressed cloacal bursa and thymus, tissue hemorrhages and reproductive tract lesions predominate.

In geese, intestinal lymphoid disks are analogous to annular bands in ducks. In a single Canada goose, lesions of the intestinal lymphoid disks resembled "button-like ulcers" (39). Similar intestinal lesions have been observed in an outbreak of DVE in Canada and Egyptian geese. In swans, diphtheritic esophagitis is a consistent lesion (37).

An outbreak caused by a low virulent strain of DVE in commercial 2- to 6-week-old white Pekin ducklings produced atypical gross lesions, including diphtheritic membranes under the tongue and in nasal and infraorbital sinuses. Esophageal mucosa had a few necrotic

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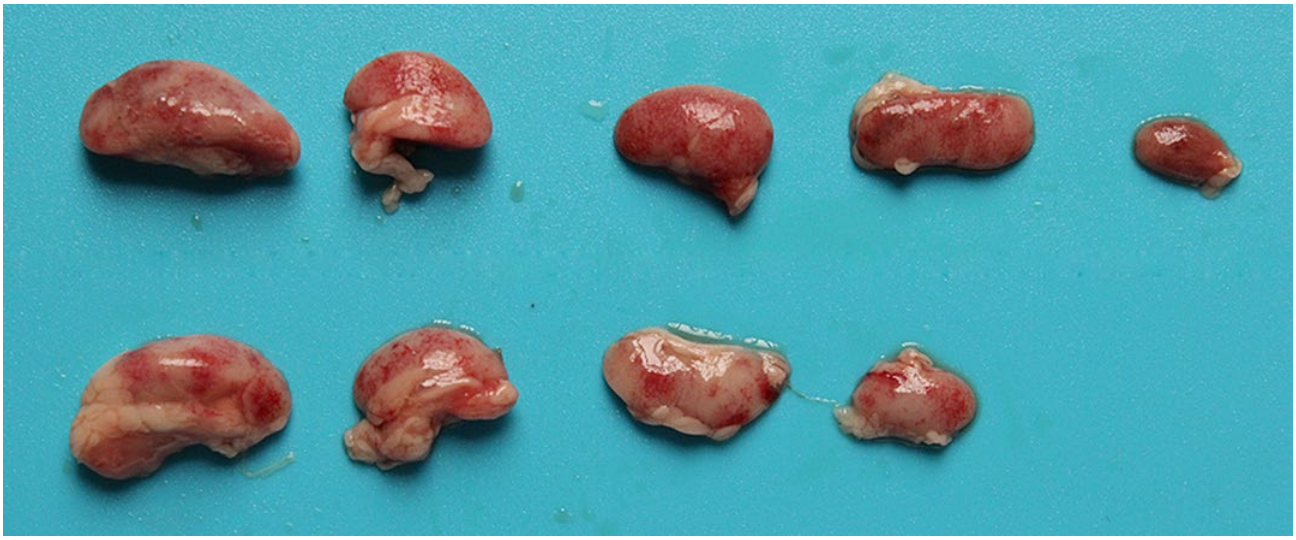


Figure 13.12 The thymus is atrophied and has multiple petechiae.



Figure 13.13 Multiple irregular pale foci in liver with duck virus enteritis (DVE). Microscopically, liver shows focal areas of necrosis filled with fibrin from duck virus enteritis (DEV).

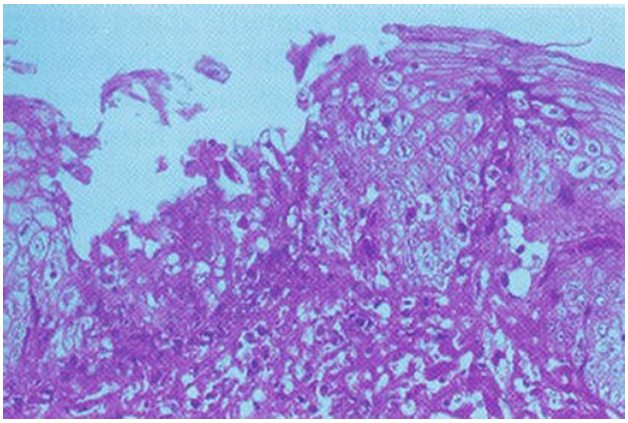


Figure 13.14 Microscopic appearance of esophageal ulcerations of duck virus enteritis (DVE). Note the lack of inflammatory response and presence of intranuclear inclusion bodies.

plaques, and cloacal mucosa was covered with necrotic, greenish, diphtheritic membranes. No lesions were seen in the intestines including annular bands. Thymus and bursa were atrophied and hemorrhagic, and the bursal lumen was filled with cheesy exudate. An experimental study showed that the bursal atrophy could last for at least 39 days PI; however, the thymus recovered after 10 days of infection (61).

Microscopic

The initial lesion occurs in the walls of blood vessels. Smaller blood vessels, venules, and capillaries, instead of larger blood vessels, are more markedly involved. The endothelial lining is disrupted, and connective tissue of the wall becomes less compact, with visible separations at points where extravasations of blood pass from the lumen through the thin ruptured wall into surrounding tissues.

Hemorrhages are especially pronounced in certain locations: interlobular venules of the proventriculus, hepatic, and portal venules at the margins of liver lobules; venules in the spaces between lung parabronchi; capillaries within intestinal villi; and star-shaped intralobular renal hemorrhages.

As a result of vascular damage, affected tissues undergo progressive degenerative changes. Microscopic changes can be found in any visceral organs including those without gross lesions.

Digestive lesions appear initially as hemorrhages of capillary arcades of submucosal papillae or folds. Hemorrhages become larger and confluent, elevating and separating the overlying mucosa. The affected epithelium above the hemorrhage becomes edematous, necrotic, and raised into the lumen above normal adjacent mucosal surfaces (Figure 13.14). Later, margins of

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necrotic epithelium separate to define the borders of elevated plaques. There is necrosis and degeneration of stratified squamous epithelium of the esophagus and cloaca (56). Eosinophilic intranuclear and cytoplasmic inclusions have been seen in epithelial cells (70).

Hemorrhage from venules and capillaries fills lymphoid tissue within intestinal annular bands or lymphoid disks and lymphoid tissue of the esophageal–proventricular sphincter and spleen. Lymphocytes undergo karyorrhexis and pyknosis. Fragments of lymphocytes appear everywhere and are engulfed by phagocytes. In addition to cellular debris and hemorrhage within lymphoid follicles, marked swelling of reticulum cells occurs, and their cytoplasm becomes subdivided and condensed into spherical and oval pale-staining bodies. Reticulum cells rupture and discharge their cytoplasmic contents into tissue spaces. An intranuclear inclusion body and delicate nuclear membrane and cell wall are the remaining vestiges of reticulum cells.

Intestinal lymphoid lesions become large hemorrhagic infarcts. A layer of free blood separates lymphoid tissue from the mucosa, which undergoes coagulation necrosis. The necrotic mucosa forms a pseudomembrane higher than adjacent normal intestinal mucosa.

In the small intestines, sheets of epithelial cells are displaced from the surface of villi, many of which are broken and cast into the lumen. Abundant blood and cellular debris fill the lumen.

Within the cloacal bursa, submucosal and interfollicular capillary hemorrhages are found. There is a severe depletion of lymphocytes in the follicles, many of which have empty hollow cavities in the medulla. Corticomedullary epithelial cells, capillary networks, and large phagocytic cells containing fragmented lymphocytes form the circumference around these cavities. Severe depletion of lymphocytes in the follicles occurs, which is replaced by eosinophilic material mixed with heterophils. There are occasional mononuclear cells that contain intranuclear inclusions. Bursal epithelial cells are hypertrophied with vacuolated cytoplasm and contain both intranuclear and intracytoplasmic inclusions (61).

In the thymus, free blood fills interfollicular spaces. Coagulation necrosis of central medullary reticulum cells and destruction of cortical lymphocytes are pronounced.

In mature female breeder ducks, congestive, hemorrhagic, and necrotic alterations occur in the oviduct. Follicles may be misshapen and blood stained. In the ovary of immature female breeder ducks, focal intestinal hemorrhages from capillaries and venules may be found.

In mature breeder drakes, focal capillary hemorrhages occur in interstitial tissues between seminiferous tubules. In parenchymatous organs such as liver, pancreas, and kidneys, hemorrhages and focal necrosis are found surrounding blood vessels.

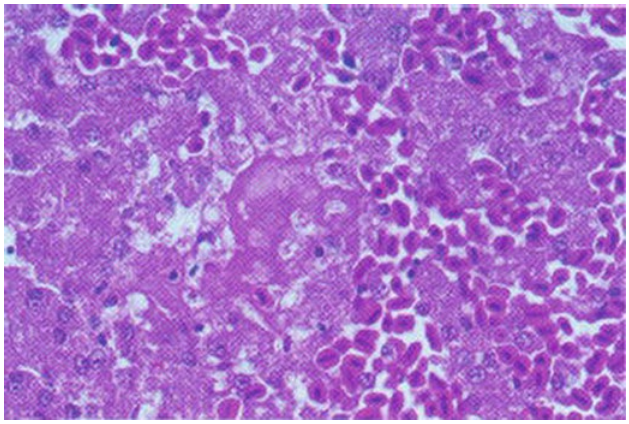


Figure 13.15 Microscopically, liver shows focal areas of necrosis filled with fibrin from duck virus enteritis (DVE). Intranuclear inclusion bodies can be seen in hepatocytes near areas of necrosis.

Within necrotic foci in the liver, hepatic cords show a variety of changes including detachment and disassociation of hepatocytes from each other and their surrounding structure. A few necrotic liver cells become swollen or subdivided and discharge their cytoplasmic contents through a ruptured cell surface and are represented only by intranuclear inclusion bodies. Focal areas of necrosis may be filled with fibrin (Figure 13.15). Similar, but more limited, changes occur in pancreas and kidney (40).

Immunity

Active Immunity

Active immunity has been demonstrated following the use of a modified live virus vaccine (27), inactivated tissue culture vaccine (63), and bivalent recombinant live attenuated vaccine (48, 75, 85). It is assumed that both humoral and cell-mediated immunity are involved in protection (73). Field observations suggest that recovered birds are immune to re-infection with DEV.

Passive Immunity

Maternal immunity has been reported in ducklings that declines fast and may interfere with response to live virus vaccines. Ducklings from those breeder ducks that are vaccinated with a live-attenuated vaccine are fully susceptible. However, ducklings from breeders that had been vaccinated and challenged with a virulent virus were found protected at 4 days of age, and less than 40% were protected at 13 days of age (72). In an experimental study (8), superinfection of persistently infected mallard ducks resulted in death, indicating that protection depends on the route of exposure, strain leading to persistent infection, and strain of superinfecting virus.

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Diagnosis

Isolation and Identification of DEV

Duck enteritis virus mainly attacks the immune organs. Samples recommended for virus isolation are liver, spleen, bursa, kidneys, peripheral blood lymphocytes (PBL), and cloacal swabs. Virus isolation is carried out by inoculation of susceptible 1-day-old Muscovy ducklings, white Pekin ducklings, or onto CAM of 9- to 14-day-old embryonating duck eggs. Characteristic lesions and mortality in inoculated ducklings are highly suggestive of DEV. Virus also can be isolated and propagated in white Pekin or Muscovy duck embryo fibroblasts, primary cell culture of liver and kidney, and Vero cell lines, but inoculation of infected tissue to susceptible ducklings is considered more sensitive than the virus isolation in cell culture. Although a presumptive diagnosis can be made on the basis of gross and histopathologic lesions, isolation and identification of DEV confirms the diagnosis even in the absence of typical lesions when coupled with serological tests and molecular diagnosis.

Serological Diagnosis

Virus neutralization assay is the most classical method for identification of DVE. Increase in virus neutralization (35) titers following convalescence from DEV will demonstrate progress of the disease within a flock. A VN index of 1.75 or more indicates infection with DEV (12). A VN index of 0–1.5 has been found in sera of domestic and wild waterfowl not exposed to the disease. The use of chicken embryo-adapted virus in chicken eggs for VN studies is safer and more convenient than the use of field-strain viruses inoculated onto the CAM of duck eggs (12).

Immunofluorescence tests can be used to detect viral antigens in cell cultures or tissue sections (65). Other serologic procedures for detecting antibodies include a microtiter plate isolation and neutralization test using duck embryo fibroblasts, a reverse passive hemagglutination test (14), agar gel immunodiffusion test, and ELISA (31, 77, 82). A Dot-ELISA and passive hemagglutination assays have been developed for detection of DEV antibodies; however, the specificity and sensitivity of these two assays were shown to be moderate (51). An immunochromatographic strip test has been developed for use as a field test for monitoring flock immunity post vaccination and detection of exposure to DEV in unvaccinated flocks. This test is easy to perform, rapid (15 minutes), specific, and with equal sensitivity as the antibody ELISA (64).

Molecular Diagnosis

Duck enteritis virus-specific DNA segments can be amplified by PCR from infected cell culture supernatant and tissues from esophagus, liver, kidney, and spleen. By comparison, qRT-PCR gives an idea regarding the load of DVE viral DNA in the body tissues of infected ducklings which can further be correlated with the dissemination of virus and progression of disease in different organs (58). In situ hybridization can detect the presence of DEV DNA through specific oligonucleotide probes in tissue sections of various organs and can provide information about localization of the viral DNA in case of quick diagnosis of the viral infection (11). With advances in designing diagnostic procedures, LAMP-based nucleic acid amplification methods for DEV detection has proven to be a rapid, simple, accurate, specific, and sensitive method for the diagnosis of DEV and has been considered to be a good choice for on-farm disease diagnosis (30).

Differential Diagnosis

Differential diagnosis requires consideration of other diseases producing hemorrhagic and necrotic lesions in Anseriformes. In domestic ducks, common diseases producing such changes are duck virus hepatitis, fowl cholera, necrotic enteritis, coccidiosis, and specific intoxications. Although Newcastle disease, fowl pox, and fowl plague are reported to produce similar changes in Anseriformes, these diseases have been infrequently reported.

Intervention Strategies

Management Procedures

Prevention is achieved by maintaining susceptible birds in environments free from exposure to the virus. These measures include the addition of stock known to be free from infection and avoiding direct and indirect contact with possibly contaminated material. Introduction of the disease by free-flying Anseriformes and contaminated aquatic environments must be prevented. All possible measures should be taken to prevent dissemination of virus by free-flowing water. After DEV has been introduced, control can be achieved by depopulation, removal of birds from the contaminated environments, sanitation, disinfection, and vaccination of all susceptible ducklings.

In countries where the disease is not enzootic and is truly exotic, effective quarantine of imported or clinically suspected Anseriformes should be done. Accordingly, surveillance of ornamental bird collections, zoos, and domestic growers of Anseriformes should be performed by using efficient detection assay of DEV in laboratory.

Vaccination and Types of Vaccines

Vaccination has been used as a preventive measure and also for controlling disease outbreaks. Currently, both

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live attenuated and inactivated vaccines are being used in broiler and breeder ducks that are over two weeks of age (84). Live attenuated vaccines are considered most effective against DEV hence it is of foremost importance to maintain the vaccine at optimum physical and physiological conditions of temperature, salt, and pH to prevent any loss in the activity of vaccine candidate molecule (50). The vaccinated ducks could excrete the virus thus demands revaccination of the entire flock (26). By comparison, the inactivated DEV vaccine is effective in protecting domestic and captive waterfowl from the virulent strain infection (63) without the risk of introducing a live virus.

DNA vaccine is a promising strategy for protection ducks against DEV infection. Vaccination with plasmids or carrier *E. coli* for expressing DEV gB/gD/gC/UL24/tgB genes induced potent cellular and humoral immunity against DEV in ducks (3, 44, 49, 87). However, their relatively low immunogenicity is an obstacle to their use.

Recent studies supported the use of attenuated DEV vaccine strain as an efficient vector for developing polyvalent live attenuated vaccine against high pathogenic avian influenza virus (AIV) strain H5N1 (47, 75) and H9N2 (69), Duck Hepatitis A virus type 1 and 3 (88), and duck tembusu virus (DTMUV) (10). This vaccine provided speedy immunological protection for long duration against delivering pathogens and DEV infection by a single dose inoculation. It also suggested DEV has the potential to be utilized as a promising viral vector candidate for developing vaccines for poultry and aquatic birds.

Field Vaccination Protocols and Regimes

The vaccine can be used in the face of an outbreak, because it provides protection immediately after vaccination due to an interference phenomenon (67). It should be noted, however, that birds in the period of incubation may not be protected. A naturally apathogenic and immunogenic strain of DVE was reported to be successful for active and passive immunization of ducks (45, 46).

Attenuated live virus vaccine is administered by subcutaneous or intramuscular routes in domestic ducklings more than two weeks of age. Normally, the breeding flocks are vaccinated. Flocks maintained for more than a year are revaccinated annually. Apparently, vaccinated ducklings do not excrete inoculated virus to a degree that would be sufficient to bring about contact immunization (28, 71).

Treatment

There is no specific treatment of DEV infection.

Hemorrhagic Nephritis Enteritis of Geese

Jean-Luc Guérin

Summary

Agent, Infection, and Disease. Hemorrhagic nephritis enteritis of Geese (HNEG) is one of the major viral diseases of geese. The causative agent is a polyomavirus, namely *Goose hemorrhagic polyomavirus* (GHPV). Goslings are susceptible up to 14 weeks of age, while ducks are healthy viral carriers. The main clinical signs are lameness and prostration, leading to death. Main necropsic signs are edema, ascites, and nephritis. In chronic forms of HNEG, visceral and/or articular gout is frequent.

Diagnosis. Mortality and lesions (edema, ascites, and nephritis) on goslings are suggestive of HNEG. Confirmation is routinely based on polymerase chain reaction (PCR).

Intervention. Prevention is based on biosecurity, including prevention of contact with ducks which may be healthy carriers of goose polyomavirus. Experimental vaccines have been developed but are not commercially available.

Introduction

Definition and Synonyms

Hemorrhagic nephritis enteritis of Geese (HNEG) is one of the major viral diseases of geese. Due to confusion with goose parvovirus infection HNEG had long been named "young geese disease" or "late form of Derzsy's disease". According to its etiology, a more relevant denomination should be "goose polyomavirosis." This systemic, frequently lethal disease is to date the only condition associated with a polyomavirus in a poultry species.

Public Health Significance

Polyomaviruses have a very narrow host range. This is supported by evidence of codivergence of mammalian and avian polyomaviruses with their respective hosts (7). Hemorrhagic nephritis enteritis of Geese is thought to have no public health implication.

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History

Hemorrhagic nephritis enteritis of Geese was first described in 1969 in Hungary (1) and was described a few years later in Germany, then in France (16). For many years, HNEG was suspected to correspond to a late evolution of Derzsy's disease. Etiology of HNEG was clarified in France in 2000 (6). More recently, it was shown that ducks are actually largely healthy carriers of the virus (3).

Etiology

Classification

The agent of HNEG, namely *Goose hemorrhagic polyomavirus* (GHPV), is a member of the Polyomaviridae family. It has been recently assigned into the *Gammapolyomavirus* genus with all other avian polyomaviruses (9). This virus is significantly divergent from *Budgerigar fledgling polyomavirus* (BFPV), the prototype avian polyomavirus infecting psittacines, falconiformes, and passerines (9).

Morphology

Virus particles are naked and spherical, and show icosahedral symmetry. Their size ranges from 40–50 nm in diameter (6). Buoyant density of virions is of 1.20 g/cm^{-3} in sucrose gradient (6), which corresponds to 1.34–1.35 g/cm^{-3} in CsCl.

Chemical Composition

The GHPV genome is a circular, double-stranded DNA of 5,256 base pairs (8). Genome organization of all polyomaviruses shares common features, with a set of early genes encoding polymerases (t and T antigens) and late genes, encoding structural proteins: VP1, the main capsid protein, and two other structural proteins, VP2 and VP3 (9). As for avian polyomaviruses, an additional VP4 has been demonstrated, the precise functions of which still remain to be clarified (8).

Virus Replication

Replication of GHPV occurs in the nucleus (6). Infected cells show a huge concentration of viral material in the nucleus, both in cultured cells and tissues of infected goslings Virus is easily detected in the nuclei by electron microscopy of immunofluorescence (Figure 13.16). Releasing of virions implicates disruption of cell membrane.

Susceptibility to Chemical and Physical Agents

Goose hemorrhagic polyomavirus shows a great resistance to heating: virus is still fully virulent even two hours after incubation at 55°C (6). The virus also resists freezing–thawing cycles and lipid solvents: treatment with 1% phenol has no effect on its viability. Avian polyomaviruses are mostly sensitive to chloride-derived products (15).

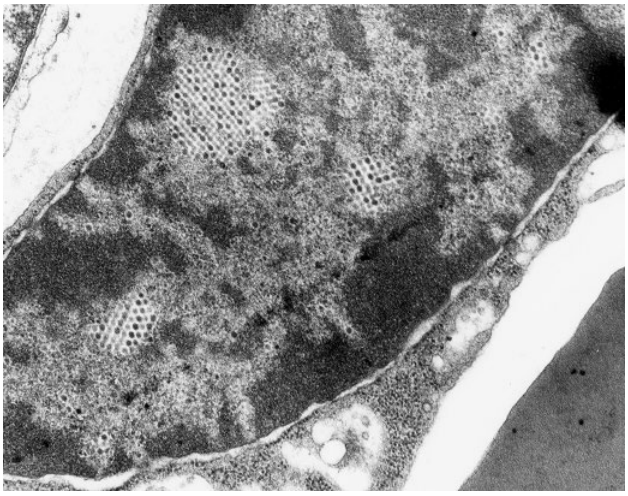


Figure 13.16 Electron micrograph of a goose hemorrhagic polyomavirus (GHPV)-infected cell. Notice many naked virions in the nucleus and peripheral accumulation of chromatin. ×25,000.

Strain Classification

Genetic variability among field isolates has been poorly investigated so far. Nevertheless, polyomavirus genomes are generally highly conserved and phylogenic analysis of GHPV confirmed that VP1 is remarkably conserved among isolates from different countries (14). Moreover, duck GHPV strains do not show distinctive genetic features compared to the goose strains (3). No cross-neutralization experiment has been performed so far on GHPV field isolates.

Laboratory Host System

Hemorrhagic nephritis enteritis of Geese is successfully reproduced by parenteral inoculation on 1-day-old goslings. Clinical end points are reached between 6 and 8 days postinoculation (PI), with peracute disease. Goslings are susceptible to inoculation either by subcutaneous or intraperitoneal routes. All attempts to adapt the HNEG virus to duck fibroblasts or embryos, as well as goose fibroblasts, remained unsuccessful (5, 6). Propagation of GHPV on goose embryos has been reported: 14-day-old goose embryos inoculated onto the chorioallantoic membrane (CAM) died from 8–10 days PI, with lesions similar to those described in goslings (1). Virus propagation can be accomplished by propagation on epithelial primary cells derived from 1-day-old gosling kidneys. Cytopathic effects appear by day 5 PI; granulations and

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vesicles are distinguished in the cytoplasm, followed by budding of the cell, and finally cell detachment from the monolayer (6). Cell-based dilution titration procedures are seldom done, because cytopathic effect appears late after infection. Alternatively, detection and quantification of virus yields from cell cultures or tissues can be determined by quantitative real-time PCR (3, 5, 12).

Pathobiology and Epizootiology

Incidence and Distribution

Until now, HNEG has been mostly described in Hungary, Germany, France, and Poland (2, 4, 6, 10, 14, 16). Occurrence of the disease in other countries seems likely, although there are no published reports confirming this. Cases are frequently observed in winter, probably due to climatic conditions or weakness of the goslings hatched from light-conditioned breeders.

Natural and Experimental Hosts

Hemorrhagic nephritis enteritis of Geese has only been described to date in growing geese. Subclinical infections have been reported in migrating wild geese (6). Other waterfowl species, such as mule or Muscovy ducklings, are clinically refractory to GHPV inoculation, but can be infected at high replication levels. Field surveys have suggested that ducks may constitute a significant subclinical reservoir of GHPV (3).

Transmission, Carriers, and Vectors

Infected geese and ducks excrete significant amounts of virus in their droppings, resulting in dissemination of contagious material to the environment and easy direct and indirect contamination. Vertical transmission of the virus through the egg has never been confirmed, but cannot be excluded. The experimental infection of goose embryos has been shown, although this does not formally demonstrate the field occurrence of vertical transmission (1). No biologic vector seems to be involved in GHPV transmission.

Incubation Period

The incubation period is mostly age dependent. Inoculation of day-old goslings results in death within 6–8 days (11). In contrast, in 3-week-old goslings, the incubation period could last for up to 15 days (14). After four weeks, inoculation results mostly in nonclinical infection. Although clinical signs rarely start before five or six weeks of age, contamination from infected birds could possibly occur early in life.

Clinical Signs, Morbidity, and Mortality

Hemorrhagic nephritis enteritis of Geese has been described in goslings from 4–14 weeks of age. In affected flocks, morbidity ranges from 10–80% and death is the most common outcome (6). Clinical signs develop rapidly only a few hours before death; birds sit alone, away from the flock, stay in a coma, and die (2, 10). Nervous signs, such as opisthotonos, are only observed after experimental or iatrogenic infections of goslings (11). Chronic evolution of the disease leads to urate deposits on viscera and in joints, resulting in lameness. In these late forms, mortality may be limited to a few birds every day up to the age of 12 weeks.

Pathology

Necropsic findings in goose include edema of subcutaneous connective tissues, gelatinous ascites, inflammation of the kidneys (Figure 13.17), and less frequently, hemorrhagic enteritis. Renal dysfunction leads to an increase of blood uric acid concentration. Geese that die after a chronic infection show visceral gout and deposition of urates in the joints (Figures 13.18 and 13.19) (10, 11, 16). Histopathologically, the most obvious features are: (1) an interstitial nephritis and necrosis of the kidney tubular epithelium, and (2) a moderate to severe lymphocytosis in cortical and medullary regions of bursal follicles, suggestive of B-lymphocyte depletion (6, 11). Gross lesions of enteritis are associated with necrosis of intestinal epithelium. Hemorrhagic foci also are observed in most tissues, particularly in acute infections (11, 14). No inclusion could be detected in tissues of birds diagnosed with HNEG (6, 11). Electron microscopy examination of infected tissues shows aggregated virions in nuclei (Figure 13.16) and large vesicles of dense material, including optically clear centers, in the cytoplasm of about 20% of the infected cells (6). In infected ducks, no macroscopic or microscopic lesion has been observed (3).



Figure 13.17 Gosling affected by hemorrhagic nephritis enteritis of geese (HNEG), showing edema and swelling of kidneys.

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Figure 13.18 Chronic form of hemorrhagic nephritis enteritis of geese (HNEG), visceral gout with deposition of urates.



Figure 13.19 Chronic form of hemorrhagic nephritis enteritis of geese (HNEG), urates deposition in the tibio-metatarsal joints.

Pathogenesis of the Infectious Process

During the course of infection, GHPV seems to replicate first in endothelial cells; nuclear enlargement of endothelial cells and arteriolitis are the first lesions noticed (11, 14). These histological findings suggest a selective tropism for endothelial cells, which might be of great relevance in pathogenesis of HNEG. Endothelial cells are indeed known to play a critical role in many biological pathways, resulting in vascular dysfunctions as ascites or edema. Another main target of GHPV is lymphoid cells; virions are observed in many bursal lymphoid cells, and cloacal bursa (bursa of Fabricius) systematically shows significant lympholysis. However, thymic lymphoid cells are less or not affected. This feature is fairly relevant with the well documented tropism of polyomaviruses to B-lymphocytes, suggesting immunodepressive effects in subclinical infections (7).

Immunity

Immunological aspects of HNEG have so far received little attention. Neutralizing antibodies are detected in previously infected birds and their transmission to the progeny seems very efficient, because goslings hatched from infected breeders are refractory to experimental infections with huge viral load (5, 17). The duration of maternal immunity has not been fully determined, but serological monitoring assays on goslings from vaccinated breeders suggest a complete disappearance of maternal antibodies within three weeks of life (5).

Diagnosis

Isolation and Identification of Causative Agent

Goose hemorrhagic polyomavirus can be detected in clinical material from geese showing clinical disease, as well as from nonclinical carrier birds. Isolation could be based on either kidney cell culture (6) or goose embryo inoculation (1), but these methods are time consuming and can hardly be applied to routine diagnosis of HNEG. Detection of the GHPV genome is therefore a more reliable way to detect the virus; end-point or real-time PCR detection of DNA extracted from infected tissues (liver, spleen, kidney) with primers designed on VP1 gene is efficient and reliable (6, 12). In subclinically infected carriers, PCR assays can be advantageously performed on blood samples, spleen, or cloacal swabs (12). A loop-mediated isothermal amplification (LAMP) assay allows

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a rapid molecular diagnosis (18). Serology appears of limited value to detect infection by a polyomavirus, because serologic response is greatly variable, while virus may persist in infected birds for months, if not years (12, 14). ELISA has been developed using viral antigens (5) or recombinant VP1.

Differential Diagnosis

Lesions of ascites, subcutaneous edema, visceral urate deposition, and nephritis in 4- to 10-week-old goslings are all suggestive of HNEG. However, similar lesions also may be associated with goose parvovirus infections. Histopathological, virological, or serological procedures may be helpful in confirming the etiology. Hemorrhagic nephritis enteritis of Geese is probably underdiagnosed because of the existing confusion with Derzsy's disease.

Intervention Strategies

Management Procedures

Goose polyomavirus spreads from carriers and clinically affected birds, mostly by the fecal route (6, 11). Disinfection procedures should be thoroughly observed; that is, complete removal of organic material, followed by the use of an appropriate disinfectant, is required to prevent or interrupt a disease outbreak. Chloride derived products are considered efficient to inactivate polyomaviruses, but are particularly sensitive to the presence of organic debris (15). Because infected birds have a viremia, needles used for administration of vaccines should be sterilized between uses. Though transmission of HNEG virus through the egg is not clarified, biosecurity practices should be respected in the hatchery to limit potential early contamination of goslings before they reach the farm. When goslings are infected by the virus, occurrence of clinical signs may greatly depend on management factors such as cold and/or stress. Oil-adjuvant vaccines should be administered with extreme caution to flocks affected by the disease. Because ducks can be healthy carriers of GHPV, mixed goose and duck farming systems should be carefully reconsidered and biosecurity between these species enforced (3).

Vaccination

Management procedures are unlikely to be sufficient for the control of HNEG infection. Vaccination of breeders could be used to provide maternal immunity to goslings when they are critically sensitive to virus contamination (5). An inactivated vaccine, based on viral antigen produced by propagation on goose kidney cells, inactivated with β-propiolactone, and adjuvanted with carbopol, has been the subject of a trial on breeding geese and induced maternal antibodies, providing protection to goslings against a viral challenge (5). This vaccine, when administered to growing goslings, induced a significant serological response for several weeks.

A rational vaccination schedule could rely on: (1) administration to breeders before each laying period and (2) vaccination of growing goslings to induce an active immunity covering the whole economic life of birds.

A subunit vaccine, based on recombinant VP1 protein produced in *E. coli*, also induced protection in goslings against a viral challenge, confirming that VP1 is the major antigenic determinant of polyomaviruses and can be expressed in subunit or vectored vaccines (13).

Treatment

There is no effective treatment. Technical management of an infected flock should be adapted to prevent stress; this may be helpful in preventing nonclinically infected birds from developing HNEG.

Acknowledgement

Grateful acknowledgment is made to Dr. Richard E, Gough, the author of previous chapters, for his contributions included in this text.

Parvovirus Infections of Waterfowl

Vilmos J. Palya

Summary

Agent, Infection, and Disease. *Anseriform dependoparvovirus 1* can cause a highly contagious fatal disease of young geese and Muscovy ducks, and less severe chronic disease in mule and Pekin ducks. Severity of the disease decreases with age. The chronic form is characterized by growth retardation and loss of feathers. The virus is transmitted both vertically and horizontally. All geographical areas of the world with intensive waterfowl production are affected with the disease.

Diagnosis. The preferred diagnostic test is molecular detection of the agent by polymerase chain reaction (PCR) and virus isolation. Antibodies can be detected by

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virus neutralization or enzyme-linked immunosorbent assay (ELISA).

Intervention. Vaccination of breeders with live and/or inactivated vaccines provides passive immunity to the progeny to prevent severe disease in young birds. Vaccination of progeny provides life long immunity.

Introduction

Definition and Synonyms

Waterfowl parvoviruses can cause different disease conditions depending on the species and the age of affected waterfowl. The highly contagious fatal disease affecting young geese and Muscovy ducks (*Cairina moschata)*, caused by goose parvovirus (GPV) was described under different names depending on the major clinical–pathological features that were observed (41). To eliminate the discrepancies in nomenclature, the World Poultry Association in 1974 agreed to name the disease in geese as Derzsy's disease to acknowledge the pioneering work of the Hungarian researcher Domokos Derzsy. The disease caused by the duck parvovirus (DPV) in Muscovy duck is often referred to as parvoviruses of Muscovy ducks. A syndrome associated with parvovirus infection causing short beak and dwarfism syndrome (SBDS), also called as beak atrophy and dwarfism syndrome (BADS), in Muscovy, mule, and Pekin ducks was also described (36, 39, 44). It has been shown that waterfowl parvoviruses can be divided into GPV and DPV species, the latter frequently referred to as Muscovy duck parvovirus (MDPV) (30, 68). Most recent classification assigned both GPV and DPV into a single species, namely *Anseriform dependoparvovirus 1* (9).

Economic Significance

In countries where intensive farming of geese and ducks is practiced, the disease has important economic significance. Introduction of vaccination has greatly reduced the impact of the disease.

Public Health Significance

There is no known public health risk associated with infection of waterfowl parvoviruses.

History

In the late 1950s and early 1960s a highly contagious and fatal disease of young goslings was reported in China and several European countries where intensive goose farming was present (41). A few decades later the occurrence of a new waterfowl parvovirus strain, named Muscovy duck parvovirus (MDPV or DPV), was reported in France and Taiwan (41). Short beak and dwarfism syndrome was first reported in mule and Muscovy ducks in France and Poland in the early 1970s and later in mule, Muscovy, and Pekin ducks in Taiwan (36, 41, 44). In recent years a similar disease affecting Cherry Valley and mule ducks has been observed frequently in China (67).

Etiology

In early reports describing the disease several etiological agents have been proposed, including bacteria, reoviruses, and adenoviruses as they were frequently isolated or detected from diseased goslings (41). In subsequent studies, however, it has been confirmed that the etiological agent is a parvovirus (27, 41).

Classification

According to the International Committee on Taxonomy of Viruses (ICTV), waterfowl parvoviruses are classified as autonomous members of the *Dependovirus* genus in the Parvoviridae family. All GPV and DPV belong to a single species, *Anseriform dependoparvovirus 1* (9).

Morphology

Waterfowl parvoviruses have small nonenveloped virions with icosahedral symmetry (Figure 13.20) and a diameter of 20–22 nm. The density of the virus in cesium chloride is approximately 1.38 g/mL (15).

Chemical Composition

The single-stranded DNA genome of GPV comprises of 5,106 nucleotides, and DPV is 5,132 nucleotides in length including the inverted terminal repeats with U-shaped hairpin structures at both ends. Both plus- and minus-strand genomes are encapsidated into the virions. The genome contains two large open reading frames (ORF). The 5' open reading frame encodes the non-structural NS1 (or Rep1) protein as well as several smaller proteins generated after mRNA splicing (33). The 3' open reading frame encodes three related proteins referred to as VP1, VP2, and VP3. These proteins are generated from the use of different initiation codons and through proteolytic cleavage (41, 46). These three capsid proteins constitute the icosahedral capsid in a ratio of approximately 1:1:8 (17).

Virus Replication

The replication of GPV and DPV, similar to that of other parvoviruses, occurs in the nucleus of host cells by using

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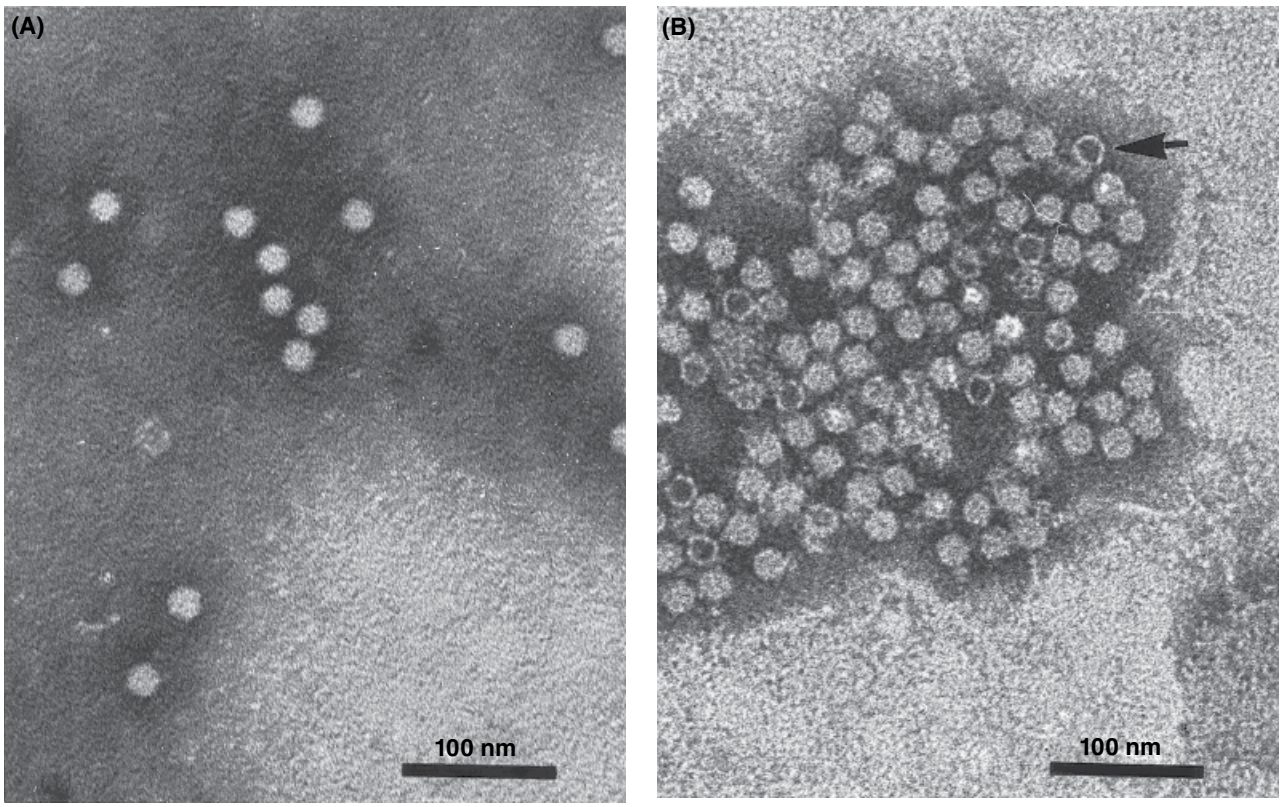


Figure 13.20 Electron micrograph of purified goose parvovirus. (A) Purified virions. (B) Virions in the feces of a naturally infected 10-day-old gosling showing intact and hollow particles (arrow).

the apparatus of the cells without the presence of helper virus (1, 10). Parvoviruses in general can only replicate productively in actively mitotic host cell populations. Accordingly, pathogenic or lethal infections typically occur in fetal or neonatal hosts, which have many dividing cell populations, or involve adult tissues that remain actively dividing in later life (10). The expression strategy of goose parvovirus exhibits features of both the *Dependovirus* and *Parvovirus* genera (33, 46).

Susceptibility to Chemical and Physical Agents

Goose parvovirus is very resistant to chemical and physical inactivation. The virus is stable at 65°C for 30 minutes and at pH 3.0 for 1 hour at 37°C. However, treatment with 0.5% formaldehyde can destroy the infectivity of the virus (41).

Strain Classification

Antigenicity

Using cross-neutralization tests, it was demonstrated that GPV differs antigenically from DPV (23). However, because of the high level of amino acid sequence identity between GPV and DPV, there is at least one NS1 epitope and three VP1 epitopes that might cross-react between GPV and DPV and cause a certain level of cross-immunity between them (34).

Genetic Characteristics

Phylogenetic analysis based on the structural proteins verified the existence of two monophyletic groups of waterfowl parvoviruses, the genetic linages of GPV and DPV (41). Molecular analysis of the genomes of GPV and DPV identified approximately 80% (2, 7, 68) nucleotide sequence homology between the two linages. The nucleotide sequences of GPV and DPV viral capsid genes share 77% similarity at DNA, and 84.6% at amino acids level. The most variable region resides in the N-terminal of VP2 before the initiation codon of VP3 with 35% amino acids divergence between GPV and DPV, causing major and minor changes on surface-exposed residues of VP3 (7).

Among the GPV strains, separate monophyletic subgroups can be differentiated based on the geographical origin and pathogenic nature of the strain (57) (Figure 13.21). A DPV strain belonging to another branch was isolated in the United States, showing only 85% identity with both GPV and DPV strains (45). Phylogenetic analysis of certain duck parvovirus strains circulating in Muscovy duck flocks suggest that they are recombinant of DPV and GPV (50, 69). Strains isolated from SBDS cases belong to a distinct linage of GPV (44).

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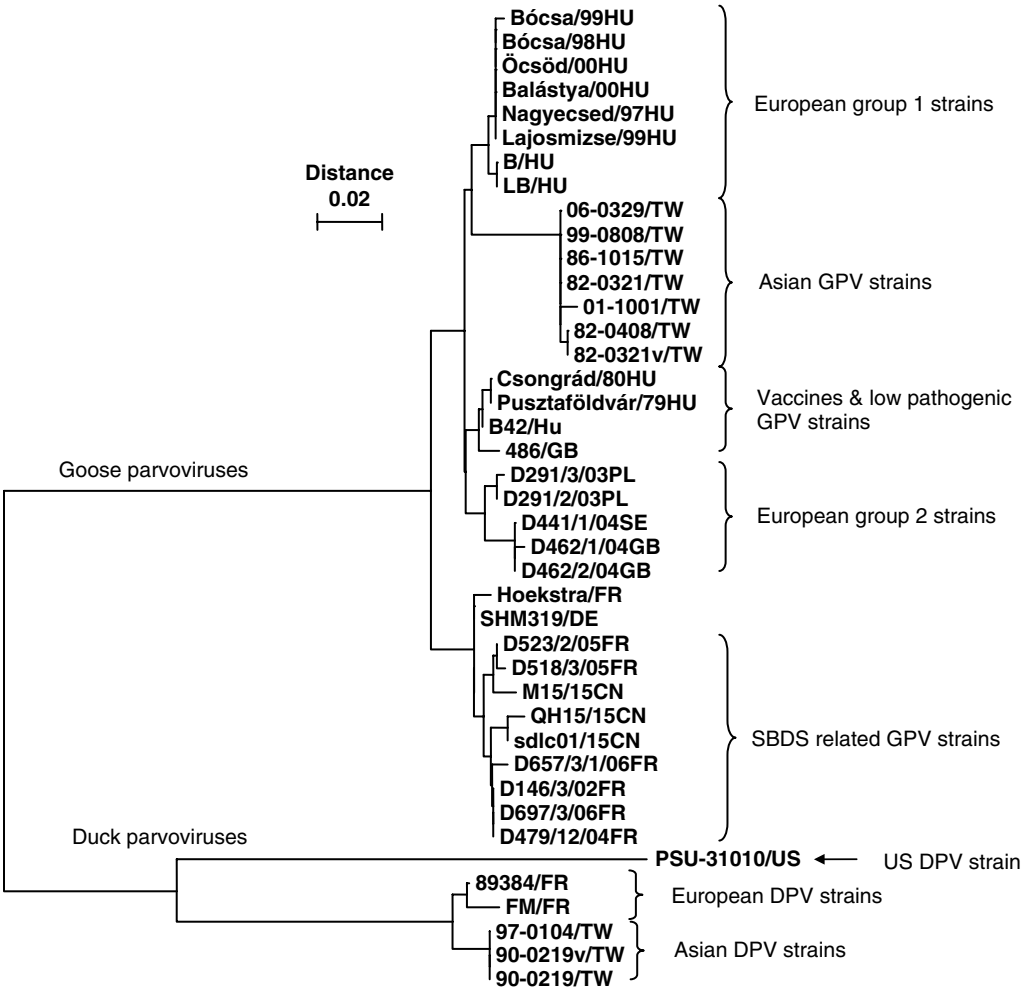


Figure 13.21 Phylogenetic relationship of goose and duck parvoviruses. The tree is based on a 493 bp region of the VP1 protein. GenBank accession numbers of the strains included: Bócsa/99HU (AY496898), Bócsa/98HU, Öcsöd/00HU, Balástya/00HU, Nagyecsed/97HU, and Lajosmizse/99HU had the same accession number (AY496897), B/HU (U25749), LB/69HU (AY496900), 06-0329 (EU583391), 99-0808 (AY382888), 86-1015 (AY382887), 82-0321 (AY382884), 01-1001 (AY382889), 82-0408 (AY382886), 82-0321v (AY382885), GPV486/GB (AY496904), B42 (AY496901), Csongrád/80HU and Pusztaföldvár/79HU had the same accession number (AY496903), D291/2/03PL (DQ862009), D291/3/03PL (DQ862010), D441/1/04SE (DQ862008), D462/1/04GB (DQ86011), D462/2/04GB (DQ862012), SHM319/DE (U34761), Hoekstra/FR (AY496907), D146/3/02FR (AY496906), D479/12/04FR (EU938702), D518/3/05FR (EU938703), D523/2/05/FR (EU938704), D657/3/06FR (EU938705), D697/3/06FR (EU938706), PSU-31010 (DQ413026), 89384 (Z68272), FM (U22967), 97-0104 (AY382893), 90-0219 (AY382892), 90-0219v (AY382890), sdlc01 (KT343253), QH15 (KT751090) and M15 (KU844283).

Compared with the full-length genomes of other classical goose and duck parvovirus field isolates, the inverted terminal repeat of GPV causing the SBDS contains several fragments. These characteristics are similar to those of certain attenuated GPV vaccine strain (4).

Pathogenicity

The two linages of waterfowl parvoviruses differ from each other regarding their pathogenicity and host range. Goose parvovirus can cause disease both in goslings and ducklings, whereas the antigenically distinct DPV causes disease only in Muscovy ducks (13, 23). Normally, Cherry Valley ducks and mule ducks are resistant to classical goose parvovirus infection. However, a distinct GPV-related parvovirus, isolated from mule and Cherry Valley duck, could produce typical symptoms of SBDS, with high morbidity and low mortality rates (6, 32, 44).

Laboratory Host Systems

Goose parvovirus and DPV can be propagated in embryonating goose or Muscovy duck eggs or young susceptible birds of the same species and primary cell cultures prepared from the embryos of these species. After a certain level of adaptation the virus can be grown in Pekin duck embryos as well (20). Certain strains of GPV and MDPV could be propagated in continuous cell lines from Muscovy ducks (24).

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Pathobiology and Epizootiology

Incidence and Distribution

Waterfowl parvoviruses have been reported from all major goose and Muscovy duck farming countries of Europe and Asia (41). There have been reports of DPV outbreaks in Muscovy ducks in the United States (45, 62) as well. Short beak and dwarfism syndrome in mule and Pekin ducks has been observed in France, Poland, Taiwan, and similar disease was described in meat type Pekin ducks in China (3, 6, 36, 41, 67).

Natural and Experimental Hosts

All breeds of domestic geese, Muscovy, mule, and Pekin ducks, and some other members of the Anatidae family are the only species in which clinical disease has been observed (41). Muscovy, mule, Tsaiya, and Pekin ducks are susceptible to GPV strains causing SBDS (4), and a genetically closely related GPV was detected in other duck species in Poland (mandarin, wood, falcated, and silver teal ducks) (49). The embryo-adapted GPV given by parenteral route can also infect Pekin ducks. Other breeds of domestic poultry appear refractory to experimental infection with GPV (20).

In a serological survey (19), some species of wild geese also tested positive for neutralizing antibody to GPV. Disease caused by GPV has been reported in Canada geese (*Branta canadensis*) and snow geese (*Chen hypoborea atlantica*) as a consequence of accidental infection (47).

Age of Host Commonly Affected

The diseases caused by waterfowl parvoviruses are strictly age dependent. Losses decrease with age, reaching a negligible level when infection occurs in birds older than five to six weeks (41). Although older birds do not show clinical signs of infection, they respond immunologically (29).

Transmission, Carriers, and Vectors

Infection of birds may occur both by vertical and horizontal routes. The most serious outbreaks occur in susceptible goslings following vertical transmission of the virus. In horizontal transmission, ingestion of virus-contaminated feed and water is of prime importance. Goose parvovirus and DPV are similar in that the two viruses re-excreted in large amounts in the feces of infected waterfowl, subsequently spreading rapidly to susceptible birds by both direct and indirect routes (41).

Due to its high resistance to extreme physical and chemical effects (heat, cold, dryness, disinfectants), the virus can remain infectious for long periods in the contaminated environment of animal houses. Birds that survive infection or those that are subclinically infected frequently become carriers and transmit the virus both horizontally and vertically. Latently infected breeders play an important role in the transmission of virus through their eggs to susceptible goslings or ducklings in the hatchery (5, 12, 41, 48). Duck parvovirus might cause asymptomatic infection in geese and they can shed virus from cloaca for one to four weeks post-inoculation, therefore geese can be a source for infection of DPV (66).

Wild geese visiting the same pasture or water pond as domestic geese and Muscovy ducks could also be a source of infection and may play a role in the introduction of the infection to a disease-free country or flocks (22). No biological vectors have been identified.

Incubation Period

Infection of susceptible birds during the first week of life results in the appearance of clinical signs within 3–5 days. In 2- to 3-week-old birds, the incubation period may vary between 5 and 10 days (13, 20, 48). In general the older the bird at infection, the more time passes between infection and the appearance of clinical signs. In mule and Pekin ducks the incubation period is considerably longer. Following infection of one-day-old ducklings, it can take 2–3 weeks before the characteristic signs of SBDS can be seen (4, 44).

Clinical Signs

Depending on the age and the immune status of goslings and Muscovy ducklings when GPV infection occurs, the disease may be present either in acute, subacute, or chronic forms, representing multiple pathological features of the disease (41). In the acute form, the course of the disease is usually rapid. The birds develop signs of illness with anorexia, polydipsia, weakness, watery diarrhea, and prostration, followed by death within a few days. Many birds show nasal and ocular discharge, conjunctivitis, profuse white diarrhea, and loss of natal down (Figure 13.22). Some of these birds have mucosal necrosis and a fibrinous pseudomembrane covering the tongue and oral cavity. In birds infected at an older age, between one and three weeks, the course of the disease can be either subacute or a more prolonged. In birds that survive the acute phase of the disease and in those that develop the chronic form of the disease, as a consequence of infection at an older age, the birds lose their feathers, especially on the back, neck, and wing, and have profound growth retardation (Figure 13.23). Deaths may occur between the third and tenth weeks of age (11).

Duck parvovirus affects only Muscovy ducks and the course of disease is similar the one of Derzsy's disease in geese (13). In Muscovy ducks, locomotor problems

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including weakness, lateral recumbency, and inability to walk are usually evident.

In SBDS parvovirus-infected duck flocks the disease is characterized with high morbidity and low mortality. The disease mostly affects young mule and Cherry Valley ducklings, characterized by a notably shortened beak and leg bones, protruded swollen tongue, stunted growth, fractured feathers (Figure 13.24). The pathogenicity of SBDS parvovirus is lower than that of classical waterfowl parvoviruses (4, 6, 44).

Morbidity and Mortality

Infection in the hatcheries or at less than one week of age can result in 100% mortality in susceptible goslings and ducklings (41). Infection of susceptible birds during the first 2–3 weeks of life still results in almost 100% morbidity and high mortality (10–60%). Resistance against the disease increases with age; infection later than 3–4 weeks of age usually remains subclinical (44). However, in birds with impaired immune systems, as a consequence of concomitant infection with immunosuppressive viruses (e.g., reovirus, circovirus) or due to nutritional (mycotoxins) and environmental factors the age of susceptibility may extend leading to the "late form" of the disease, causing significant economic losses



Figure 13.22 Gosling infected with goose parvovirus (GPV) showing weakness, prostration and loss of natal down.



Figure 13.23 Derzsy's disease. Gosling that survived the acute phase of the disease showing growth retardation and loss of feathers on the back and neck (natural case).

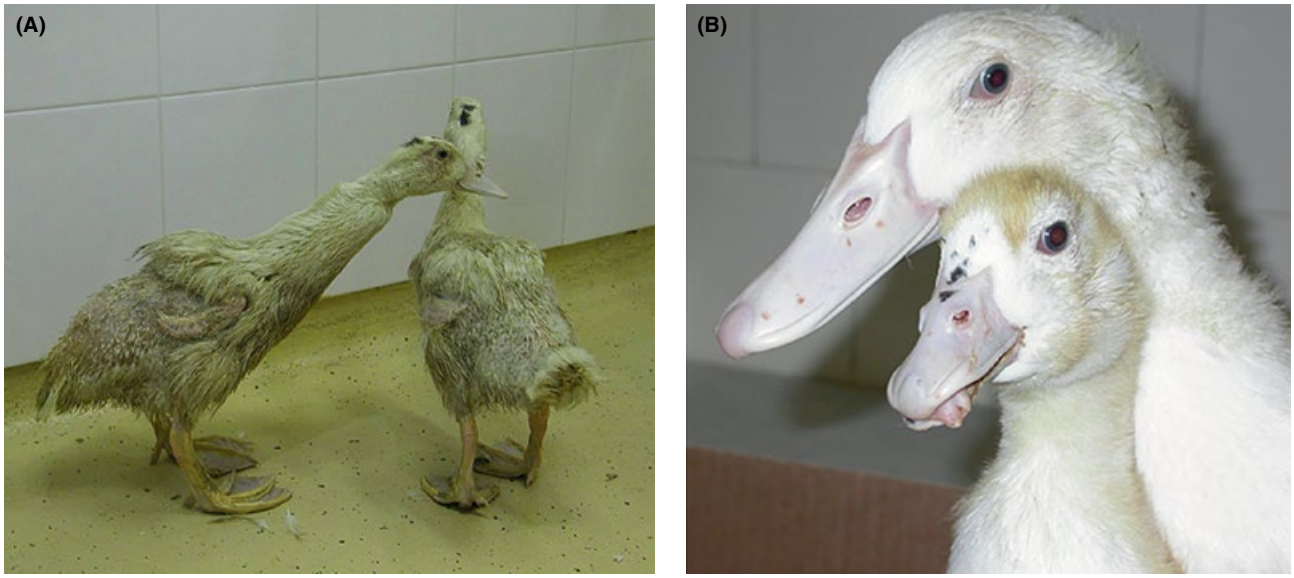


Figure 13.24 Short beak and dwarfism syndrome (SBDS) in Mule duck infected with goose parvovirus (GPV) at one day of age (experimental infection). (A) Growth retardation, shortening of tibia and fractured feathers on the back. (B) Short beak and protruded tongue. For comparison, an uninfected duck of the same age is shown next to the diseased one.

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up to 6–10 weeks of age. The presence of maternally-derived antibodies can considerably influence the course of the disease and consequently the rate of morbidity and mortality (41).

In SBDS cases, typical clinical signs usually appeared in 15-day-old ducks, and population of diseased ducks is continually growing with age until the day of slaughter. The feed conversion ratio (FCR) of infected ducks is worse than that of healthy ducks (4).

Pathology

Gross Lesions

In acute cases, the lesions considered characteristic in geese include enlarged and congested liver with fibrinous membrane on the surface (Figure 13.25B), pale myocardium, and dilatation of the heart. Typically, a serofibrinous perihepatitis with large volumes of straw-colored fluid in the abdominal cavity and pale dilated heart (Figure 13.25A) are present (41). In the acute enteric form of the disease, severe necrotic enteritis with diphtheritic lesion in the small intestine (Figure 13.26) is the predominant postmortem finding (21). Diphtheritic and ulcerative lesions may be observed in the mouth and pharynx as well.

In Muscovy ducks, the gross pathological lesions include pale thigh and heart muscles, increased pericardial fluid, serofibrinous perihepatitis, and ascites. In prolonged cases, the birds become stunted, have chronic congestion of liver, and ascites (13, 23).

Usually no gross lesions of the internal organs can be seen in SBDS. In a few cases, when the bird dies at a very young age following infection, fibrinous perihepatitis, hydropericardium, and ascites are observed (44).

Microscopic Lesions

The specific lesions observed in the affected geese and ducks include myopathy of skeletal muscle, hepatitis, myocarditis, sciatic neuritis, and polioencephalomyelitis. Other commonly observed lesions include atrophy of lymphoid organs (bursa of Fabricius, spleen, and thymus) (13, 55).

Both GPV and DPV infections produce extensive degenerative changes of myocardial cells, loss of striation of myocardial fibers, and the presence of scattered Cowdry type-A intranuclear inclusions. In liver, the predominant lesions are vacuolic degeneration of hepatocytes (Figure 13.27), multifocal single-cell necrosis, and occasional eosinophilic intranuclear inclusion bodies. In the case of the predominantly enteric form of the disease, necrosis of intestinal epithelium, especially the crypt-lining epithelium, can be seen in the duodenum and proximal jejunum. Intranuclear inclusions may be found in the damaged epithelium cells. In Muscovy ducklings, degeneration and necrosis of muscle fiber with lympho-histiocytic infiltration, together with mild sciatic neuritis and polioencephalomyelitis (Figure 13.28) are frequent findings (13, 62). The pathological features, however, vary depending on the clinical course of the disease. The main histological lesions in SBDS include

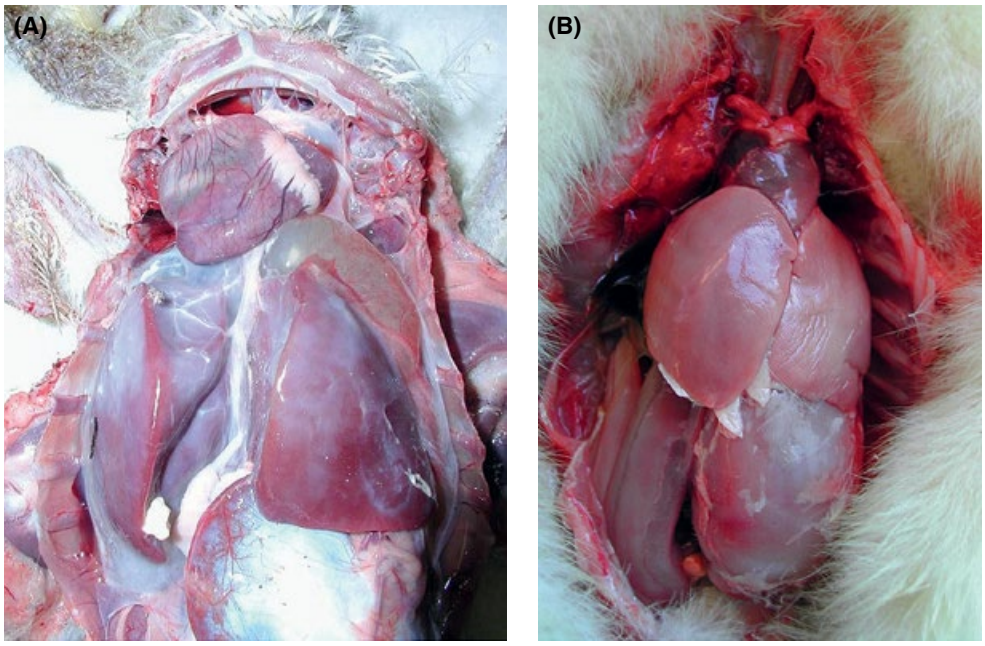


Figure 13.25 Derzsy's disease. Gross pathology of gosling got infected during the first week of life. (A) Enlarged and congested liver with fibrinous exudate on the surface, ascites, pale myocardium, and dilatation of heart (natural case). (B) Fibrinous membrane on the surface of liver.

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hemorrhage of the thymus, calcification of the tip of the tongue and degeneration of the liver, whereas, no other obvious lesions could be observed in other organs or tissues.

Pathogenesis of the Infection Process

Detailed studies on the pathogenesis of waterfowl parvovirus are not available. The most accepted theory is that following infection, replication occurs in the intestinal wall. The virus enters the bloodstream and reaches secondary target organs, including the liver and heart, where the most severe pathological changes occur (28). Another possibility is that the virus enters the bloodstream through the nasopharyngeal lymphoid tissues and reaches the secondary target organs more rapidly (26). This latter theory is supported by recent data obtained by quantification of GPV in different organs of experimentally infected goslings. Goose parvovirus was first detected at 4 hours postinfection in blood, heart, liver, spleen, kidney, and lymphoid organs, but not in any part of the gastrointestinal and respiratory system. From 8 hours to 9 days postinfection, the virus was detected in all organs tested, with the highest amount in the liver, spleen, kidney, and lymphoid organs (65). The level of GPV and MDPV replication and distribution plays a significant role in the parvoviral infection progress and is strictly correlated to clinical symptoms. Analysis of quantitative real-time PCR results revealed correlation

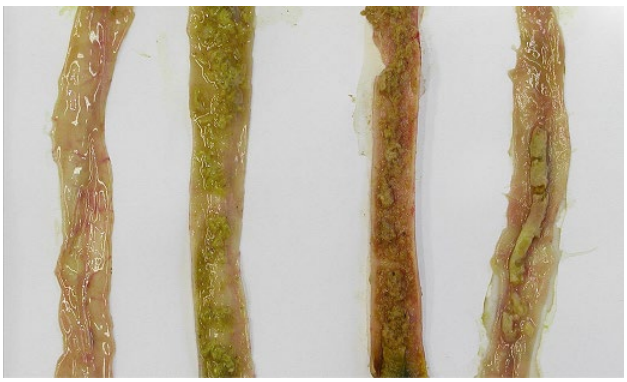


Figure 13.26 Enteric form of Derzsy's disease. Haemorrhagic-necrotic enteritis with pseudomembrane formation in the small intestine.

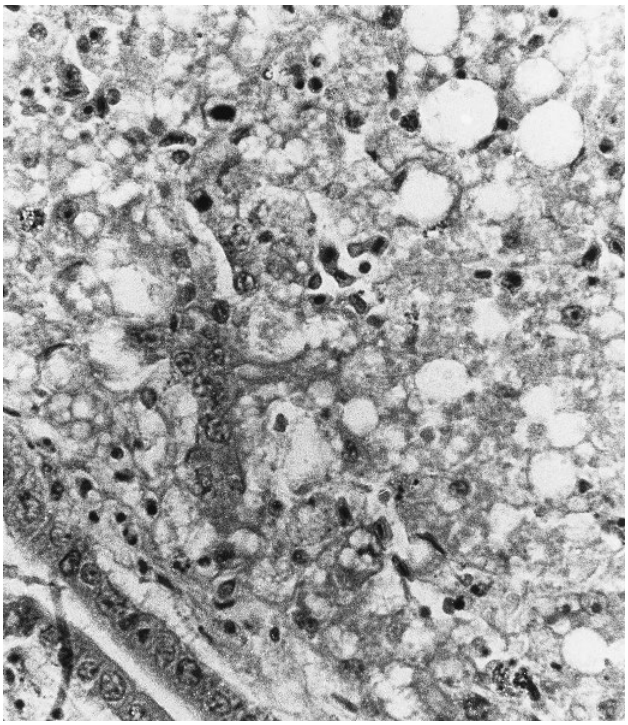


Figure 13.27 Liver section from a 10-day-old gosling infected with goose parvovirus (GPV) showing widespread vacuolation and degeneration of hepatocytes.

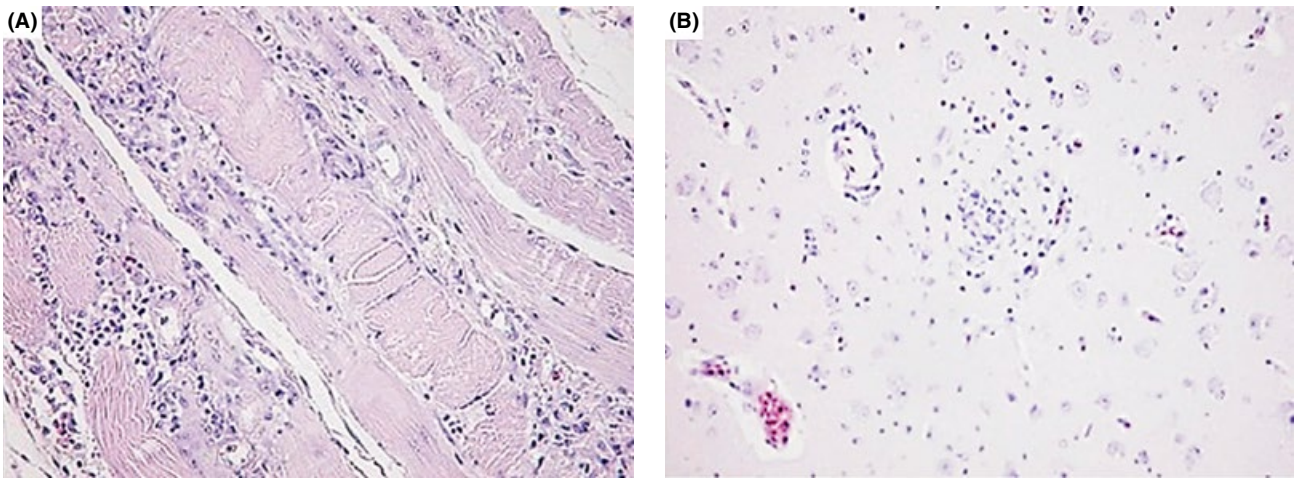


Figure 13.28 Parvovirosis of Muscovy duck. (A) Zenker's necrosis of muscle fiber with lympho-histiocytic infiltration. (B) Focal lymphocytic infiltration and gliosis in the cerebrum.

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between the age of the infected birds, the observed clinical symptoms, and DNA copy number of GPV and MDPV in the different organs (63).

Immunity

Active Immunity

Following infection or vaccination, humoral immune response develops, which is characterized by the initial production of IgM and then IgY-type immunoglobulin (29). Using virus neutralization (VN) and agar gel precipitin (AGP) tests, high and persistent antibody levels could be detected for several months after infection (16). Although much less is known about the role of cell-mediated immunity, it is not thought to play a significant role in immunity to waterfowl parvovirus.

Passive Immunity

From adult breeding geese and ducks that have been vaccinated or naturally infected with parvovirus, transfer of maternal antibody occur via the egg yolk to their progeny (16, 20, 28). This passively acquired antibody may persist until about 2–3 weeks of age based on the starting level at hatch (14, 29).

Diagnosis

Isolation and Identification of the Causative Agent

Waterfowl parvovirus can be isolated from a variety of organs of affected animals using embryonated goose or Muscovy duck eggs or tissue cultures prepared from them (41). Ten- to 15-day-old embryonated goose or Muscovy duck eggs should be inoculated via the allantoic cavity. Embryo mortality occur 5–10 days postinoculation with liver lesions and hemorrhages on the skin. Inoculation of goose or Muscovy duck embryo fibroblast cultures should be done before they reach confluency. The virus produces a well-defined cytopathic effect at 3–5 days postinfection, although several blind passages may be required before a detectable CPE is observed. In infected cultures Cowdry type-A intranuclear inclusions are often present. The presence of the virus can be confirmed by immune-staining or molecular methods (41).

Direct Detection of Viral Antigens

Immunofluorescence has been used to detect the presence of viral antigen in goslings, embryonated goose eggs, and infected cell cultures. Flow cytometry assay has been used to detect virus-infected cells in the spleen. Other methods, including immunoperoxidase techniques, also have been developed (41).

Molecular Identification

The classical detection of GPV and MDPV by virus isolation in gosling or duckling embryos, cell cultures, and serological assays like seroneutralization test (SN) is time consuming and dependent on the availability of SPF gosling embryos and tissue cultures. These limitations resulted in the application of PCR based techniques which allowed fast detection and identification of both GPV and MDPV. Primers have been designed to amplify conserved regions of the capsid protein genes (2, 57) or the nonstructural gene (59). Differentiation of GPV and DPV can be done by restriction fragment length polymorphism (RFLP) or nucleic acid sequencing, the latter being the most distinctive (2, 35, 45, 52, 57). Nucleotide sequence analysis of capsid protein genes of GPV allows the differentiation of vaccine and wild strains (51, 57, 58). Another fast molecular method for the detection of GPV and DPV is the loop-mediated isothermal amplification assay (LAMP) that could remarkably simplify the detection of both viruses (64). Quantification of GPV and DPV by real-time PCR has also been described (63, 65). The Real-time PCR method was developed for the rapid detection of novel duck-origin goose parvovirus as well (40, 61).

Serology

Serology is useful for evaluating the immune status of breeding flocks and their progeny. It is widely used to confirm recent infection, efficiency of vaccination, and determine the level of maternally-derived antibodies (MDA) in newly hatched birds.

A number of serological methods have been developed for detection of antibodies against GPV and DPV, including AGP, VN, and plaque neutralization assay, ELISA using whole virus or recombinant antigens, and indirect fluorescent antibody test (41). Viral proteins expressed by *Escherichia coli* proved to be suitable antigens for western blotting or multiscreen western blotting assays (60). Nonstructural (NS) protein-based serological tests may be used for differentiation of birds that have been vaccinated with subunit vaccines from those that are naturally infected (60).

The VN test performed in primary goose or Muscovy duck embryo fibroblast cell cultures is the most widely used method to detect the presence of antibodies (14, 29). Cross-neutralization tests can be used to differentiate between GPV and DPV antibodies (23). Duck-origin GPV shows stronger cross-reaction with GVP than with DPV (67). The AGP test is less sensitive than the VN test and does not differentiate between antibodies against GPV and DPV, but is still useful method for testing large numbers of sera (14).

Differential Diagnosis

Goose parvovirus can be differentiated from DPV by using serological and molecular methods. Very few pathogens of geese and ducks exist that show the strict age-relatedness

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of waterfowl parvoviruses. The diseases to be considered for differential diagnosis include duck virus enteritis, duck hepatitis, HNEG, reovirus, adenovirus, circovirus, and *Riemerella anatipestifer* infections.

The herpesvirus of duck viral enteritis produces disease with high mortality in geese and ducks of all ages. Isolation and identification of the causal virus clearly differentiate it from parvoviruses. Duck hepatitis viruses also cause fatal diseases in ducks under the age of six weeks, but these viruses are not pathogenic for geese.

Hemorrhagic nephritis and enteritis of geese usually affects geese from three weeks of age; however, the virus generally causes only subclinical infection of ducks (8). Mortality ranges from a few percentage points to 50–70%. On postmortem, apart from enteritis and nephritis, edema and hemorrhages of the subcutaneous connective tissue and hydropericardium and ascites can be seen (43). Diagnosis can be confirmed by the detection of GHPV with specific PCR (18, 43).

Reovirus diseases have been described in young, 2- to 6-week-old Muscovy duck and geese. The disease is characterized by splenitis with miliary necrotic foci during the acute phase and epicarditis, arthritis, and tenosynovitis during the subacute/chronic phase (37, 42).

Circovirus infection of geese and ducks may result in growth retardation and feathering disorders without causing significant mortality. Histopathological examination shows lymphocyte depletion, necrosis, and histiocytosis in the bursa of Fabricius. Globular or coarse granular inclusion bodies can be detected in the cells of bursa follicles (53, 54).

Adenovirus infection causing hepatitis and hydropericardium in young (2–3 weeks old) geese is characterized by the accumulation of clear fluid in the dilated pericardial sac and enlarged liver with multiple necrotic foci. Histologically, basophilic intranuclear inclusion bodies can be found in the liver cells around the necrotic foci (21).

Riemerella anatipestifer may also cause high mortality in goslings and Muscovy ducklings. Treatment of birds with appropriate antibiotics and isolation of the etiologic agent in suitable media will enable differentiation from waterfowl parvovirus.

In most cases the course of the disease, clinical signs and gross and histopathological lesions help to differentiate these diseases from those caused by waterfowl parvoviruses; however, in questionable cases molecular methods should be used to detect and identify the causal agents (15).

Intervention Strategies

Management Procedures

Because outbreaks of waterfowl parvovirus are frequently attributed to transmission of the infection by vertically infected birds during hatching, the practice of incubating and hatching eggs that have originated from different breeding flocks should be avoided. Only eggs from flocks with the same parvovirus status should be incubated together and good hatchery hygiene should be maintained. The practice of breeding from parent stocks that have survived the disease when young also should be discouraged, since these birds are potential carriers of the virus.

Vaccination

Types of Vaccines

Both live and killed oil emulsion vaccines, containing either whole inactivated virus or baculovirus expressed VP2 capsid protein, are available and are widely used in countries where the disease is endemic. Vaccination against Derzsy's disease and SBDS relies on the use of attenuated live and inactivated GPV-based vaccines, while bivalent inactivated vaccines containing both GPV and DPV antigens are used to ensure protection against the two waterfowl parvoviruses causing disease in Muscovy ducks (41). Recently a live attenuated parvovirosis vaccine, consisting of a live DPV strain, attenuated by passages on duck embryo cells has been developed. This vaccine induces a protective immunity against both GPV (Derzsy's disease) and DPV (38).

Live vaccines containing attenuated goose and duck parvovirus can stimulate rapid immune response and protection in MDA-free birds (41). Maternally-derived antibodies, even at a very low level, can neutralize the live vaccine, thus preventing the virus from stimulating immune responses. Inactivated vaccines containing the whole parvovirus antigens, either in the monovalent (GPV) or bivalent (GPV and DPV) forms have the disadvantage of being relatively slow to induce immune response, but they are less sensitive to the interference with maternal antibodies than live vaccines (56).

Recombinant subunit vaccines have also been developed both from GPV and DPV using the baculovirus expression system (41). The structural protein(s) expressed were able to self-assemble into virus-like particles (VLP), and vaccines formulated from these VLPs in oil emulsion proved to be comparable to inactivated whole virus containing vaccines in Muscovy ducks (31) or geese (25).

Field Vaccination Protocols and Regimes

Historically, hyperimmune or convalescence serum injected subcutaneously in day-old goslings was used to avoid heavy losses in flocks exposed to an early parvovirus infection (12, 20). This technique was effective but presented the risk of carrying over undetected infectious agents by the contaminated serum. Currently, prophylaxis based on vaccination is the preferred choice.

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An optimal vaccination strategy must take into account the presence or absence of MDA, their levels and heterogeneity within a flock, and the susceptible period of goslings and ducklings to the disease. Breeder geese and ducks that have been naturally infected or vaccinated transfer MDA to their progeny, which may persist until two to four weeks of age depending on the antibody levels of individual birds at hatch. Because the disease is confined to young age, control measures have been aimed at providing adequate immunity during the first six to eight weeks of life. To achieve this, different methods have been applied. These include: passive immunization of newly hatched birds with convalescence or hyperimmune serum and the use of attenuated vaccine alone or in combination with an inactivated one for the active immunization of both adult and young animals.

Immunization of breeders has two objectives: (1) protection of breeders from infection, and thus prevention of virus transmission to the progenies, and (2) supplying the progenies with passive immunity. An optimal vaccination strategy must protect goslings and ducklings against both the early and the late forms of the disease. Therefore, knowing the antibody titers against GPV and DPV in parent flocks or the level of MDA in the day-old birds is fundamental to establishing an adequate vaccination strategy. The development of an early immune response is crucial when the birds are reared at a farm contaminated with a parvovirus. Any gap between the waning of MDA and vaccination in a contaminated environment is likely to lead to disease. In order to extend the protection after MDA declines to nonprotective levels, the vaccination of goslings and ducklings around one to two weeks of age is essential to stimulate active immunity in face of still persisting residual maternal antibodies. This can be best achieved by the use of inactivated vaccine with high antigen content.

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14

Other Viral Infections

Yehia M. Saif

Introduction

This chapter has traditionally included viral infections that did not fit into virus groupings used in a given edition of *Diseases of Poultry*. Yet, the grouping of viral infections into different chapters has changed in the different editions for a variety of reasons.

The etiology of some conditions such as proventriculitis and hypoglycemia-spiking mortality in chickens continue to be elusive and are presented in Chapter 33 in this edition. A viral etiology is suspected in both conditions. A subsection on pseudorabies infection of birds was not included in the 14th edition, and readers are referred to the 13th edition. This herpesvirus is not known to naturally cause disease in birds.

Columbid alphaherpesvirus-1 (Pigeon herpesvirus 1)

Didier Marlier

Summary

Agent, Infection, and Disease. Columbid alphaherpesvirus-1 (CoHV-1) infection, also known as pigeon herpesvirus 1, is a common disease of pigeons (*Columba livia*) that are the natural host. CoHV-1 has a worldwide distribution and all CoHV-1 strains analyzed seem to belong to the same serotype. The clinical signs are mainly respiratory (yellow to grey caruncles, sneezing, conjunctivitis, obstruction of nostrils with nasal mucus, laryngo–pharyngeal congestion and pharyngeal ulceration). Mature birds can be asymptomatic carriers that infect squabs very early in life because of re-excretion at the time of gorging.

Diagnosis. Clinical diagnosis is based on the observation of typical gross lesions and clinical signs.

Intervention. Control is based on improving loft environmental factors and addressing secondary parasitological and bacteriological infections. One commercial vaccine is available in Europe that may reduce clinical disease within a flock. Chemotherapy has not generally been effective for reducing disease.

Introduction

Definition and Synonyms

Columbid alphaherpesvirus-1, also known as pigeon herpesvirus 1, is the only viral infection causing a primary respiratory tract disease in pigeons. Clinical respiratory signs are limited or totally lacking in cases of pigeon avian paramyxovirus-1 or avian influenza virus infection. Pigeons are resistant to experimental infection with infectious bronchitis virus (22) and infectious laryngotracheitis virus (*Gallid* herpesvirus 1) (22). They are resistant to infectious bursal disease virus (21) and are considered to be refractory to Marek's disease virus (40).

Economic Significance

The economic burden of respiratory diseases in pigeons either directly (costs of healthcare) or indirectly (lost production in meat type pigeons—poor performance in racing pigeons) has never been fully studied. One case report had three-week-old meat type White King pigeons that suffered from respiratory diseases barely weighing 280 g at slaughter compared to the average 400 g of healthy pigeons (22).

Diseases of Poultry, Fourteenth Edition. Editor-in-chief David E. Swayne.
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Public Health Significance

Columbid alphaherpesvirus-1 and respiratory disorders of pigeons as a whole have no public health significance.

History

Respiratory disorders are a major cause of poor race performance of homing pigeons (*Columba livia*) and are a frequent condition in fancy and meat-type pigeons also. Initially, the etiology of "respiratory disease" was wrongly attributed to diphtheroid bacteria or Chlamydial infections (14, 38) before the essential role of columbid alphaherpesvirus-1 (CoHV-1) was demonstrated (30). The first available scientific description of a putative herpesvirus infection in pigeons dates back to 1945 (16) when researchers first described eosinophilic intranuclear inclusion bodies in the liver of US army pigeons that died during a presumed "psittacosis" epidemic. This new "intranuclear inclusion agent" was characterized as a herpesvirus (3). Since then, the CoHV-1 has been reported in many different countries (42). Columbid alphaherpesvirus-1 infection has been described in hawks, owls, and falcons (7, 13, 39), with suspected infection associated with consumption of herpesvirus-infected pigeons (13).

Etiology

Classification

The CoHV-1 (1, 5) previously known as Columbid herpesvirus-1 or pigeon herpesvirus belongs to the *Mardivirus* genus within the subfamily Alphaherpesvirinae and the family Herpesviridae.

Morphology

Columbid alphaherpesvirus-1 has the typical herpesvirus morphology and shares the main physico-chemical properties with other herpesviruses. It is an enveloped virus of about 180 nm of diameter. The icosahedral nucleocapsid has 162 capsomers of elongated shape.

Susceptibility to Chemical and Physical Agents

Columbid alphaherpesvirus-1 is inactivated at an acidic pH (pH 2.8, 2 hours, 26°C) at 50°C for 10 minutes or at 60°C for 2 minutes. It is inactivated by ether treatment and chloroform treatment. The virus at −70°C remains infectious for years, but infectivity may be lost in a few weeks when stored at above −20°C (10, 18, 22).

Strain Classification

Antigenicity

Most if not all CoHV-1 strains seem antigenically similar and have the same culture characteristics supporting only 1 CoHV-1 type (2, 9, 22, 25). Columbid alphaherpesvirus-1 cannot be serologically distinguished from the falconid and the owl herpesviruses.

Genetic or Molecular

The complete genome sequence of a CoHV-1 strain (strain HLJ) isolated from a feral pigeon in China was recently published (8). The CoHV-1 genome was 204237 bp in length, with an overall G/C base composition of 61.5%. It encodes approximately 130 putative protein-coding genes and has a class E structural characteristics similar to *Falconid* herpesvirus 1 (FaHV-1), *Gallid* herpesvirus 2, *Gallid* herpesvirus 3 and *Meleagrid* herpesvirus 1, but distinct from *Gallid* herpesvirus 1, *Anatid* herpesvirus 1, and *Psittacid* herpesvirus, which contain class D genomes (8). The CoHV-1 genome had the largest genome of any avian alphaherpesvirus sequenced to date. Columbid alphaherpesvirus-1 and FaHV-1 are a monophyletic group. HLJ strain isolated from pigeon was found closely related to strains isolated from a peregrine falcon (*Falco peregrinus*) in Poland and an owl (*Bubo virginianus*) in the USA (8).

Laboratory Host Systems

Most, if not all studies made on CoHV-1 infection were done in pigeons (*Columba livia*). Pharyngeal painting and intraperitoneal injection are the two common inoculation routes. Pharyngeal painting results in a localized disease and intraperitoneal injection leads to a systemic infection (32, 34). Columbid alphaherpesvirus-1 has also been isolated from budgerigars (*Nymphicus hollandicus*) infected after close contact with pigeons (25, 27) and disease was successfully reproduced after intranasal inoculation (25).

Pathobiology and Epizootiology

Incidence and Distribution

Columbid alphaherpesvirus-1 has a worldwide distribution and all CoHV-1 strains analyzed seem to belong to the same serotype (22). In Belgium, greater than 50% of pigeons possess CoHV-1 specific antibodies; CoHV-1 can be isolated from the pharynx of 82% of pigeons with acute coryza and the presence of CoHV1 can be demonstrated in 60% of lofts in which pigeons were recurrently affected with acute coryza (22).

Natural and Experimental Hosts

Pigeons are the natural hosts of CoHV-1 and after acute infection the virus remains latent (22), Chickens, ducks, turkey, canaries, house sparrow, lovebirds, albino swiss mouse, hamsters, guinea pig, and rabbits are resistant to infection (3, 10, 19, 22, 26).

Transmission, Carriers, Vectors

In CoHV-1 infected flocks, mature birds remain latently infected and are asymptomatic carriers and may intermittently shed virus (34). The virus is not egg-transmitted but squabs are contaminated early in life from shedding adults during the feeding process where the squabs are fed with crop milk from parent (24, 31). Squabs are protected from severe disease or a death by maternal immunity conferred through the egg yolk; they survive but become asymptomatic carriers.

Incubation Period

Virus excretion begins 24 hours after experimental inoculation and lasts 7–10 days. Later virus shedding can occur spontaneously without clinical signs even in the presence of high specific antibody titers. Likewise, recurrent episodes are not more frequent in pigeons with low specific antibody titers (34). In experimental conditions, cyclophosphamide treatment successfully induces CoHV-1 re-excretion sometimes with typical lesions (34). Corticosteroids treatments failed to induce CoHV-1 re-excretion (Vindevogel, personal communication).

Clinical Signs

In acute cases, pigeon caruncles turn from white to yellow–grey and pigeons sneeze frequently either spontaneously or because of exacerbated sensibility when caruncles are pressed. Conjunctivitis in one or both eyes is frequent. Nostrils are generally obstructed with nasal mucus and moisture, there is clear laryngo–pharyngeal congestion and, in severe cases, the mucous membranes of the mouth, pharynx, and larynx are covered with foci of necrosis and small ulcers. If supervening secondary bacterial infections develop the whole respiratory tract from sinus to air sacs may be involved leading to typical clinical signs and gross lesions of chronic respiratory disease (sinusitis, pericarditis, airsacculitis) (22). In young pigeons not protected by maternal immunity, a general infection with hepatitis may also develop (32).

Pathology

Gross

Gross lesions are usually those of an acute to chronic infection of the upper to lower respiratory tract. In the acute form, mucous membranes of the mouth, pharynx, and larynx turn red due to acute congestion and inflammation. Then foci of necrosis and small ulcers may develop mainly in severe cases. If supervening bacterial (*Staphylococcus intermedius*, *Pasteurella multocida*, *Escherichia coli*, *Streptococccus β haemolytic*, *Pasteurella haemolitica*) or parasitical (*Trichomonas columbae*) infections develop, diphtheric membranes covering the mucous membrane of the pharynx may be seen. In the final stage of the disease typical gross lesions of chronic respiratory disease, mainly chronic airsacculitis and chronic pericarditis, can occur. In the rare case of systemic infections, white foci of necrosis disseminated throughout the liver parenchyma can be observed (22, 23, 31, 32, 34).

Microscopic

Microscopic lesions reflect gross lesions. In localized CoHV-1 infections, foci of necrosis containing cells at different stages of degeneration and necrosis are found in pharyngeal stratified squamous epithelium, salivary glands, laryngeal epithelium, and tracheal epithelium. Ulcerations due to fusion of foci may be observed too (32). In disseminated cases, foci of necrosis are found in the liver and intranuclear inclusion bodies are present in many hepatic cells (32, 35). Occasionally lesions are found in pancreas and brain (3, 4).

Pathogenesis of the Infectious Process

Columbid alphaherpesvirus-1 infection is usually limited to the upper respiratory/digestive tracts but virus dissemination by viremia or by tissue contiguity from the natural infection site may happen leading to COHV-1 infection of liver, brain, trachea, kidneys, and spleen. These tissues are infected during the transient viremia seen during primary infection (4, 31, 32) or after cyclophosphamide treatment (32). Since 1990, pigeon circovirus infections have spread in European racing pigeons (12, 17). Nowadays they are widespread in European racing pigeons with a 65% estimated prevalence of the infection. Because of the virus-induced deep immunosuppression (12), it was suggested that severity of clinical signs and mortalities linked with CoHV-1 infections might strongly increase. This assertion has never been confirmed to date.

Immunity

Active

Neutralizing antibodies appear in squabs about one week after infection and peaks at about three weeks after infection. Then antibody titers slowly decrease. High

antibody titers do not prevent CoHV-1 re-excretion nor the re-onset of clinical signs (22). The CoHV-1 cell-mediated immunity has not been studied to date.

Diagnosis

Isolation and Identification of Causative Agent

Columbid alphaherpesvirus-1 is usually isolated in chicken embryo fibroblasts (CEF) from pharyngeal swabs of infected pigeons. In CEF, cytopathic effect starts about 12 hours postinoculation (HPI) and is obvious at 24 HPI. Cells are rounded with some cytoplasmic stranding and nuclear enlargement. Multinucleated syncytial cells (two to four nuclei) associated with intranuclear inclusion bodies develops and cell lysis eventually occurs. Cytopathic effect may appear earlier and may be more obvious after successive passages in cell culture. Columbid alphaherpesvirus-1 can be grown in chicken embryo hepatic cells, chicken embryonic kidney cells, duck embryo fibroblasts, and pigeon embryo fibroblasts but cytopathic effects differ (4, 28, 29, 32). Columbid alphaherpesvirus-1 can be grown on the chorioallantoic membrane of embryonating chicken egg in which it produces typical pocks. However, CoHV-1 has never been grown in mammalian cell lines with the exception of the baby hamster kidney cell line (29).

Molecular biological techniques such as uniplex PCR (15), multiplex PCR (6), and loop-mediated isothermal amplification (LAMP) (41) were developed. Uniplex and multiplex PCR may not be the best tools to identify virus carriers but may be useful in the diagnosis of diseased animals. A detection limit of 10 genome equivalents was reported (6). Loop-mediated isothermal amplification allows simple and rapid detection of pathogens without the need of sophisticated laboratory equipment (41).

Antibody detection is by virus-neutralization or by indirect immunofluorescence methods (22, 23).

Differential Diagnosis

Clinical diagnosis is based on the observation of typical gross lesions and clinical signs. The differential diagnosis of acute CoHV-1 infections includes lentogenic strains of pneumotropic avian paramyxovirus type 1 (APMV1). The differential diagnosis of chronic bacterial or parasite complicated CoHV-1 infection must be distinguished from acute diphtheritic pox virus infection (12). The pseudo-membranes are much less adherent in CoHV-1 than in pox infection and leave no large ulcers when removed. Diagnosis is often by clinical signs alone, and confirmative diagnostics, although recommended, are seldom performed at least in everyday practice.

Intervention Strategies

Management Procedures

Environmental factors, CoHV-1 infection, and secondary parasitological and bacteriological infections contribute to the development and severity of acute and chronic respiratory diseases. General environmental conditions (number of pigeons in the loft, loft orientation, dust levels) must be discussed with the owner and improved if required. The final diagnosis should always include specific parasitological (*Trichomonas columbae*) and bacteriological (*Staphylococcus intermedius*, *Pasteurella multocida*, *Escherichia coli*, etc.) examinations since primary CoHV-1 infections are frequently complicated by these agents, which must be considered if support treatment is to be successful (11, 12, 22).

Vaccination

Types of Vaccine

Experimental inactivated or attenuated vaccines have been tried, and primary viral excretion and clinical signs after challenge may be reduced. However, the vaccines were unable to prevent pigeons becoming carriers and vaccinated pigeons re-excreted the virus when immuno-suppressed (36, 37). However, vaccination reduce spontaneous CoHV-1 re-excretion and can lower CoHV-1 spread inside the loft or between lofts. There is one marketed CoHV1 inactivated vaccine in Europe (Pharmavac columbi 2, Pharmagal-Bio) that is a combination vaccine that also includes avian paramyxovirus 1.

Field Vaccination Protocols and Regimes

No studies of CoHV-1 vaccine field efficacy is available to date, but based on the author's own experience, CoHV-1 vaccines seem to improve the situation in racing pigeons lofts that had issues of chronic respiratory problems in previous years.

Treatment

Chemotherapy trials with trisodium phosphonoformate and acylguanosine failed to prevent infection (20, 33). Treatment of secondary parasitological (*Trichomonas columbae*) and bacteriological (*S. intermedius*, *P. multocida*, *E. coli*, etc.) infections may provide some reduction in clinical disease (11, 12, 22).

Avian Nephritis

Victoria J. Smyth and Amir H. Noormohammadi

Summary

Agent, Infection, and Disease. Avian nephritis virus (ANV), an astrovirus, is the main cause of nephritis in young broiler chickens although infectious bronchitis virus (IBV) and chicken astrovirus (CAstV) can also cause nephritis. Avian nephritis virus is widespread in broiler flocks globally and while most infections are subclinical, coinfections with other enteric viruses are common. Avian nephritis virus is transmitted via the fecal–oral route and can be difficult to eliminate from a poultry house. There are many strains of ANV with varying pathogenicities which are not associated with mortality in chickens but cause economic losses. Avian nephritis virus is also known to infect ducks, turkeys, and wild birds.

Diagnosis. Reverse transcriptase-polymerase chain reaction (RT-PCR) and histopathology is the primary method of detection of the virus.

Intervention. There are no ANV commercial vaccines available, so management practices and biosecurity are important.

Introduction

Avian nephritis is an acute, highly contagious, typically subclinical disease of young broiler chickens and is caused by ANV, which is a member of the Astrovirus family. Avian nephritis virus was first isolated in chicken kidney (CK) cell cultures from the rectal contents of apparently normal one-week-old broiler chickens in Japan in 1976 (40). Avian nephritis virus strains vary in terms of their pathogenicity and their antigenicity exhibiting a high level of capsid protein sequence diversity (39). The extent of the disease problems caused by ANV is largely unknown although infections are prevalent and widespread in commercial chickens (23, 37). Diagnosis of ANV is by RT-PCR and histopathology, while serological diagnosis is more complicated.

Etiology

Classification

Avian nephritis virus was originally considered to be a picornavirus and in older papers may be referred to as an enterovirus-like virus (ELV) (25–28). However, it was characterized as an astrovirus in 2000 on the basis of its genome sequence (20) and is classified as a member of the genus *Avastrovirus*, together with other avian astrovirus species including turkey astrovirus types 1 and 2 (TAstV-1, TAstV-2), duck astrovirus (DAstV), and CAstV (2). Avian nephritis virus is distinguishable from CAstV by serological assays and RT-PCR (refer to Diagnosis section for further details).

Morphology

Avian nephritis virus shares many characteristics with other astroviruses including a particle size of ~28 nm in diameter (40) and a solid capsid protein coat bearing spikes, which may not be apparent by electron microscopy. Due to its fragility in cesium chloride, its buoyant density is undetermined (20). The unenveloped icosahedral capsid encapsidating the RNA genome has $T = 3$ symmetry.

Chemical Composition

The ANV has a single-stranded, positive sense RNA genome, 6,927 nucleotides long (20) that contains a small, untranslated region (UTR) at the 5′ terminus and a longer 3′ UTR of 307 nucleotides. Between the UTRs lie three overlapping open reading frames (ORFs), designated ORF 1a, 1b, and 2 (Figure 14.1) (20). Open reading frame 1a and 1b encode the nonstructural 3C-like serine protease and RNA-dependent RNA polymerase respectively, while ORF 2, which adjoins the 3′-UTR, encodes the capsid precursor polyprotein. There is a messenger RNA-like poly A tail after the 3′-UTR.

Virus Replication

The cellular receptor for ANV is undetermined, but the virus has been detected in the kidneys, jejunum, rectum, spleen, and bursa of Fabricius (15). The virus replicates in the cytoplasm (40) producing both genomic and subgenomic RNAs, the latter of which codes for ORF 2. The capsid precursor protein is cleaved posttranslationally by cellular caspases leading to the formation of three viral proteins which together form the capsomer subunits that comprise the capsid. Encapsidation of genomic RNA leads to production of mature virions, which are then released from the cell.

Susceptibility to Chemical and Physical Agents

Its infectivity is resistant to ethyl ether, chloroform, trypsin, and acid (pH 3.0), and is relatively heat labile,

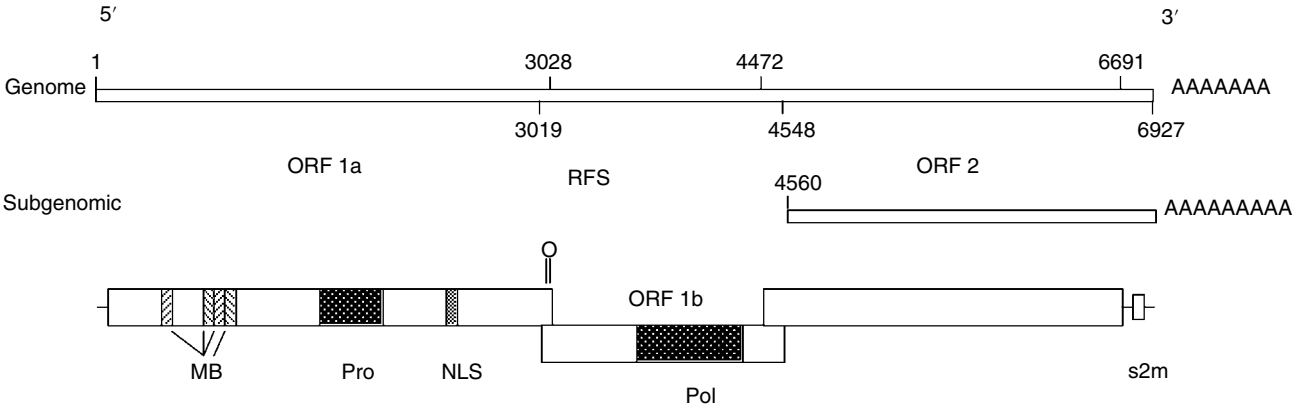


Figure 14.1 Schematic representation of the avian nephritis virus (ANV) (G-4260 strain) genome. Open boxes, open reading frames (ORFs). The locations of three ORFs, predicted transmembrane helices (MB), protease (Pro), nuclear localization signal (NLS), ribosomal frameshift structure (RFS), RNA-dependent RNA polymerase (Pol), and stem-loop II-like motif (s2m) are indicated. Numbering is according to the ANV genomic sequence (accession no. AB033998).

although it shows partial stabilization at 50°C by molar magnesium chloride (20, 40).

Strain Classification

Analyses of ANVs from the United Kingdom and Europe (10, 39), Japan (20), China (41, 42) and Australia (5) showed that the capsid proteins of ANVs exhibit substantial amino acid sequence diversity, with pairwise amino identities as low as 52% being observed and segregating into nine tentative genogroups (Figure 14.2).

Laboratory Host Systems

Avian nephritis viruses differ in their ability to grow in laboratory hosts, and are difficult to culture. Some ANV strains may grow in chicken embryos via yolk sac, chorioallantoic membrane, or allantoic cavity inoculation while other strains may propagate in primary CK cells or in Leghorn male hepatoma (LMH) cells, a chicken hepatocellular carcinoma cell line. For more detailed information please refer to previous editions of *Diseases of Poultry*.

Pathogenicity

Field viruses exhibit different degrees of pathogenicity in chickens and some ANV strains have different tissue tropisms and can vary in their ability to produce illness and death (9, 10, 12, 23, 31, 33, 34, 37). Recently a novel ANV which was isolated from chickens and turkeys affected by runting and stunting and/or locomotory problems, produced stunting, mortality, and nephritis following inoculation of 3-week-old specific pathogen free (SPF) chickens. Histological lesions were detected in the pancreas, intestine, and kidney, but not in the joints (10). A different ANV strain was shown to cause substantial growth retardation and severe, long-lasting intestinal lesions following experimental infection (9). Lesions were detected in the pancreas and proventriculus and were absent in the kidney tissue. Avian nephritis virus had no apparent effect on egg production or egg quality in laying hens (19). The detection of ANV in hatched ducklings and dead-in-shell duck embryos has led to speculation that ANV infections may be associated with reduced hatchability in ducks (1).

Pathobiology and Epizootiology

Incidence and Distribution

The diagnosis of ANV infections was achieved originally by virus isolation and serology but more recently RT-PCR is used. In addition, IBV and CAstV, which can also cause nephritis, should be excluded by molecular testing. Evidence indicates that ANV infections are highly prevalent in commercial chickens worldwide (7, 13, 23, 39, 41). Since 2007, ANV also has been detected in turkeys, ducks, pigeons, including wood pigeons, and guinea fowl (1, 4, 22, 39, 42). Using serology, ANV has been shown to be widely distributed in chicken flocks in Japan (16) and in some European countries (6, 8). Antibody to ANV has also been detected in SPF flocks and in turkeys (27). The prevalence of ANV infections and ANV-related disease problems in other avian species is unknown.

Age of Host Commonly Infected

Infections may occur from embryo to slaughter with younger birds more commonly affected. The severity of clinical disease and kidney lesions following experimental infection of SPF chickens at 1 day of age was greater than that observed following infection at 14 days of age,

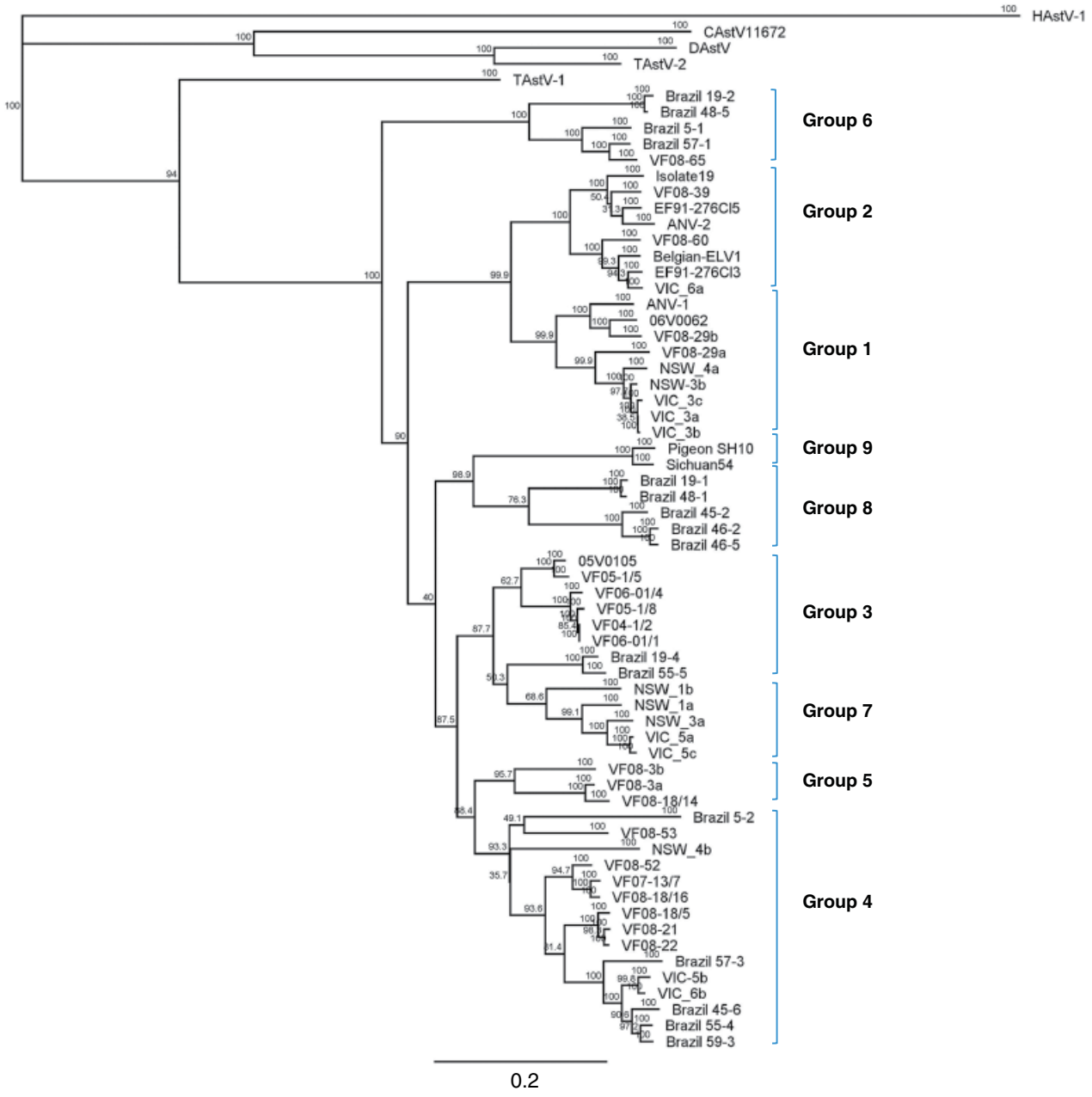


Figure 14.2 Phylogenetic tree of avian nephritis virus (ANVs), other avian astroviruses, and human astrovirus (HAstV) based on capsid amino acid sequences. The tree was constructed using Geneious version 6.1.8 (https://www.geneious.com, Kearse et al. [21]) using the Neighbor-Joining method and 1,000 bootstrap replicates (bootstrap values are shown on the tree) and rooted using HAstV. The scale bar denotes the number of nucleotide substitutions per site. Data relating to the origin of the ANVs, including the Genbank accession numbers, are described (5, 10, 11, 20, 39, 41, 42).

suggesting that there may be increasing disease resistance with age of chicken (12, 17, 29).

Transmission

Horizontal transmission readily occurs by direct or indirect contact (17), with the fecal–oral route thought to be predominant. Vertical transmission via the egg has been suggested based on field observations (6, 37), and virus has been detected in dead embryos from ducks (1). The virus is widely distributed, with maximum titers in the kidney and jejunum and lower titers in the cloacal bursa, spleen, and liver. The virus was consistently isolated from kidney, jejunum, and cloaca, but not from brain and trachea during the first ten days PI (15).

Incubation Period

In day old chicks experimentally infected by the oral route, the virus was first detected in feces 1 DPI, with maximum virus shedding at 2–3 DPI (26).

Clinical Signs

Under field conditions, clinical signs associated with this virus infection in broiler chickens have varied from none (subclinical) to outbreaks of the so-called runting syndrome, baby chick nephropathy, and visceral gout (Figure 14.3) (16, 23, 25, 26, 34, 36, 37, 40). Nothing is known about clinical signs in turkeys; however, there is some evidence that ANV infections may cause embryo deaths in ducks (1).

Pathology

Under natural conditions, ANV has been found to cause swelling and paleness of kidneys and urate deposits in the ureters of young birds (8–11 days of age). Microscopic lesions included interstitial infiltration of lymphomonocytic cells, tubular degeneration, and accumulation of urate crystals (13). Under experimental conditions (12, 17–19, 29, 31, 33, 34), histological renal lesions consisted of degeneration/necrosis of epithelial cells of the proximal convoluted tubules associated with infiltration of granulocytes, interstitial lymphocyte infiltration, and moderate fibrosis (Figure 14.4). Avian nephritis virus particles and viral antigens were demonstrated in the degenerating epithelium by electron microscopy (Figure 14.5) and IF, respectively. Virus-specific antigens also were recognized by IF in the jejunum, but distinct microscopic lesions were not observed in the small intestine. The chicks that died revealed many urate tophi in the serosa and parenchyma throughout the body, including the kidneys.

Pathogenesis of the Infection

The virus has a rapid replication cycle. *In vitro* studies have detected clusters of virus particles in the cytoplasm of infected cells by 18 hours postinoculation (40). Under *in vivo* conditions, viral antigen is detectable in the renal tubular epithelium at 24 hours postinoculation and renal histological changes were visible by 3 days postinoculation (15). The detailed mechanism by which ANV interacts with the target cells (especially epithelial cells of the convoluted tubules in kidney) and induces cytopathic effects is not studied so far.



Figure 14.3 Visceral urate deposits in a chick that died 10 days postinfection (PI). Chalk-like urate crystals were deposited on the surface of the peritoneum and liver, although those on the surface of the liver were mostly removed during necropsy. The heart is white due to heavy urate deposits on the epicardium.

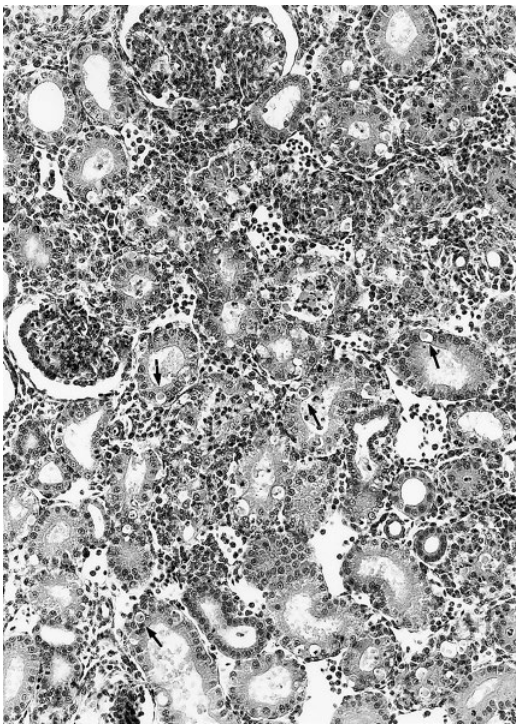


Figure 14.4 Degenerated proximal convoluted tubules containing acidophilic granules (arrows) in epithelial cell cytoplasm, and lymphocytic infiltration in interstitium, 5 days postinfection (PI). H&E, ×300.

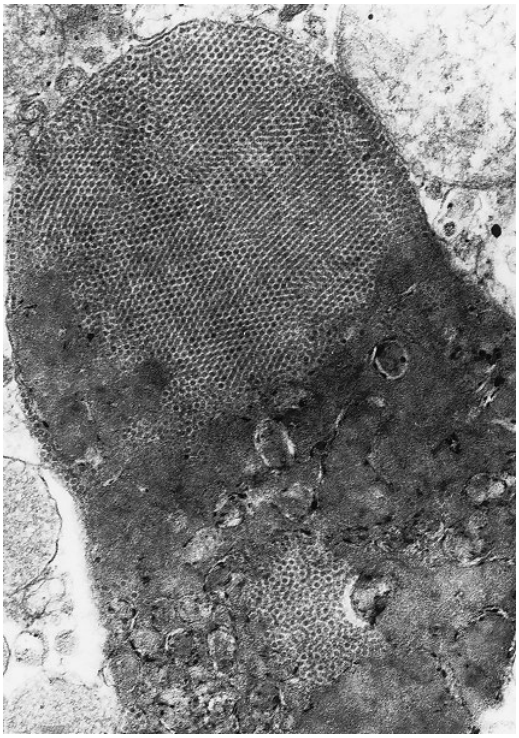


Figure 14.5 Crystalline array of virus particles in the cytoplasm of a kidney epithelial cell, 3 days postinfection (PI). ×30,000.

Immunity

There appear to be at least three different serotypes, typified by the G-4260 (serotype 1, ANV-1), M8 (serotype 2, ANV-2), and WG3 (serotype 3) isolates (12, 33, 34, 37, 40). Natural immunity to ANV is not well characterized although experimental studies would suggest that antibodies against epitopes in ORF 2 will detect and, to a lesser degree, neutralize strains from more than one genogroup (14).

Diagnosis

Detection of ANV

Three conventional RT-PCR tests have been described, each amplifying different regions of the ANV genome, including ORF 1a (24), ORF 1b (7), and the 3′ UTR (38) Since the severity of the pathogenic effect produced by an ANV infection is likely to increase with increasing levels of virus replication, a real time, quantitative RT-PCR test (36) was designed which gives an estimate of the amount of ANV present in a sample which may prove useful in differentiating cases in which ANV is having a pathogenic effect in either the intestine or the kidney. A specialized ANV real-time RT-PCR test has been developed using high-resolution, melting curve analysis which can distinguish between serotypes (5). A positive RT-PCR result should be interpreted in conjunction with suggestive gross and histopathological lesions in kidneys.

Serology

Natural and experimental infections with ANV elicit a virus-specific antibody response in chickens, which can be measured with a conventional VN test, an indirect IF test, and an enzyme-linked immunosorbent assay (ELISA) (8). There are no commercial serological tests available that will screen antisera for multiple ANV serotypes. Some laboratories may offer immunofluorescent antibody tests for ANV-1 or ANV-2. An indirect ELISA based on the C-terminal region of the capsid protein appears to have potential in detecting antibodies to different ANV serotypes tested (14).

Differential Diagnosis

Because certain nephrotoxic strains of IBV cause interstitial nephritis and certain strains of CAstV (3), it is difficult to differentiate the causal virus on the basis of the histological lesions (35). New strain identification techniques for IBV can determine whether it is a virulent strain that might be causing the nephritis lesions detected. The possibility that flocks may be infected simultaneously with ANV and IBV and/or CAstV should not be overlooked and specific RT-PCR tests can determine coinfection status.

Intervention Strategies

Management Procedures

There is no specific treatment. The common and widespread occurrence of ANV in commercial poultry and in wild birds (22), when combined with its capacity for vertical transmission, strongly suggests that its eradication from commercial poultry is not feasible. In addition, astroviruses are stable in the environment and may be resistant to inactivation by some routinely used disinfectants, which may make virus elimination from infected premises more difficult (32). Strict biosecurity, increased down time between flocks, and effective disinfection of the premises including fumigation help reduce the likelihood of exposing young chicks to substantial ANV challenges.

Vaccination

Currently, there are no commercially available ANV vaccines nor specific treatments for ANV infections.

Arbovirus Infections

James S. Guy

Summary

Agent, Infection, and Disease. Eastern equine encephalitis (EEE) virus, western equine encephalitis (WEE) virus, Highlands J (HJ) virus, West Nile (WN) virus, Israel turkey meningoencephalitis (IT) virus, and Tembusu (TMU) virus are arthropod-borne viruses and potential causes of disease in domestic poultry and farm-reared game birds. Neurological disease is the most common clinical outcome; however, these viruses also may result in decreased egg production and myocarditis.

Diagnosis. Laboratory diagnosis is by virus isolation, serology, or detection of viral antigen or RNA in tissues.

Intervention. These infections are best prevented and controlled by measures aimed at reducing vector populations and/or locating production facilities away from vector habitats. Vaccines rarely are utilized for prevention of these diseases in avian species.

Introduction

The term *arbovirus* is an abbreviation of *arthropod-borne-virus.* This term identifies those viruses that replicate in hematophagous (bloodsucking) arthropods, and share the property of bite transmission to vertebrate hosts. Over 100 arboviruses have been isolated from avian species or ornithophilic arthropod vectors. However, only six arboviruses—eastern equine encephalitis (EEE) virus, western equine encephalitis (WEE) virus, Highlands J (HJ) virus, West Nile (WN) virus, Israel turkey meningoencephalitis (IT) virus, and Tembusu (TMB) virus have been identified as causes of disease in domestic poultry and farm-reared game birds.

Public Health Significance

Eastern equine encephalitis virus, WEE virus, and WN virus are zoonotic agents and potential causes of significant neurological disease in human beings; these infections may progress to paralysis, convulsions, coma, and death. The case fatality rate for EEE virus in human beings is 50–75% (91). Western equine encephalitis virus and WN virus are less severe, with most infections being subclinical. The case-fatality rate for WEE virus and WN virus is approximately 3–7% and 4–11%, respectively (70, 91). Human infection usually is acquired by mosquito bite; however, care should be taken to avoid contact or droplet exposure when handling suspect infected birds. Highlands J virus, IT virus, and TBM virus are not known to be pathogenic for human beings.

Etiology

Classification

The arboviruses comprise a large, diverse group of viruses, with members in 12 different virus families; however, only the *Togaviridae* and *Flaviviridae*, contain viruses that cause disease in poultry and game birds. The principal characteristics of the *Togaviridae* and *Flaviviridae* are presented below.

Togaviridae

Togaviruses are spherical enveloped viruses approximately 70 nm in diameter (Figure 14.6). The genome consists of a single molecule of positive-sense, single-stranded RNA of 9.7 to 11.8 kilobases (kb), enclosed within a 40 nm diameter icosahedral nucleocapsid (93). Some togaviruses exhibit pH-dependent hemagglutinating activity.

Togaviridae comprise two genera, *Alphavirus* and *Rubivirus*, but only the *Alphavirus* genus contains arboviruses. The alphavirus genus comprises 29 viruses, including EEE virus, WEE virus, and HJ virus. Based on serologic cross-reactivity and genetic similarity (63, 93),

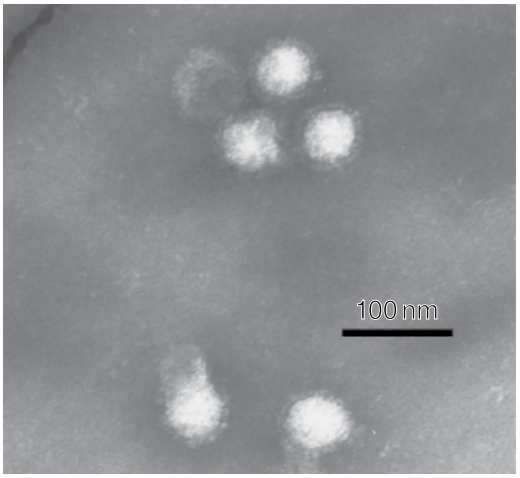


Figure 14.6 Negative-contrast electron micrograph of eastern equine encephalitis virus. ×150,000.

alphaviruses have been subdivided into eight serogroups. The EEE serogroup includes EEE virus; the WEE serogroup includes WEE virus and HJ virus.

Flaviviridae

Flaviviruses are spherical enveloped viruses approximately 50 nm in diameter (105). The genome consists of a single molecule of positive-sense, single-stranded RNA of approximately 11 kb. Flaviviruses exhibit pH-dependent hemagglutinating activity.

The *Flaviviridae* comprise three genera, *Flavivirus*, *Pestivirus*, and *Hepacivirus*, but only the *Flavivirus* genus contains arboviruses (105). The *Flavivirus* genus contains about 70 virus members grouped into multiple serogroups (19, 105). The Japanese encephalitis group includes WN virus; the Ntaya group includes IT virus, Bagaza virus, and TMU virus (19, 105).

Laboratory Host Systems

Day-old chickens, newborn and baby mice are highly susceptible to arboviruses when inoculated by the intracerebral (IC) route and some are susceptible following inoculation by peripheral routes (89, 104). Intracerebral inoculation of newborn mice, one to four days of age, is the preferred method for isolation of these viruses. Arboviruses also propagate in embryonating chicken eggs and in a variety of vertebrate and arthropod cell cultures. Vero cells, BHK-21 cells, and primary cultures of chicken and duck cells frequently are used for virus propagation. Arboviruses produce readily discernable cytopathic effects in vertebrate cell cultures; these effects are not always produced in arthropod cell cultures.

Intervention Strategies

Arbovirus infections of poultry and farm-reared game birds are best prevented and controlled by measures aimed at reducing vector populations. Such measures include reduction of vector habitats by modifications of the environment or by chemical spraying. If feasible, farms that raise susceptible avian species should be located away from wetlands and other areas that provide habitat for vectors. Viral vaccines rarely are utilized for prevention of these infections in avian species.

Eastern Equine Encephalitis

History

Eastern equine encephalitis virus was first isolated in 1933 from the brain of a horse with encephalitis (114). It was subsequently identified as a cause of disease in pheasants, pigeons, chukar partridges, ducks, and turkeys (28, 36, 84, 107, 117).

Pathobiology and Epizootiology

Incidence and Distribution

Eastern equine encephalitis occurs most commonly as a disease of horses. In avian species, this disease occurs most commonly in farm-raised ring-neck pheasants and chukar partridges; it occurs only sporadically in other species of poultry and game birds. The disease occurs primarily in the eastern parts of North America, throughout Central America and the Caribbean, and in eastern parts of South America. In the USA, EEE has been identified in most states east of the Mississippi River, as well as Louisiana and Texas; it occurs most often in Atlantic seaboard states and Gulf Coast states. Isolations of EEE virus in Europe and Asia have not been confirmed.

Eastern equine encephalitis outbreaks most commonly occur in late summer and fall, a consequence of increasing numbers of mosquito vectors.

Natural and Experimental Hosts

Outbreaks of EEE in avian species have been reported primarily in pheasants (60, 117); however, outbreaks in pigeons (36), chukar partridges (84, 95), turkeys (34, 107, 119), and ducks (28) have also been reported. Clinical disease in chickens and quail has not been reported, but both species are highly susceptible to experimental infection (116, 117).

Transmission, Carriers, Vectors

Culiseta melanura, an ornithophilic mosquito, is the principal vector of EEE virus in North America (20, 53). The virus also has been identified in a variety of other mosquitoes including *Aedes sollicitans, Coquilletia perturbans, Culex (Cx) pancossa, Cx. dunni,* and *Cx. sacchettae,* as well as mites, lice, simuliid flies, and culicoides (24, 120, 121). *Coquilletia melanura* is the likely vector responsible for transmission to poultry and game birds; transmission to mammalian species most likely occurs by other mosquitoes such as *Aedes* spp. and *Coquillettia* spp., which feed on birds but also have a propensity to bite mammals (82).

Wild birds, primarily the smaller species of Passeriformes, are the principal vertebrate hosts of EEE virus (68, 83, 123). These birds rarely become ill but serve as maintenance and amplifying hosts for the virus in the transmission cycle. In experimental studies, a variety of wild birds were shown to develop viremia lasting up to four days; small passeriform birds were shown to develop viremias with lethal-dose-50% (LD_{50}) titers greater than 10^6 mL (68).

Transmission of EEE virus occurs principally by mosquitoes, but direct transmission occurs among pheasants due to feather picking and cannibalism (52). Additionally, pheasants are susceptible to experimental infection by

oral inoculation (99). Epornitics of EEE virus infection in pheasants likely are initiated by mosquito-borne infection, with subsequent spread occurring due to feather picking and cannibalism.

Transmission of EEE virus by semen also has been demonstrated (43); virus was shed in the semen of experimentally infected tom turkeys on days 1 to 5 postinfection (PI). Semen collected from infected tom turkeys at 1–2 days PI resulted in transmission to breeder hens after artificial insemination.

Clinical Signs and Pathology

Clinical disease produced by EEE virus in poultry and game birds usually is attributed to central nervous system (CNS) infection with or without involvement of viscera. However, EEE virus also may produce visceral infections with little or no involvement of CNS tissues.

Pheasants

Naturally infected pheasants develop signs of neurologic dysfunction consisting of depression, leg paralysis, torticollis, and tremors (9, 117). Clinical signs occurred in 40–100% of experimentally infected pheasants with mortality of 25–100% (45, 67, 99). Mortality rates up to 80% characterize naturally occurring outbreaks.

Gross lesions are not observed in infected pheasants; however, histopathologic changes in the CNS consist of vasculitis, patchy necrosis, neuronal degeneration, and meningeal inflammation (62, 117).

Turkeys

Outbreaks of EEE in turkeys were characterized by drowsiness, incoordination, progressive weakness, paralysis of legs and wings, and low mortality (107). Affected turkeys had neurologic lesions consisting primarily of calcification of blood vessel walls in the cerebral cortex, the cerebellar folia, and the basal part of the medulla. Neurological lesions in intracerebrally inoculated birds included lymphocytic perivascular infiltration, neuronal degeneration, and endothelial cell swelling.

Serology was used to identify EEE virus as the cause of high mortality in young (1- to 4-week-old) turkeys (34). Subsequent experimental studies demonstrated susceptibility of young turkeys to experimental infection (40). Two-week-old turkeys experimentally infected with EEE virus exhibited depression, somnolence, and high mortality. Viremia was detected in infected turkeys on days 1 and 2 PI, with peak viremia of $10^{5.5}$ plaque-forming units per mL (PFU/mL) detected on day 1 PI. Pathologic changes consisted of multifocal necrosis in heart (Figure 14.7A), kidney, and pancreas, and lymphoid necrosis and depletion in thymus (Figure 14.7B), spleen, and bursa of Fabricius (Figure 14.7C). No lesions were detected in the brain.

Acute drops in egg production in turkey breeder hens due to EEE virus infection have also been reported (119). Decreased egg production in affected flocks was characterized by sudden onset with production of white, thin-shelled, and shell-less eggs. No increase in mortality was observed, and acute ovarian regression was the only gross lesion. Experimental infection of turkey hens with EEE virus reproduced the disease observed in naturally affected flocks with the hens exhibiting mild depression and inappetence on day 1 PI (42). A precipitous decline in egg production began on day 2 PI, and production remained depressed for 15 days; no mortality was observed. Viremia of short duration (1–2 days), peaking at $10^{5.8}$ PFU/mL on day 1 PI, was detected in EEE virus-infected hens.

Chukar Partridges

Chukar partridges infected with EEE virus exhibited clinical signs of depression, somnolence, and high mortality (30–80%) (95). Pale, focal areas were present in hearts of affected birds, and spleens were mottled and enlarged. Microscopic lesions consisted of gliosis, satellitosis, and perivascular lymphocytic infiltration in brains, and myocardial necrosis with lymphocytic infiltration.

Ducks

White Pekin ducklings infected with EEE virus developed a paralytic disease characterized by sudden onset, posterior paresis, and paralysis (28). Mortality rates in EEE virus-affected flocks ranged from 2–60%. Histopathologic lesions consisted of edema of spinal cord white matter, lymphocytic meningitis, and microgliosis.

Chickens

Newly hatched chickens are highly susceptible to EEE virus and succumb rapidly to infection, often without showing signs of CNS involvement. Susceptibility of chickens to lethal EEE virus infection declined rapidly with age and chickens became refractory to lethal infection by 14 days of age (16). In contrast to these findings, other investigators demonstrated susceptibility to lethal infection in 3- to 13-day-old chickens, and in 14-day-old chickens (41, 116). The different findings from these studies likely are due to differences in host genetics and/or differences in virulence of the EEE viruses used in these studies.

Experimental infection of young chickens, 1–14 days of age, caused depression, somnolence, and high mortality; paralysis was infrequently observed (41, 116). The principal lesion, and the presumed cause of death, was myocarditis. Heart lesions consisted of multifocal necrosis with fragmentation of myocardial fibers, and infiltration with lymphocytes, plasma cells, and macrophages (Figure 14.7E). Central nervous system lesions in infected

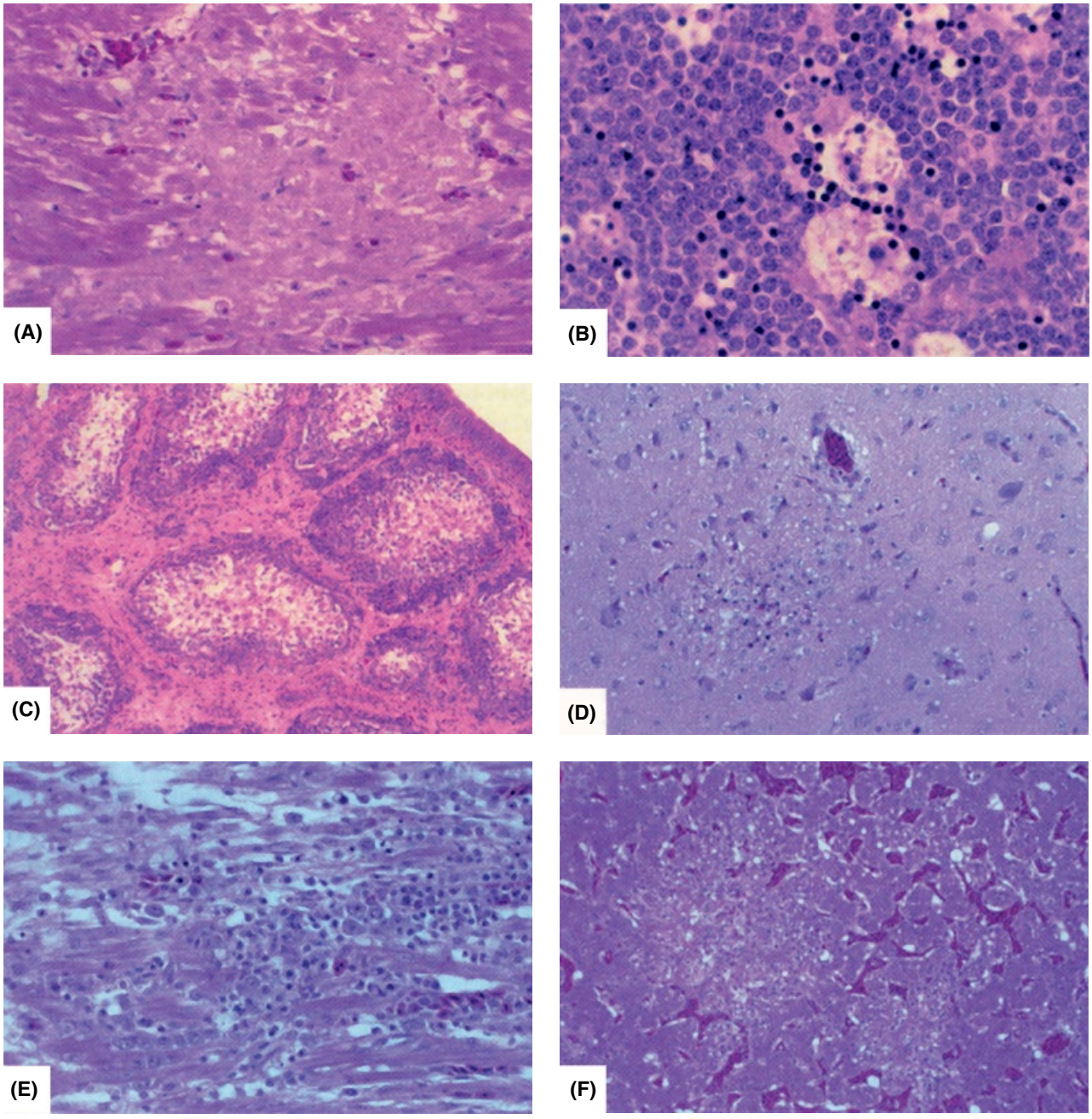


Figure 14.7 Microscopic lesions in turkeys and chickens experimentally infected with eastern equine encephalitis (EEE) virus. (A) Heart of turkey, 3 days postexposure. A large focal area of myocardial necrosis is present, with no inflammatory reaction. (B) Thymus of turkey, 3 days postexposure. Aggregates of pyknotic nuclei within clear spaces indicate acute lymphocyte necrosis. (C) Bursa of Fabricius of turkey, 3 days postexposure. Atrophy of bursal follicles with marked lymphoid depletion is present. (D) Brain of chicken, 2 days postexposure. A focal area of necrosis is present with mild perivascular cuffing. Note emigration of mononuclear cells from an adjacent venule distended with erythrocytes. (E) Heart of chicken, 5 days postexposure. Myocardial degeneration and necrosis with a mononuclear cell infiltrate. (F) Liver of chicken, 5 days postexposure. Focal necrosis is present with minimal inflammatory cell response.

chickens were inconsistently observed (41, 116). In brains, microscopic lesions consisted of occasional small foci of necrosis and mild perivascular cuffing (Figure 14.7D). Multifocal necrosis of the liver (Figure 14.7F) and lymphoid depletion and necrosis in the thymus, spleen, and bursa of Fabricius also were present in EEE virus-infected chickens (41). Ascites and right ventricular dilatation of the heart was detected in chickens that survived the acute effects of EEE virus infection; these effects likely occur due to myocardial damage (41).

Diagnosis

Diagnosis of EEE virus may be accomplished by isolation and identification of the virus, detection of viral antigens using antigen-capture enzyme-linked immunosorbent assays (ELISAs) (49, 50, 100, 101), or immunohistochemistry (124), detection of viral RNA using reverse transcriptase-polymerase chain reaction (RT-PCR) procedures (118), and serologic testing (104). The virus can be isolated by inoculation of blood or tissue homogenates (brain, spleen, liver, heart) into newborn mice by intracerebral route, day-old chickens by subcutaneous or intramuscular routes, and 5- to 7-day-old embryonated chicken eggs by yolk sac route (89, 104). In addition, a variety of cell cultures may be utilized for virus isolation; Vero, BHK-21, and chicken or duck embryo cells are highly susceptible. Newborn mice and 1-day-old chickens generally die of encephalitis in 2–5 days. Chicken embryos generally die within 18–72 hours and have a hemorrhagic appearance. Cell cultures develop cytopathic effects (CPE) within 24–48 hours, and plaques develop under agar within 36–48 hours. Identification of EEE virus in inoculated animals, embryonated eggs, or cell cultures generally is accomplished by virus-neutralization (VN) tests or complement fixation (CF) tests.

Antigen-capture ELISA and immunohistochemistry procedures may be utilized for detection of EEE virus antigens (14, 49, 50, 86, 100, 101, 124). Reverse transcriptase -PCR procedures (118) may be utilized for detection of EEE viral RNA. These procedures are rapid, sensitive, and specific methods for detection of EEE virus in tissues. Additionally, these diagnostic procedures reduce the human health risks inherent with virus isolation and identification procedures.

Serology

Serological diagnosis of EEE virus is accomplished using VN, hemagglutination-inhibition (HI), ELISA, and CF. Of these, VN and HI tests are most commonly utilized. The HI test is rapid and relatively simple; it requires either goose or 1-day-old chicken erythrocytes, and antigen prepared from infected suckling mouse brains by the sucrose–acetone extraction method (21, 104). Avian serum contains nonspecific inhibitors of hemagglutination and these must be removed by kaolin adsorption before use in HI tests. A presumptive serologic diagnosis may be obtained by detection of EEE virus antibodies in serum collected from recovered birds. A definitive diagnosis is achieved by demonstrating a rising antibody titer in serum samples collected soon after onset of clinical signs and 1–2 week later.

Serology was shown to be particularly important for diagnosis of EEE virus-induced episodes of decreased egg production in turkey breeder hens (42). In experimentally infected breeder hens, viremia was present for only a very brief period (on days 1–2 PI) following experimental inoculation, yet marked drops in egg production became apparent only after day 2 PI.

Differential Diagnosis

Eastern equine encephalitis must be distinguished from other causes of neurologic disease in poultry and game birds such as HJ virus, Newcastle disease virus, avian encephalomyelitis virus, botulism, and listeriosis. In cases of egg-production drops in turkeys, EEE virus, WEE virus, HJ virus, Newcastle disease virus, avian influenza virus, avian encephalomyelitis virus, paramyxovirus type 3, turkey coronavirus, and turkey rhinotracheitis virus must be considered.

Vaccination

Formalin-inactivated EEE vaccines, prepared for use in horses, have been used to protect pheasants against EEE epornitics (110), although their efficacy has been questioned (30).

Western Equine Encephalitis

Western equine encephalitis virus has many characteristics in common with EEE virus; however, unlike EEE virus, WEE virus is rarely associated with disease in avian species. In 1957, WEE virus was first identified as the cause of encephalitis and high mortality in turkeys in Wisconsin with affected turkeys exhibited somnolence, tremors, and leg paralysis (125). Isolation of WEE virus from the brain of a pheasant and as the cause of high mortality in chukar partridges has also been reported (32, 95), but the identification of WEE virus in these instances is tenuous. It is now generally accepted that WEE virus rarely occurs in the eastern United States, and that all WEE-related alphaviruses isolated in the eastern United States are strains of HJ virus (see below) (17, 115).

Western equine encephalitis virus was identified as a cause of decreased egg production in turkey breeder hens in California (22). Affected flocks experienced decreased egg production with production of small, white-shelled, and shell-less eggs. No increase in mortality and no clinical signs were observed. A WEE virus isolated from affected breeder hens was evaluated for pathogenicity in 2-week-old turkeys (23). The isolate failed to produce clinically apparent disease in inoculated turkeys, but infection resulted in mild to moderate lymphoid necrosis in bursa of Fabricius and thymus.

Western equine encephalitis is present mainly in western parts of the North America, Central America, and South America. It is transmitted principally by *Culiseta*

tarsalis, a mosquito vector that is relatively common in the United States west of the Mississippi River (20). Laboratory diagnosis is accomplished using the same procedures that are used for EEE.

Highlands J Virus Infection

Highlands J virus initially was isolated in 1960 from blue jays in Florida (48). Since that time, the virus has been identified as a cause of disease in chukar partridges (30, 93) and turkeys (34, 40, 42, 119).

Antigenically, HJ virus is closely related to WEE virus and for many years was considered a variant of that virus (46, 48, 64). However, serologic and oligonucleotide mapping studies identified HJ virus as a distinct virus in the WEE serogroup (17, 18, 63, 115). All viruses belonging to the WEE serogroup that have been isolated in the eastern United States have been determined to be HJ virus (17).

Experimental disease was reproduced by subcutaneous inoculation of young chukars that exhibited somnolence, ruffled feathers, and recumbency prior to death and lesions primarily consisting of encephalitis and myocardial necrosis (95). A more recent outbreak of HJ virus infection in chukar partridges exhibited similar clinical signs and high mortality (35%); myocarditis was a consistent finding in affected birds, but lesions in the brain were uncommon (31).

In turkey breeder hens, HJ virus was the cause of an acute drops in egg production (119). In addition, these viruses were serologically associated with mortality in young turkeys (34). Experimental infection of breeder hens with HJ virus produced precipitous egg-production drops (42), but was only mildly pathogenic for young turkeys (40). The clinical and pathologic characteristics of HJ virus infection in turkeys closely resemble those of EEE virus infection (see above).

Laboratory diagnosis of HJ virus infection is accomplished using the same procedures used for EEE virus and WEE virus (34, 42, 122). Highlands J virus is readily distinguished from WEE virus by a variety of serologic procedures using polyclonal and monoclonal antibodies (65).

Israel Turkey Meningoencephalitis

History

Israel turkey meningoencephalitis (IT) was first described in 1960 (72). In 1961, the etiologic agent was identified as a virus belonging to the Flaviviridae (92). In 2014, IT virus and Bagaza virus, members of the Ntaya serogroup, were determined to be the same virus species based on phylogenetic studies; Bagaza virus previously was determined to be a cause of disease in pheasants and partridges (1, 33).

Pathobiology and Epizootiology

Incidence and Distribution

Israel turkey meningoencephalitis virus has been identified in Israel, South Africa, and Spain (1, 8, 72). Outbreaks of disease in turkeys occur seasonally in Israel, corresponding with activity of arthropod vectors; outbreaks generally begin in late summer, peak in October, and disappear in early winter (54).

Natural and Experimental Hosts

Field cases of IT rarely are observed in turkeys less than 10 weeks of age, but younger birds are susceptible (95). Experimental infection of turkeys less than 10 weeks of age results in disease with an incubation period of 5 to 8 days (54). A viremia is detectable within 24 hours PI in experimentally infected turkeys and persists for 5–8 days (57).

Pheasants and partridges are susceptible to natural infection (1, 37). Newly hatched poults (55), Japanese quail (*Coturnix coturnix japonica*) (58) and suckling mice (55) are highly susceptible to IT virus inoculated by the intracerebral and intramuscular routes. Chickens, ducks, geese, and pigeons are refractory to infection (72).

Transmission, Carriers, Vectors

The seasonal incidence of IT strongly suggests that insect vectors transmit this disease. The virus has been isolated from unsorted pools of mosquitoes (*Aedes* spp. and *Culex pipiens*) and culicoides trapped near affected turkey flocks (12). Experimentally, IT virus has been shown to infect *Aedes aegypti* and *Culex molestus* mosquitoes (88). Field observations and experimental studies indicate that virus transmission does not occur by direct contact between infected and uninfected birds (57, 59).

Clinical Signs and Pathology

In field outbreaks, IT occurs with greatest incidence in turkeys 10 to 12 weeks of age. Affected turkeys exhibit neurologic dysfunction characterized by progressive paresis and paralysis, with variable mortality. Morbidity and mortality rates generally average 15–30% but may be as high as 80% (54). Affected birds initially exhibit incoordination and walk with one or both wings drooping. As the disease progresses, birds become reluctant or unable to walk, and rest on their breasts with legs extended forward and wings spread laterally. Turkey breeder hens exhibit a severe drop in egg production, but

egg quality, fertility, and hatchability are unaffected. Egg production returns to normal after recovery from infection.

Gross lesions include splenomegaly or atrophy of the spleen, catarrhal enteritis, and myocarditis (7, 56, 72). Ovarian regression, ruptured ovarian follicles, and peritonitis are observed in affected breeder hens (6). The principal microscopic lesions are nonpurulent meningoencephalitis characterized by submeningeal and perivascular lymphocytic infiltration, and focal myocardial necrosis (56, 72).

Clinical signs in pheasants and partridges include disorientation, incoordination, and ataxia (1, 37). Microscopic lesions include meningoencephalitis, myocarditis, and hemosiderosis in liver and spleen (1, 37).

Diagnosis

Diagnosis of IT virus may be accomplished by isolation and identification of the virus, detection of viral RNA using RT-PCR procedures (25), and serologic testing (8, 55, 56, 57, 90). Brain, spleen, liver, serum, and ovary are the preferred materials for virus isolation (55, 57). Tissue homogenates or undiluted serum are inoculated into 6- to 8-day-old embryonated chicken eggs by the yolk sac route, or onto monolayers of chicken embryo fibroblasts (CEF). One or more passages in embryonated chicken eggs may be required before embryo mortality occurs; embryos die 3 to 6 days PI and show a distinct cherry-red discoloration. Suckling mice inoculated by the intracerebral or intramuscular routes also may be used for virus isolation (55).

A readily recognizable CPE is produced in infected CEF cells by 3 days PI (56, 57); however, CEF cells are less sensitive than embryonated chicken eggs or suckling mice for isolation of IT virus. Identification of isolates usually is accomplished by VN tests.

Differential Diagnosis

Israel turkey meningoencephalitis must be differentiated from other causes of neurological disease in turkeys, particularly Newcastle disease virus, avian influenza virus, EEE virus, and HJ virus. The known geographic distribution of these viruses and the greater severity of paralysis observed with IT as compared with EEE and HJ are helpful in distinguishing these agents. Nervous signs caused by *Riemerella anatipestifer* and ionophore toxicity also must be distinguished from IT.

Vaccination

Vaccination is an effective method for control of IT. Live attenuated vaccines have been prepared by serial passage of IT virus in embryonated chicken eggs (56), Japanese quail kidney cells (59), and BHK-21 cells (8). The Japanese quail kidney cell-attenuated virus is highly efficacious and commercially available.

West Nile Virus

History

West Nile (WN) virus was first isolated in 1937 from the blood of a febrile woman in Uganda (106). West Nile virus was identified as a significant cause of disease in domestic avian species in 1997, when the virus was identified as a cause of neurological disease in young geese (76). In August 1999, the disease was detected for the first time in avian species, horses, and human beings in North America (108).

Pathobiology and Epizootiology

Incidence and Distribution

West Nile virus is now considered to be endemic in many countries in Europe, Asia, Africa, North America, and Central America (47, 108). Outbreaks affecting primarily human beings, horses, and geese occur sporadically in these countries and there is evidence for viral transmission bidirectionally between Africa and Europe by migrating birds (4, 38, 77, 80). Most outbreaks begin in late summer and fall and end when cold temperatures reduce mosquito vector activity.

West Nile viruses isolated from different parts of the world segregate into two distinct lineages based on genomic sequencing studies (10, 73). Lineage I contained WN viruses isolated in Europe, Africa, and North America; lineage II contained viruses isolated in Africa, Madagascar, and most recently in Central Europe (5).

Natural and Experimental Hosts

Outbreaks of WN in poultry have been reported primarily in geese (4, 6, 38, 76, 80). In naturally infected flocks, mortality rates of 10–60% have been reported (5, 38).

Ducks, chukar partridges, and pheasants are susceptible to WN virus infection but episodes of naturally occurring disease are rare (51, 126). Experimental infection of young Muscovy ducks and Aigamo ducks resulted in clinical signs and mortality (103).

Episodes of naturally occurring disease in chickens and turkeys have not been reported, but both species are susceptible to experimental infection (102, 111).

A wide variety of feral and captive birds are known to be susceptible to WN virus infection (70). In a study examining the role of various feral birds as reservoirs of WN virus in the transmission cycle, 25 species were shown to be susceptible to experimental infection (71). Based on levels of viremia, the five most competent species were Passeriform birds: blue jay (*Cyanocitta cristata*), common grackle (*Quiscalus quiscula*), house finch (*Carpodacus mexicanus*), American crow (*Corvus brachyrhynchos*), and house sparrow (*Passer domesticus*).

Transmission, Carriers, Vectors

Culex (Cx) mosquitoes are the principal vectors of WN virus. The virus has been identified in *Cx. pipiens* and *Cx. Restans* in the United States (37), *Cx. univittatus* in Africa and Middle East, and *Cx. pipiens* and *Cx. Modestus* in Europe (81). West Nile virus also has been isolated from at least 10 tick species belonging to *Amblyomma*, *Dermacentor*, *Hyalomma*, *Rhipicephalus*, *Argas*, and *Ornothodorus* genera (85).

Wild birds are the principal vertebrate hosts of WN virus (3, 79). These birds rarely become ill but serve as maintenance and amplifying hosts for the virus in the transmission cycle. The virus has been isolated from white storks, gulls, feral pigeons, crows, jays, doves, and hawks.

Mosquitoes are principally responsible for transmission of WN virus, but direct transmission has been suggested based on experimental studies (6, 79, 112).

Clinical Signs and Pathology

Geese

West Nile virus-infected geese (*Anser anser domesticus)* show various degrees of neurological involvement ranging from recumbency to leg and wing paralysis (Figure 14.8) (38, 112). Affected birds are either reluctant or unable to move when disturbed. Signs of incoordination are pronounced; some birds may fall while attempting to stand. Torticollis and opisthotonus may occur.

In 3–4 week old geese (*Anser anser domesticus)* experimentally infected by the subcutaneous or intramuscular route, viremia occurred as early as day 1 PI. Peak viremias of 10^4–10^6 tissue culture doses/mL occurred on days 2–4 PI; viral titers declined or disappeared coincident with the appearance of neutralizing antibodies. Some geese had detectable VN antibodies by day 4 PI. Viral excretion from inoculated geese was determined to be from the oropharynx and not from feces. The high viremic levels in infected geese are sufficient to transmit virus to engorging mosquitoes; geese thereby can potentially act as reservoirs for further virus circulation.

Pathological changes in WN virus-infected geese include pallor of the myocardium and occasionally the kidneys, splenomegaly, and hepatomegaly. Microscopic lesions were found mainly in the brain and consist of lymphocytic perivascular infiltration and neuronal degeneration (Figure 14.9). Small necrotic foci were present in the heart muscle, but lymphocytic infiltration was minimal.

Chickens

Day-old chickens develop neurological signs including tremors and paralysis following inoculation by a variety of routes; clinical signs appeared between 5–10days PI (96). Chickens aged 1–11 days developed viremia of



Figure 14.8 Six-week-old geese infected with West Nile virus. The bird on left is unable to stand; bird on right has spread its wings in attempt to retain its balance (Y. Weisman).

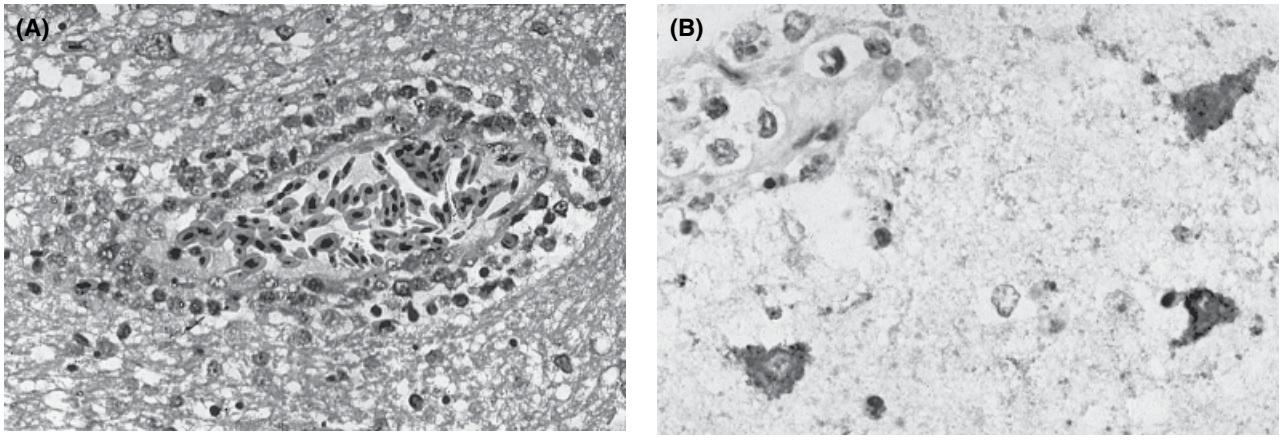


Figure 14.9 Microscopic lesions in the brain of West Nile virus-infected goose. (A) Perivascular cuffing by mononuclear cells (H&E stain) (S. Perl). (B) Immunohistochemistry. Three intensely stained neurons with viral antigen in the cytoplasm; the nuclei remain unstained. Stained granules are dispersed in the neuropil. Counter-staining with hematoxylin (S. Perl).

10^4–$10^{6.3}$ mouse infectious doses/mL following infection by mosquito bite and were capable in turn of infecting mosquitoes (113). In endemic areas chickens may be naturally infected, and sentinel chickens may play an important role in serological surveillance programs (15, 69).

Experimentally infected 7-week-old chickens developed viremia of 10^5 tissue culture doses/mL on day 5 PI that persisted until day 7 PI; this level of viremia is enough to infect engorging mosquitoes. Some chickens shed virus in their feces on days 4 and 5 PI (102). However, no clinical signs or mortality were observed in birds infected subcutaneously with WN virus (102). Experimental birds euthanatized on days 5 and 10 PI showed myocardial necrosis, nephritis, and peritonitis; nonsuppurative encephalitis was present at termination of the experiment on day 21. No transmission to in-contact chickens was detected; they remained antibody and viremia negative for 21 days.

Turkeys

No morbidity or mortality has been reported in commercial turkey flocks. No clinical signs were observed in 3-week–old turkeys experimentally inoculated subcutaneously with WN virus, however, most of them became viremic for up to 10 days PI (111). Virus was present in feces on days 4–7 PI, but in-contact poults were not infected.

Immunity

Geese rapidly develop high serum antibody titers to WN virus; however, these are not reliable indicators of protection (13). Cell-mediated immunity to WN virus has not been studied in geese; however, geese vaccinated with a live, attenuated IT vaccine were resistant to intracerebral challenge even though they failed to develop detectable VN antibodies (78). In a mouse model, B cells and antibody were shown to play critical roles in defense against disseminated infection (27).

Maternal antibodies were detected in sera collected from 1–2-week-old goslings hatched from commercial geese flocks but these did not interfere with an active response to inactivated WN virus vaccine. Based on field observations, susceptibility of geese to natural infection appears to decline with increasing age; geese older than 12 weeks of age appear to be resistant to disease.

Diagnosis

Diagnosis of WN virus may be accomplished by isolation and identification of the virus, detection of viral antigens in tissues using immunohistochemistry (108), detection of viral RNA using *in situ* hybridization or RT-PCR procedures (10, 74, 108), and serologic testing (11, 44). Tissues of choice for isolating WN virus are brain, spleen, and kidneys. Tissue homogenates are inoculated into newborn mice by the intracerebral route, into embryonated eggs by yolk sac route, or onto Vero cell cultures or mosquito cell cultures. Mice develop ataxia within 4–7 days; chick embryos die within 2–6 days PI and have a hemorrhagic appearance. Cell cultures develop a cytopathic effect within 48–72 hrs. Virus may be identified in cell cultures by indirect immunofluorescence; monoclonal antibodies are commercially available for use in this procedure.

Reverse transcriptase-PCR procedures have been described (12, 74). These procedures allow rapid detection of WN virus in avian tissues, cell cultures, and field-collected mosquitoes. Immunohistochemistry and *in situ* hybridization have been described for detecting WN virus antigens and viral RNA, respectively, in tissues of infected birds (35, 61, 108). These diagnostic procedures minimize the human health risks inherent with virus isolation and identification procedures.

Serology

Serological diagnosis is accomplished using HI, VN, or ELISA tests (21, 104). Several different ELISA procedures have been described for serologic detection of WN virus infection including indirect ELISAs, IgM capture ELISA, and competitive ELISAs (11, 29, 60, 61).

Differential Diagnosis

Nervous signs in young geese may be caused by Newcastle disease virus, avian influenza virus, *Riemerella anatipestifer*, *Streptococcus gallolyticus*, *Erysipelothrix spp.*, *Listeria spp.*, and *Salmonella spp*. Nervous signs also may be caused by *Aspergillus spp*. and ionophore intoxication.

Vaccination

West Nile vaccines have been developed primarily for vaccination of horses and several of these are commercially available for use in this species; however, none of these vaccines are commercially available for use in birds (26, 82, 87).

An inactivated mouse brain-derived WN vaccine was produced based on the procedure described for production of Japanese encephalitis virus vaccine (2). Field trials indicate that over 75% of geese vaccinated with a single dose of this vaccine at three weeks of age were protected and 94% protection was achieved with two doses spaced two weeks apart (78, 98). Duration of immunity was approximately 12 weeks. Inactivated vaccines prepared from chick embryos or Vero cells are less protective, likely because of low antigenic mass.

WN virus has been attenuated by serial passage in mosquito cell cultures (75). A single dose of this vaccine induced immunity to intracerebral challenge in young geese.

The use of IT virus vaccine has been investigated (78). A single dose given at three weeks of age produced protection in geese challenged two weeks later. This is an example of cross-protection that is known to exist within the flavivirus family (94). However, some birds vaccinated with IT virus vaccine developed a post vaccination paralytic reaction causing losses of up to 10% in some flocks.

Tembusu (TMU) Virus Infection

Tembusu virus is a cause of disease in chickens, ducks, and geese (127). This virus is present in China, Malaysia, Indonesia, and Thailand (127).

Tembusu virus most commonly is a cause of disease in laying ducks wit sudden declines in feed intake and severe drops in egg production (127). Laying ducks also may exhibit neurologic signs including incoordination, a reluctance to move, and wing and leg paralysis; mortality ranges from 5–15%. In young ducks and geese, TBM virus causes anorexia, diarrhea, retarded growth, and neurological signs including incoordination, torticollis, and opisthotonus; mortality may be as high as 20% (127).

Laboratory diagnosis of TBM is based on virus isolation and identification, detection of viral RNA using RT-PCR procedures, or detection of viral antigens using immunohistochemistry. Brain, spleen and ovary are preferred clinical samples for diagnostic analyses. Live, attenuated, and recombinant virus-vectored vaccines have been developed for prevention of TBM virus-induced disease; however, these are not commercially available (109, 128).

Turkey Viral Hepatitis

James S. Guy

Summary

Agent, Infection, and Disease. Turkey viral hepatitis (TVH) is a disease of young turkeys characterized by the presence of hepatitis with or without pancreatitis. Turkey viral hepatitis is caused by a picornavirus that is shed in feces and transmitted by both direct and indirect contact. Turkey viral hepatitis has been identified only in the United States, Canada, Italy, and Great Britain.

Diagnosis. Diagnosis of TVH may be based on histopathology, virus isolation, or detection of TVH viral RNA. A presumptive diagnosis may be obtained by histopathology, as the presence of lesions in both the liver and pancreas of turkeys is highly suggestive of this disease.

Intervention. No specific therapeutic or prophylactic measures are available.

Introduction

Turkey viral hepatitis (TVH) is a highly contagious, generally subclinical disease of turkeys. It is characterized by multifocal hepatic necrosis with or without accompanying pancreatic necrosis.

The economic significance of TVH is not known. There is no evidence to suggest that TVH virus is transmissible to human beings or other mammalian species.

History

Turkey viral hepatitis initially was described in 1959. A picornavirus was suggested as the likely etiology based on size, morphology, site of replication, and antigenic analyses (3, 4, 6, 9, 13). In 1982, MacDonald et al. (4) identified aggregates of 24 nm, picornavirus-like particles in the cytoplasm of hepatocytes in livers from TVH-affected turkeys. In 1991, Klein et al. (3) isolated a picornavirus-like virus, 26–28 nm in diameter from affected turkeys and reproduced the disease with this isolate. In 2011, TVH virus was determined to be a picornavirus based on nucleotide sequence analyses (2).

Etiology

Turkey viral hepatitis virus recently was identified as a member of the Picornaviridae (2). The Picornaviridae comprise a large family of RNA viruses that infect a wide variety of avian and mammalian species (10). They are characterized by an approximately 22–30 nm, icosahedral, non-enveloped capsid that encloses a single-stranded RNA genome, 7–8.8 kilobases (kb) in size (10).

Previous studies identified approximately 24 nm picornavirus-like particles in tissues from TVH-affected turkeys (1, 4). Additionally, TVH virus was determined to have sequence similarities with other picornaviruses, but phylogenetic analyses indicated that this virus could not be classified within presently recognized picornavirus genera (2).

Chemical Composition

The TVH virus genome consists of a single-stranded RNA molecule that is approximately 9 kb in size (2). The structural properties of viral proteins have not been determined.

Susceptibility to Chemical and Physical Agents

The virus is resistant to ether, chloroform, phenol, and creoline, but not formalin. In yolk it survives 6 hours at 60°C, 14 hours at 56°C, and 4 weeks at 37°C. It survived for 1 hour at pH 2 but not at pH 12 (12).

Laboratory Host Systems

Turkey viral hepatitis virus can be propagated and assayed in embryonating chicken eggs, embryonating turkey eggs, and turkey poults. The virus has not been propagated in cell culture (13).

The virus may be propagated by yolk sac inoculation of 5- to 7-day-old embryonating chicken eggs (6, 8, 9). Virus was demonstrated in inoculated embryonating chicken eggs at 66 hours postinoculation and peak virus titers of approximately $10^{3.5}$ EID_{50}/mL were detected at 90 hours postinoculation (11). The virus also may be propagated by yolk sac inoculation of embryonating turkey eggs up to 10 days of incubation; however, embryonating chicken eggs have been shown to be a superior host system, possibly due to presence of maternal antibody in turkey eggs (5).

Turkey poults are susceptible to infection by intraperitoneal, intravenous, and intramuscular routes of inoculation. Clinical signs seldom develop in experimentally infected poults, but infection may be demonstrated 5–10 days PI by necropsy and detection of characteristic lesions (7).

Pathobiology and Epizootiology

Incidence and Distribution

Turkey viral hepatitis has been described in Canada, the United States, Italy, and Great Britain (4–6, 9). The disease is believed to be widely distributed in North America, but the true incidence and distribution is not known owing to the frequent subclinical nature of the disease, and the absence of serological tests.

Natural and Experimental Hosts

Turkey viral hepatitis has been recognized only in turkeys. Chickens, pheasants, ducks, quails, mice, and rabbits have been shown to be refractory to infection (13).

Transmission, Carriers, Vectors

Transmission of TVH virus occurs readily by both direct and indirect contact. Feces from infected turkeys is believed to be the principal source for virus transmission; the virus could be consistently isolated from liver and feces of experimentally infected birds during the first 28 days PI, and less frequently from bile, blood, and kidney during this period. The virus could not be detected in tissues and feces after 28 days PI (11, 13). Vertical transmission via the egg has been suggested by field observations and by isolation of virus from an ovarian follicle of an experimentally infected hen (8).

Incubation Period

The incubation period in poults, as determined by the appearance of lesions, varied between 2–7 days in both intraperitoneally inoculated and in-contact poults (8, 11).

Clinical Signs

Turkey viral hepatitis is usually a subclinical infection of turkeys (4, 7). It is believed that the disease becomes apparent as a result of undefined factors such as concurrent infection and/or environmental stresses. Clinical signs in TVH-affected birds are not well defined. Variable degrees of depression may be observed in affected flocks, but more commonly field cases are characterized by sudden death of apparently normal birds. Decreased egg production, decreased fertility, and decreased hatchability in turkey breeder hen flocks has been associated with TVH virus, but an etiologic role has not been conclusively determined (7).

Morbidity and Mortality

Morbidity and mortality vary considerably among affected flocks. Morbidity rates of up to 100% have occurred in some flocks and 25% mortality was reported in one flock, but mortality is usually low (7). It is believed that severity of morbidity and mortality are influenced by other factors such as concurrent infection. Mortality in turkeys over six weeks of age has not been reported.

Pathology

Gross

Gross lesions attributable to TVH have been detected only in the liver and pancreas. Livers generally are enlarged, and lesions consist of focal, gray, sometimes depressed areas up to several millimeters in diameter (Figure 14.10). Lesion distribution is variable; birds that

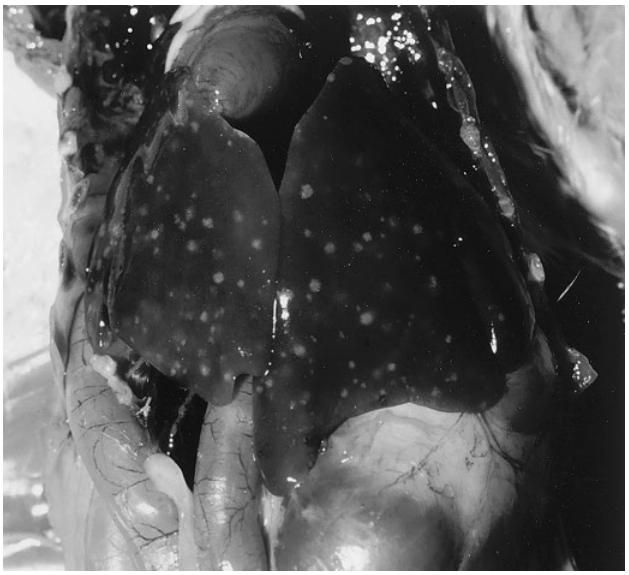


Figure 14.10 Multiple, pale tan to gray foci in the liver of a poult with turkey viral hepatitis. Lesions vary from one to several mm. They are randomly scattered throughout the liver, and roughly circular, oval, or elliptical. Lesions often have an irregular, "frayed" border, and some have a darker, slightly depressed central area. (Barnes)

die usually exhibit very extensive lesions, which often coalesce and may be partially masked by vascular congestion and focal hemorrhage. Pancreatic lesions are less consistently observed than hepatic lesions. Lesions in the pancreas generally are roughly circular, gray-pink, and may extend across a lobe.

Microscopic

Vacuolation of hepatocytes occurs early in the course of infection with dense infiltration by mononuclear leukocytes, and proliferation of bile ductules. Lesions progress to overt focal necrosis with pooling of blood around the focus; necrotic cells are scattered among infiltrating lymphocytes (Figure 14.11). Late in the course of infection, lesions are comprised of proliferating reticuloendothelial cells which frequently form giant cells (Figure 14.12).

Pancreatic lesions exhibit the same general histopathological changes as those observed in livers. Acinar cell degeneration and necrosis are observed with infiltration of macrophages and lymphocytes.

Immunity

Immunologic aspects of TVH have received little attention. Neutralizing antibodies in sera from recovered turkeys, or hyperimmunized chickens has not been detected (13). Immunity to reinfection was observed in previously infected turkeys, but the duration of immunity has not been determined (9).



Figure 14.11 Poult with turkey viral hepatitis showing prominent pancreatic foci. (Barnes)

Diagnosis

Diagnosis of TVH may be based on histopathology, virus isolation, or molecular detection of TVH viral RNA in tissue (2); serological procedures currently are not available. A presumptive diagnosis of TVH may be obtained by histopathology, as the presence of lesions in both the liver and pancreas of turkeys is highly suggestive of the disease. However, similar lesions may be produced in the liver by a variety of bacterial, viral, and protozoal agents. These include *Salmonella spp., Pasteurella multocida*, avian adenoviruses, reovirus, and *Histomonas meleagridis* (7, 8).

Isolation and Identification of Causative Agent

Virus isolation may be accomplished using a variety of tissues including liver, pancreas, spleen, kidney, or feces, but liver is the preferred sample. Tissues or feces should be homogenized in an appropriate diluent such as minimal essential medium, and clarified by centrifugation; clarified fecal suspensions should be filtered through a 0.45 um membrane filter. Homogenates of tissue or fecal

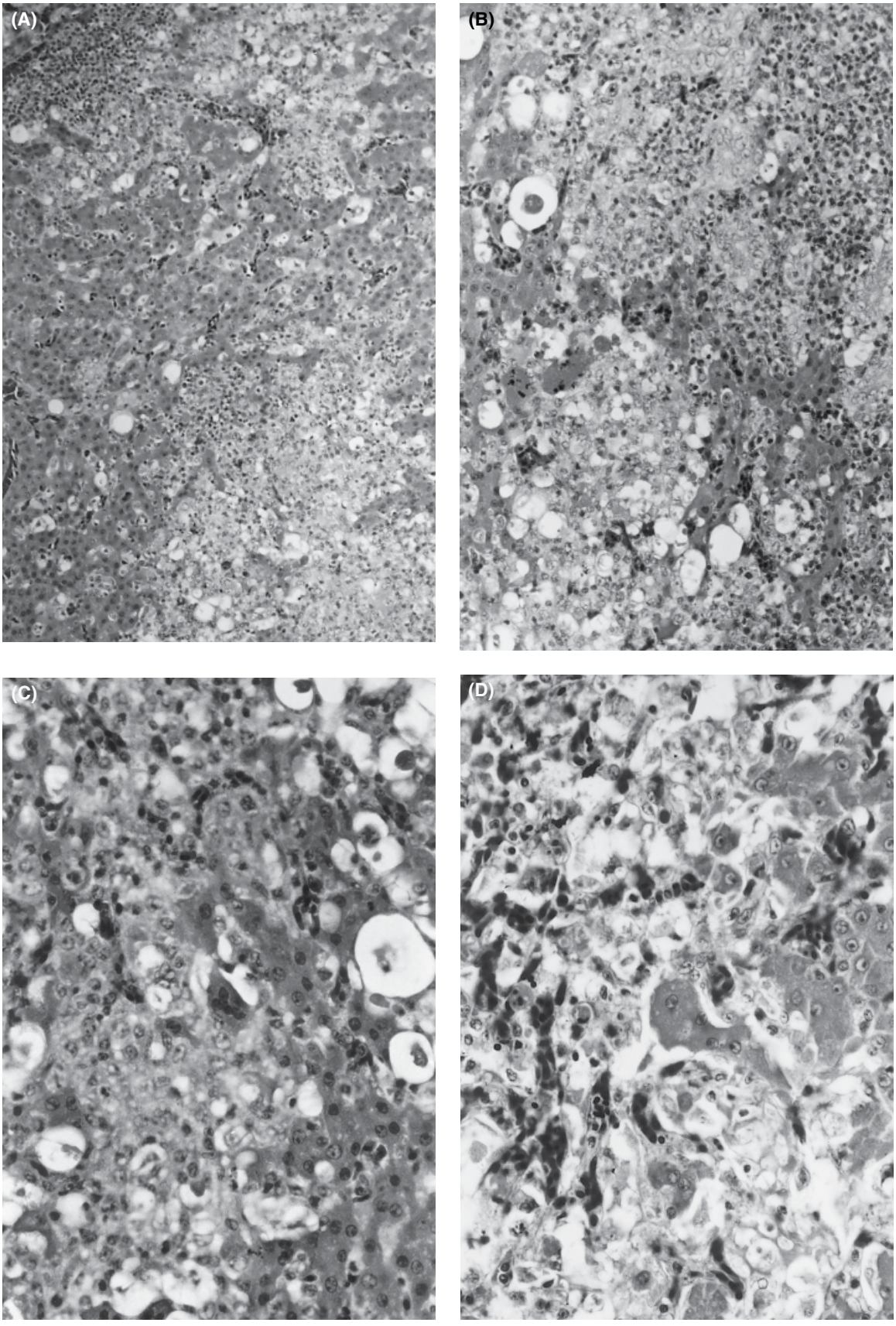


Figure 14.12 Microscopic lesions of turkey viral hepatitis. (Barnes). (A) Early lesions consist of multiple foci of vacuolar degeneration and coagulative necrosis. Cellular response primarily consists of lymphocytes and macrophages; heterophils are occasionally present but are not numerous. Pancreatic lesions are similar. In the liver, biliary hyperplasia generally is present, but the degree is highly variable among infected turkeys. (B) As lesions mature, they advance along sinusoids, often investing islands of liver cells, creating an irregular margin. (C) Frequently, liver cells within or adjacent to lesions fuse together to form syncytial cells. (D) Nuclear changes as seen here in hepatocytes adjacent to a lesion develop an appearance suggestive of inclusion bodies. Their nature is currently uncertain, but they are not believed to be of viral origin.

suspensions are inoculated into 5- to 7-day-old embryonating chicken eggs by the yolk sac route. In TVH-positive cases, embryo mortality generally occurs 4–11 days PI (9). Embryo mortality is delayed if low virus titers are present and in some cases a second passage using yolk harvest may be required. Embryos exhibit cutaneous congestion and edema; dwarfing is observed in those embryos in which mortality is delayed and less cutaneous congestion is observed in these embryos (9). Liver lesions containing necrotic foci are sometimes observed in embryos that survive to 11 days PI. Embryonic fluids do not hemagglutinate erythrocytes. Isolates may be further characterized by yolk sac or intraperitoneal inoculation of poults with yolk harvested from infected embryonating eggs; poults are examined for lesions 5–10 days PI.

Real-time RT-PCR and *in situ* hybridization procedures recently were described for detection of TVH viral RNA in infected turkeys (2). These detection procedures were demonstrated to be rapid, highly sensitive, and specific methods for detection of TVH viral RNA.

Intervention Strategies

No specific therapeutic or prophylactic measures are available.

Avian Encephalomyelitis

David L. Suarez

Summary

Agent, Infection, and Disease. Avian encephalomyelitis (AE) is a picornaviral disease affecting the central nervous system of 1–2-week-old, antibody-free chickens, turkeys, pheasants, and quail. It is mostly egg-transmitted through antibody-free, acutely infected breeders with economic losses primarily from reduction in egg production, reduced hatchability, and early chick mortality. Avian encephalomyelitis is worldwide in distribution.

Diagnosis. The clinical signs in chicks and history of egg drop in parent flock can often provide an initial diagnosis. Specific histopathological changes in the brain, pancreas, or duodenum of clinically-affected birds along with reverse transcriptase-polymerase chain reaction (RT-PCR) results and detection of antigen can confirm infection. Isolation of AE virus in 6-day embryonating chicken eggs can also be performed. Serology, typically using commercially available enzyme-linked immunosorbent assay (ELISA), can be used to monitor vaccination status.

Intervention. Vaccination of breeder hens before egg production with inactivated or live vaccines prevents disease in breeders and progeny.

Introduction

Avian encephalomyelitis (AE) is an infectious viral disease affecting young chickens, pheasants, quail, and turkeys. It is characterized by ataxia and rapid tremors, especially of the head and neck; because of the latter, it was often called "epidemic tremor."

No public health significance has been attached to this disease. The disease was of great economic importance to the commercial poultry industries prior to the widespread use of vaccines in the early 1960s.

History

Avian encephalitis was first reported in 1930 in two-week-old commercial Rhode Island red chicks showing tremors, and additional outbreaks with similar clinical disease were later observed in flocks in the Northeast United States. The disease was experimentally reproduced in 1934 using filtrates of brain material from spontaneous cases (33). It was not until the mid-1950s, however, that the first successful control of the disease by immunization (50), and the development of an orally administered vaccine (14) soon followed. Historical accounts of the control of AE and other details are available (10).

Etiology

Classification

Avian encephalomyelitis virus (AEV) is a member of the Picornaviridae family (38, 64) and the only member of the Tremovirus genus.

Morphology

Ultrastructure, Size, and Density

In purified preparations of AEV, virions are observed with hexagonal profiles lacking envelopes (21). By electron microscopic (EM) examination of purified AEV virions were 24–32 nm in diameter (21). Later EM studies

determined the mean diameter to be 26.1 ± 0.4 nm (61). Intracytoplasmic crystalline arrays observed in Purkinje cells from the brains of infected chickens had particles with diameters estimated to be 22 nm (15) or 25 nm (24).

The virus has a buoyant density of 1.31–1.33 g/mL (8, 21, 61) and a sedimentation coefficient of 148S (21).

Chemical Composition

The AEV genome is a polyadenylated, single-stranded RNA virus (64). Complete sequencing of the AEV genome determined a size is 7032 nucleotides (nt) not including the poly A tail, and has a predicted open reading frame of 6405 nt starting at nucleotide 495. The closest genetic relationship is with hepatitis A virus with a 39% overall amino acid identity to the polypeptide (38). One of the nonstructural proteins (2A) possessed conserved motifs involved in control of cell growth shared with two other picornaviruses, human parechoviruses, and Aichi virus (27).

Susceptibility to Chemical and Physical Agents

Avian encephalomyelitis virus is resistant to chloroform, acid, trypsin, pepsin, and DNase and is protected against effects of heat by divalent magnesium ions (4, 8). The virus was found susceptible to a single exposure to formaldehyde fumigation (28), and beta-propriolactone also inactivates the virus (12).

Strain Classification and Pathogenicity

Although all isolates of AEV are serologically similar, there are two distinct pathotypes of virus. One, represented by natural field strains, is enterotropic. These strains infect chickens readily via the oral route and are shed in the feces. They are relatively nonpathogenic except in susceptible chicks infected by vertical transmission or by early horizontal transmission, in which case they cause neurologic signs. Neurologic disease also occurs following experimental infection by intracerebral inoculation of susceptible chickens.

Embryo-adapted strains constitute the other pathotype. These viruses are highly neurotropic and cause severe neurologic signs following intracerebral inoculation or parenteral routes such as intramuscular or subcutaneous inoculation. They do not infect via the oral route except with high doses, and they do not spread horizontally (11, 31, 32, 41, 53, 73). Adaptation may occur after multiple passages in antibody-free chicken embryos (14, 40, 77).

Both pathotypes can replicate in embryos derived from a susceptible flock, but natural strains do not cause obvious signs or gross lesions in embryos. However, adapted strains are pathogenic for embryos, causing muscular dystrophy (Figure 14.13) and immobilization of skeletal muscles (35). The virus was detected in brains of inoculated embryos 3–4 days postinoculation (PI), and peak titers were found at 6–9 days PI (5, 35). Histopathologic changes in embryos infected with egg-adapted virus have been described as uniform in character but variable in intensity and location and consisting of encephalomalacia and muscular dystrophy (35). Muscular changes consisted primarily of eosinophilic swelling and necrosis, fragmentation and loss of striations of affected fibers with rare sarcolemma proliferation and heterophil infiltration. Neural lesions were characterized by severe local edema, gliosis, vascular proliferation, and pyknosis.

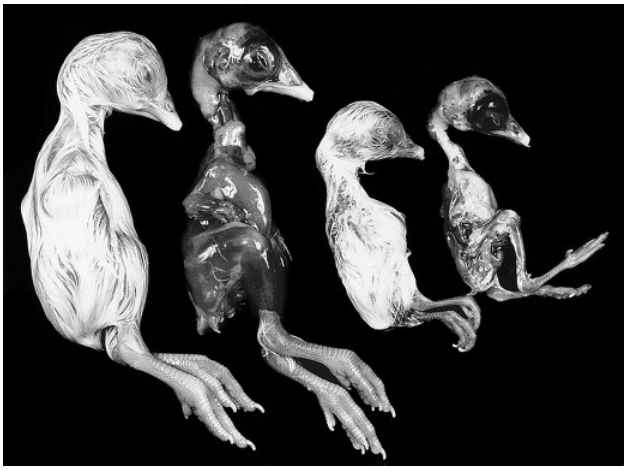


Figure 14.13 Chicken embryos on the right were inoculated via the yolk sac with the Van Roekel strain of avian encephalomyelitis virus on the sixth incubation day. Control embryos are on the left. The affected embryos, examined on the eighteenth incubation day, show extreme muscular dystrophy (most evident in the embryo with the skin removed) and rigidity of the legs.

Laboratory Host Systems

Virus may be propagated in the baby chick, chicken embryos from susceptible flocks, and a variety of cell culture systems. Chicks and embryos must be from a susceptible flock except in the case of intracerebral inoculation of chicks. Several routes of inoculation in embryos have been used (35, 58, 77), but inoculation via the yolk sac at 5–7 days of embryonation generally is considered the method of choice. Gross lesions (see previous section, Strain Classification and Pathogenicity) are observed only with adapted strains. Cell culture in fibroblasts, kidney cells, and neuroglial cells from chicken embryos and pancreatic cells from young chicks were used to cultivate both adapted and field strains of virus (60). Titers, particularly with natural strains, were generally low (rarely exceeding $10^{3.5}$ EID_{50}/mL), and cytopathic effects have not been described. Replication in cell cultures is detected by inoculation of embryos (adapted strains only) or by tests for antigen using immunofluorescence or enzyme-linked immunosorbent assays (ELISA). Chicken embryo neuroglial cells may provide

an excellent substrate for production of AEV antigen suitable for serologic tests, such as immunodiffusion and ELISA, and cell cultures can be adopted as the method of choice for titration of AE vaccine (46, 47). Efforts to demonstrate replication of AEV in a variety of established mammalian cell lines have been unsuccessful (1).

Pathobiology and Epidemiology

Incidence and Distribution

Avian encephalomyelitis occurs virtually worldwide (60, 66). Nearly all chicken flocks eventually become infected with the virus, but the incidence of clinical disease is low unless a breeder flock is not vaccinated and becomes infected after the commencement of egg production. Turkey flocks apparently also experience high rates of natural infection based on serological surveys (16). The rate of infection in pheasants and quail is not known.

Natural and Experimental Hosts

Avian encephalomyelitis virus has a limited host range. Chickens, pheasants, coturnix quail, pigeons, and turkeys have all succumbed to naturally occurring infection (6, 65, 66, 72). Experimental infection of young quail chicks (23) caused clinical signs, and the infection spread to breeding quail in the same room. Infection of the adults resulted in reduced egg production and hatchability, and clinical AE developed in chicks hatched from eggs laid during the outbreak. The naturally occurring disease in turkeys is essentially the same as that in chickens (26). Ducklings, poults, young pigeons, and guinea fowl also have been infected experimentally. Mice, guinea pigs, rabbits, and monkeys were refractory to virus introduced intracerebrally (39, 45, 67). Naturally occurring AEV antibodies has been found in serum from partridge, pheasant, and turkeys but not in serum from finches, sparrows, starlings, pigeons, jackdaws, rooks, doves, or ducks (70). The latter four species also failed to develop antibodies after oral exposure to AEV. A comparison of adult pheasants and red and gray partridges for sensitivity to intramuscular or oral–nasal inoculation with the VR strain of virus demonstrated infection in all three species, but the severity of disease based on signs and lesions was greatest in gray partridges and least in pheasants (2). Embryonating eggs from the three species were also susceptible to infection.

Transmission

The IC route of inoculation has given the most consistent results in reproducing AE in chickens. Other routes by which infection has been experimentally established are intraperitoneal, subcutaneous, intradermal, intravenous, intramuscular, intrasciatic, intraconjunctival sac, oral, and intranasal inoculation (7, 13, 50).

Under natural conditions, AE is essentially an enteric infection (13). Ingestion is the usual portal of entry (13, 25); exposure via the respiratory tract may be unimportant other than through the coincident exposure of the alimentary tract (13). Virus is shed in the feces for a period of several days, and because it is quite resistant to environmental conditions, it remains infectious for long periods of time. The period during which virus is excreted in feces is dependent in part on the age of the bird when infected. Young chicks may excrete virus for more than two weeks, whereas those infected after three weeks of age may shed virus for only about five days (52, 76). Infected litter is a source of virus easily transmitted horizontally by tracking or fomites. Infection spreads rapidly from bird to bird within a pen or house once introduced and from pen to pen on farms where no special precautions are taken to prevent spread. Birds in isolated flocks of a single age group were found to be less likely to have encountered infection than chickens on farms with multiple-age groups. Virus spread was found to be less rapid among birds in cages than in those on the floor (13, 18).

Vertical transmission is an important means of virus dissemination, based on both field evidence and experimental results (13, 34, 50, 63, 69). A serologic survey showed that 57% of breeder flocks tested in North America had been exposed to the virus by 5 months of age, and that by 13 months 96% were serologically positive (62). Although the source of infection for susceptible flocks is unknown, it is likely that it is carried from infected farms by people or fomites. When susceptible flocks are exposed after sexual maturity, the hens infect a variable proportion of their eggs, and experimentally infected embryos and chicks came from eggs laid during the period 5–13 days after infection of susceptible breeders (13). Conflicting reports on hatchability of eggs from infected flocks has been reported from no affect to a pattern of high embryo death during the last three days of incubation (34, 63). The percentage of embryos that hatched declined from a 78.6% preinfection level to 59.6% during the clinical stage and increased to 75.4% postinfection. Eggs produced just prior to and during the period of depressed egg production showed decreased hatchability and increased embryo mortality during the last three days of incubation. Furthermore, only chicks from the group with depressed hatchability showed signs of AE; chicks hatched prior to and after the affected hatch appeared normal (13, 63).

Virus transmission can also occur in the incubator (13). Chicks hatched from eggs inoculated at six days' incubation manifested signs on the first day of age; by the sixth day, 49 of 52 showed clinical evidence of AE. Chicks

from uninoculated eggs hatched with the infected birds first manifested signs on the tenth day, and 15 of 18 chicks developed clinical signs. An isolated control group of 19 chicks remained negative. The possibility of a carrier status is unknown.

Incubation Period

The incubation period in chicks infected by embryo transmission was 1–7 days, whereas chicks infected by contact transmission or oral administration had a minimum incubation period of 10 days (13).

Clinical Signs

Avian encephalomyelitis presents an interesting syndrome. In naturally occurring outbreaks, it usually makes its appearance when chicks are 1–2 weeks of age, although affected chicks have been observed at the time of hatching. Affected chicks first show a slightly dull expression of the eyes, followed by a progressive ataxia from incoordination of the muscles, which may be detected readily by exercising the chicks. As the ataxia grows more pronounced, chicks show an inclination to sit on their hocks. When disturbed, they may move about, exhibiting little control over speed and gait; finally, they come to rest or fall on their sides. Some may refuse to move or may walk on their hocks and shanks. The dull expression becomes more pronounced and is accompanied by a weakened cry. Fine tremors of the head and neck may become evident, the frequency and magnitude of which may vary. Exciting or disturbing the chicks may bring on the tremor, which may continue for variable periods and recur at irregular intervals. Ataxic signs usually, but not always, appear before the tremor. In some cases, only tremor has been observed. Ataxia usually progresses until the chick is incapable of moving about, and this stage is followed by inanition, prostration, and finally death. Chicks with marked ataxia and prostration are frequently trampled by their penmates. Some chicks with definite signs of AE may survive and grow to maturity, and in some instances signs may disappear completely. Survivors may later develop blindness from an opacity giving a bluish discoloration to the lens (48).

There is a marked age resistance to clinical signs in birds exposed after they are 2–3 weeks of age (see Pathogenesis of the Infectious Process). However, sporadic reports of older pullets showing neurologic signs 1–2 weeks after vaccination have been reported as recently as 2016 (51). Mature birds may experience a temporary drop in egg production (5–10%) but do not develop neurologic signs.

Morbidity and Mortality

Morbidity from the naturally occurring disease has been observed only in young birds. The usual morbidity rate is 40–60% if all the chicks come from the infected flock. Mortality averages 25% and may exceed 50%. These rates are considerably lower if many of the chicks comprising the flock originate from breeder flocks of immune birds.

Pathology

Gross

The only gross lesions associated with AE in chicks are whitish areas (due to masses of infiltrating lymphocytes) in the muscularis of the ventriculus. These are subtle changes and require favorable conditions to be discerned. No changes have been described for infected adult birds, other than the lens opacities described in Clinical Signs.

Microscopic

The principal changes are in the CNS and some viscera. The peripheral nervous system is not involved—a point of importance in differential diagnosis.

In the CNS, the lesions are those of a disseminated, nonpurulent encephalomyelitis and a ganglionitis of the dorsal root ganglia. The most frequently encountered addition is a striking perivascular infiltrate seeming to occur in all portions of the brain and spinal cord (Figures 14.14 and 14.15), except the cerebellum, where it is confined to the nucleus (n.) cerebellaris. Infiltrating

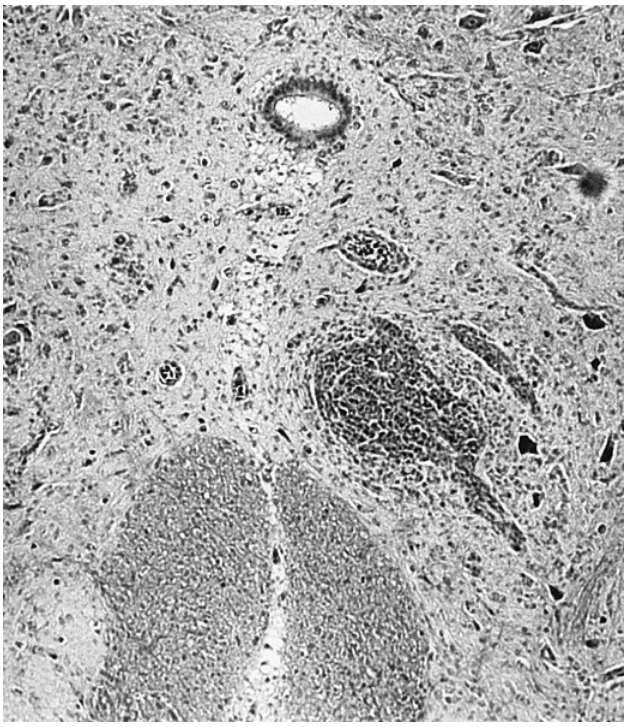


Figure 14.14 Spinal cord at the lumbar level of chick. Large glial nodule and several perivascular infiltrates of lymphocytes are in gray matter. The central canal is at the top. H&E. ×75.

small lymphocytes may pile up several layers to form an impressive perivascular cuff.

Microgliosis occurs as diffuse and nodular aggregates. The glial lesion is seen chiefly in the cerebellar molecular layer, where it tends to be compact (Figure 14.16). A loose gliosis usually is found in the n. cerebellaris, brain stem, midbrain, and optic lobes and less often in the corpus striatum. In the midbrain, two nuclei, Cn. rotundus and n. ovoidalis, are invariably affected with a loose microgliosis that can be considered pathognomonic. Another lesion of pathognomonic significance is central chromatolysis (axonal reaction) of the neurons in the nuclei of the brain stem, particularly those of the medulla oblongata (Figure 14.17). If several sagittal sections are made, one can almost always find this alteration. The dying neuron is surrounded by satellite oligodendroglia, and, later, microglia phagocytize the remains; the central chromatolysis is never seen without an attending cellular reaction.

Brain and spinal cord lesions from experimentally infected chicks on a sequential basis using light- and electron-microscopy and immunofluorescence techniques showed the most characteristic changes to be degeneration of Purkinje neurons in the cerebellum and motor neurons in the medulla oblongata and spinal cord (24). The central chromatolysis observed in the motor neurons was thought to be reversible, whereas affected Purkinje neurons always became necrotic. Purkinje neurons contained abundant viral antigen and crystalline arrays of virus particles in the cytoplasm (15). Degenerated neuronal cells showed dilatation of rough-surfaced endoplasmic reticulum, a reduction in ribosomes, and mitochondrial degeneration (15, 24, 79).



Figure 14.15 Perivascular infiltration and gliosis are seen in the nucleus cerebellaris. H&E, ×63. (Jakowski)

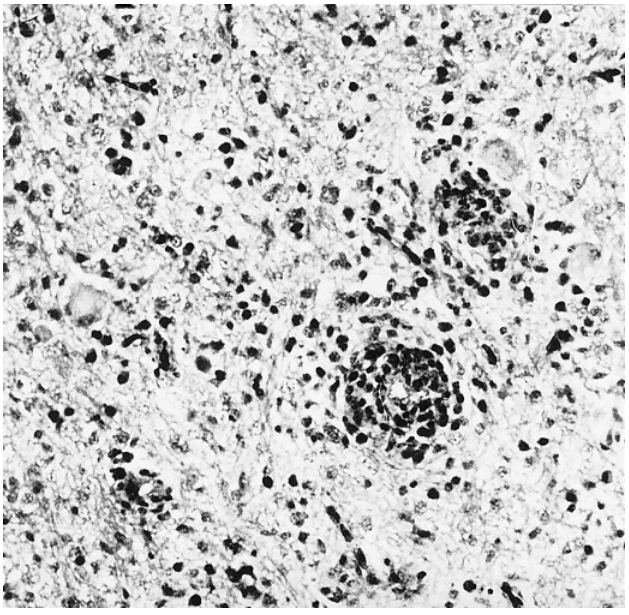


Figure 14.16 Cerebellum of a chick. Glial foci common in avian encephalomyelitis are in the molecular layer. H&E. ×75.

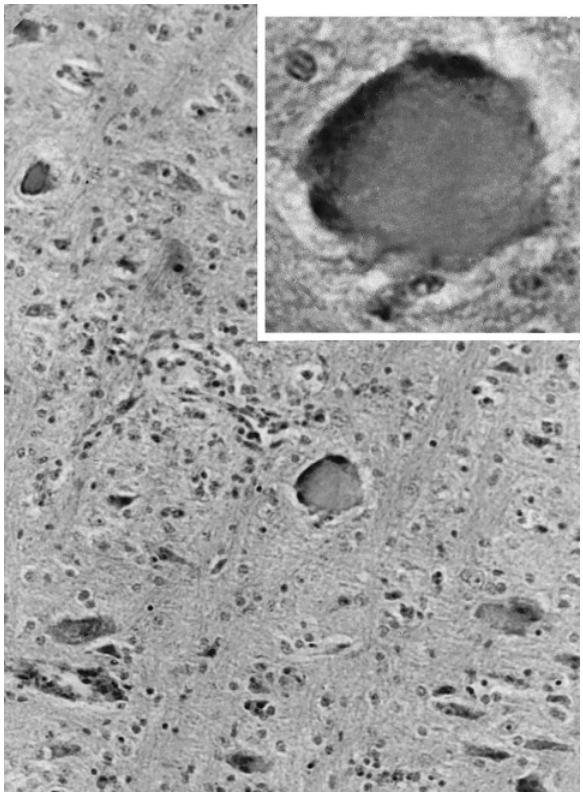


Figure 14.17 Medulla oblongata of chick. There is diffuse gliosis, and in the center a neuron is undergoing central chromatolysis. H&E. ×75. Inset shows tigrolysis and loss of nucleus. ×480.

The dorsal root ganglia often contain rather tight aggregates of small lymphocytes amid the neurons. The lesion is always confined to the ganglion and never enters the nerves (Figure 14.18). In general, signs cannot be correlated with severity of lesions or distribution in the CNS.

Visceral lesions appear to be hyperplasia of the lymphocytic aggregates scattered in a random fashion throughout the bird. In the proventriculus, aggregates of a few small lymphocytes normally are within the muscular wall; in AE, these are obvious dense nodules that are certainly pathognomonic (Figure 14.19). Similar lesions occur in the ventriculus muscle, but unfortunately, they also occur in Marek's disease. In the pancreas, circumscribed lymphocytic follicles are normal (37), but in AE the number increases several times (Figure 14.20). In the myocardium and particularly the atrium, aggregates of lymphocytes are considered to be the result of AE (56).

There appears to be an excellent correlation between clinical signs and histologic lesions in the nervous system. In one study, 11% had signs but no lesions, and 8% had lesions but no signs (34). Experimentally-inoculated chicks killed in sequential fashion invariably yield lesions 1–2 days before clinical signs. Recovered birds free from clinical signs still have CNS lesions for at least one week and probably much longer.

Pathogenesis of the Infectious Process

Significant differences exist between embryo-adapted AEV and field strains of the virus in terms of pathogenesis. This is largely because the adapted strains generally lose the enterotropic properties that characterize the natural strains. Consequently, adapted strains are relatively noninfectious by the oral route of exposure, do not replicate in the intestine, and are not excreted in the feces following infection by parenteral inoculation (11, 13).

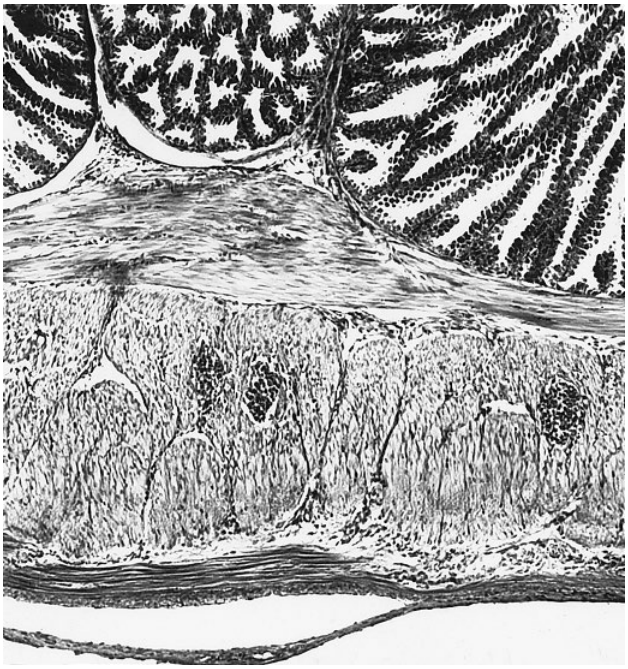


Figure 14.19 Proventriculus of a chick. Dense lymphocytic foci are in the muscular wall. This lesion is pathognomonic. H&E, ×30.

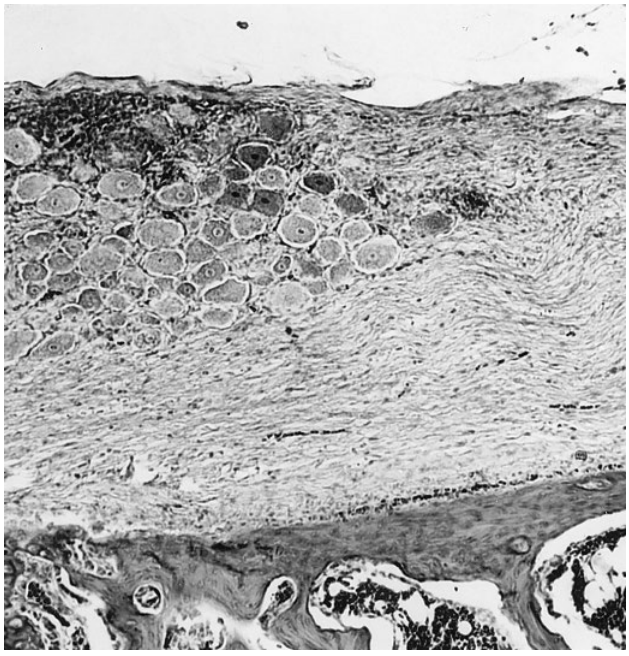


Figure 14.18 Dorsal root ganglion of lumbar level of a chick. Dense infiltrate of lymphocytes is confined to ganglion. The sciatic nerve is unaffected. H&E, ×75.

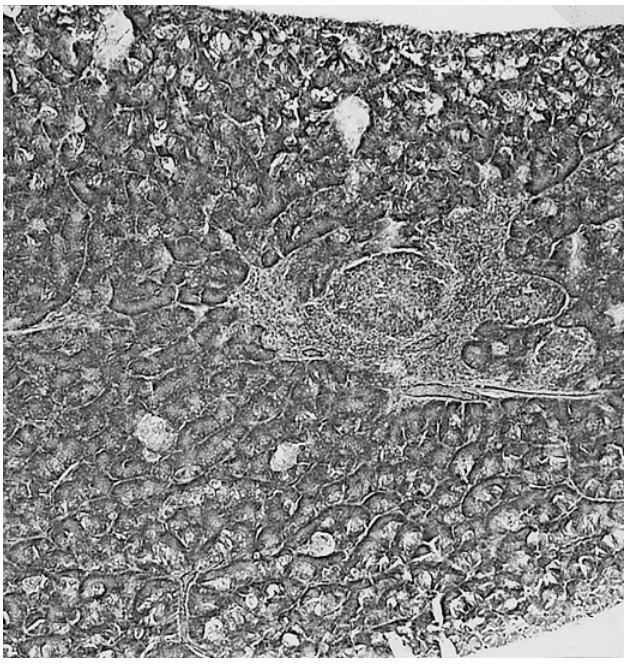


Figure 14.20 Pancreas of a young chick. Several follicles of lymphocytes are present. This lesion is significant only when abnormal numbers of follicles are present. H&E, ×30.

In young chicks exposed orally to field strains of AEV, primary infection of the alimentary tract, especially in the duodenum, is rapidly followed by a viremia and subsequent infection of the pancreas and other visceral organs (liver, heart, kidney, spleen) and skeletal muscle, and finally the CNS. Alimentary tract infections involve muscular layers, and pancreatic infections are found in both the acinar and islet cells, persisting more in the latter. Viral antigen is relatively abundant in the CNS where Purkinje neurons and the molecular layer of the cerebellum are apparently favored sites of virus replication (3, 29, 31, 42–44, 52, 53, 66). Neuroglial cells are probably also infected given the report of their susceptibility to AEV *in vitro* (46). Chicks with clinical signs at 10–30 days of age tend to have viral antigen mostly in the CNS and pancreas; lesser amounts of antigen have been seen in heart and kidney; and only small amounts have been seen in liver and spleen. Persistence of the virus infection is common in the CNS, alimentary tract, and pancreas. Interestingly, the CNS and the pancreas are the only sites uniformly infected by embryo-adapted strains of AEV, although small amounts of virus may be found transiently in other tissues including the liver, heart, and spleen.

In the intestinal tract of hens infected orally with a field strain of AEV, viral antigen was found in the epithelial tunica mucosa, circular muscle layer, and/or muscularis mucosa and in the tunica propria mucosa, but the detection rate was lower than has been reported for young chicks. No viral antigen was found in the CNS; presumably this lack of infection correlates with the absence of clinical disease in infected adults (43). As in young chicks, infection of older birds with embryo-adapted AEV has a more limited tissue distribution and/or lower titers of AEV in tissues other than those of the CNS, when compared with infection with field strains (29, 30).

Age at exposure is especially important for pathogenesis with birds that are infected at 1 day of age generally died, whereas those infected at 8 days developed paresis but usually recovered, and infection at 28 days caused no clinical signs (15, 73–75). Bursectomy but not thymectomy abrogated the age resistance (76). Young birds with lower immunologic competence may have an extended viremia persistence of virus in the brain, and development of clinical disease (113). Presumably, the immune response of an immunologically competent bird would stop the spread of infection before it reached the CNS (15, 73). Age resistance was not expressed when experimental infection was induced by IC inoculation of virus.

Immunity

Birds recovered from naturally occurring and experimental infection develop circulating antibodies capable of neutralizing the virus (see reviews [6, 60]). Antibodies to the VP1 protein appear to be the most important neutralizing epitopes, with only limited protection being induced from VP3 and VP0 proteins (71).

It has been clearly shown that humoral, but not cellular, immunity was important in curtailing infection. If the response is rapid, as is usual in birds greater than 21 days of age, the CNS infection apparently does not progress to the point where clinical signs may develop (15, 74).

Active

Positive virus neutralization (VN) antibody tests (i.e., those with a neutralization index (NI) of 1.1 or greater), can be found after 11–14 days PI (14, 75), and positive immunodiffusion (ID) tests as early as 4–10 days PI (31). Flocks of chickens with positive serology rarely if ever have recurrent outbreaks of AE.

Passive

Antibodies are transferred to progeny from the dam via the embryo and can be demonstrated in the egg yolk (59). Birds from immune dams were not fully susceptible to oral inoculation until 8–10 weeks of age, and antibodies were demonstrated in the serum until 4–6 weeks of age (14). Passively acquired antibodies can prevent development of disease and prevent or reduce the period of virus excretion in feces (13, 76). They also render embryonating eggs resistant to virus inoculated via the yolk sac, forming the basis for the embryo-susceptibility test.

Diagnosis

Isolation and Identification of Causative Agents

Two different RT-PCR tests have been developed that are being used for AEV RNA detection (36, 78). Although neither test was validated with a diverse set of AEV strains, clinically they were valuable in confirmation of outbreaks in several different countries (17, 22, 51, 80). Based on the clinical history and histologic examination (lesions as described in Pathology), particularly of the brain, pancreas, and proventriculus, an initial diagnosis of AEV infection can be made with confirmation by RT-PCR or virus isolation. The brain is an excellent source of virus for RT-PCR or isolation, although other tissues and organs induce the disease when injected into chicks (33, 68).

Historical methods of virus titration using inoculation of virus embryos, immunohistochemistry, or detection of antigen by IF tests are described in earlier editions of this chapter (57).

Serology

Chickens naturally exposed to AEV or vaccinated develop antibodies that can be measured by a variety of

methods. However, commercial ELISA tests are most commonly used to measure the antibody response and the other methods are rarely used. See previous editions of this chapter for more detailed information (57).

Antibodies induced to the VP1 protein, a structural viral antigen, are the most important for the detection of AE experimentally (71). The ELISA also appears to correlate well with the embryo susceptibility test and was used to diagnose active infections with AEV by an increase in titer with sequential serum samples (55, 80). The ELISA titers in hens have also been correlated with the resistance of progeny embryos to challenge with AEV (19).

Differential Diagnosis

In spontaneous cases, a tentative and frequently definite diagnosis of disease can be made when a complete history of the flock and typical specimens are provided for histopathology. Histopathologic evidence of gliosis, lymphocytic perivascular infiltration, axonal type of neuronal degeneration in the CNS, and hyperplasia of the lymphoid follicles in certain visceral tissues usually can be considered as a basis for a positive diagnosis. Virus isolation, RT-PCR, or a rise in titer with serologic tests gives a more specific diagnosis.

Avian encephalomyelitis should not be confused with other avian diseases manifesting similar clinical signs, such as Newcastle disease, equine encephalomyelitis infection, nutritional disturbances (rickets, encephalomalacia, riboflavin deficiency), and Marek's disease.

Avian encephalomyelitis is predominantly a disease of one- to three-week-old chicks. Because Newcastle disease may strike at this time, a problem of differential diagnosis can arise. Certain histological lesions are peculiar to AE: central chromatolysis as opposed to peripheral chromatolysis of Newcastle disease, gliosis in the n. rotundus and n. ovoidalis that is not observed in Newcastle disease, lymphocytic foci in the muscular wall of the proventriculus, and circumscribed lymphocytic follicles in the pancreas. Newcastle disease rarely causes an interstitial pancreatitis.

Encephalomalacia generally appears 2–3 weeks later than AE, and from the standpoint of clinical history, the signs should be no problem. Histologically, it causes severe degenerative lesions in no way similar to AE.

Marek's disease, which occurs still later, presents little difficulty. The peripheral nerve involvement and state of lymphomatosis of the viscera are two criteria not seen in AE.

Intervention Strategies

No satisfactory treatment is known for acute outbreaks in young chicks. Removal and segregation of affected chicks may be indicated under certain conditions, but they generally will not develop into profitable stock. After a flock has experienced an outbreak of AE, no further evidence of it is likely to be observed (50).

Vaccination

Control of AE is achieved by vaccination of breeder flocks during the growing period to ensure that they do not become infected after maturity, thereby preventing dissemination of the virus by the egg-borne route. Maternal antibodies also protect progeny against contact to AEV during the critical first 2–3 weeks. Vaccination may also be used with commercial egg-laying flocks to prevent a temporary drop in egg production associated with AE. Vaccines used to control AE in chickens have been shown to be efficacious in turkeys as well (16).

The development of AE vaccination strategies has been detailed by Calnek and Jehnich (10). Inactivated vaccines have been developed (9, 12) and may be useful in flocks already in production or where the use of a live virus is contraindicated. Most flocks, however, are vaccinated with a live, embryo-propagated virus, such as strain 1143 (14), which can be administered by naturally occurring routes such as via drinking water or by spraying (14, 18). Live virus vaccines, which can be stored frozen or after lyophilization (4, 49), are similar to field virus in that they spread readily within a flock. This allows for administration per os to a small percentage of the birds in a flock, which then spreads infection to others, although this method is generally unsatisfactory for birds in wire cages (18). The serologic responses to vaccine administered conjunctivally to 10% (but not 5%) of a flock were as good as those following drinking-water administration of virus to the entire flock (54). Vaccination by wing-web inoculation of AEV is also practiced in many flocks, but this method may carry some risk of clinical signs (20, 51). Generally, vaccination is done after eight weeks of age and at least four weeks before egg production.

It is important that embryo adaptation of strains used for live virus vaccines does not occur because: (1) adapted virus loses its ability to infect via the intestinal tract and is, therefore, no longer efficacious when administered by naturally occurring routes (14); and (2) adapted virus, like field strains, can cause clinical disease when administered by the wing-web route (11). Adaptation is detected by careful monitoring of inoculated embryos used in the production of vaccine for characteristic signs (see Etiology), and any adapted virus can be eliminated from vaccine seed virus stocks by passage in susceptible chicks inoculated orally.

Avian Hepatitis E Virus Infections

X.J. Meng and H.L. Shivaprasad

Summary

Agent, Infection, and Disease. Avian hepatitis E virus (avian HEV) is the primary causative agent of hepatitis–splenomegaly (HS) syndrome. Outbreaks of HS syndrome, characterized by decreased egg production and increased mortality in layers and broiler-breeders, have been reported in many countries including Australia and the United States. Avian HEV infection in chickens is widespread worldwide, although the majority of the infection is subclinical. Avian HEV belongs to the family Hepeviridae, and infects chickens, common kestrel, and red-footed falcon.

Diagnosis. Avian HEV cannot be propagated in cell culture, and diagnosis of avian HEV infection is primarily based on detection of viral RNA in feces, bile, and sera by reverse transcriptase-polymerase chain reaction (RT-PCR).

Intervention. A vaccine against avian HEV is not yet available, and strict biosecurity in chicken farms may limit the spread of virus.

Introduction

Hepatitis-splenomegaly (HS) syndrome is a disease of layer and broiler-breeder chickens characterized by increased mortality and decreased egg production and is caused by avian hepatitis E virus (avian HEV) (21, 35, 37). Dead birds have red fluid or clotted blood in their abdomens, and enlarged livers and spleens. Although first described as HS syndrome, the disease is also referred to as big liver and spleen (BLS) disease, necrotic hemorrhage hepatitis-splenomegaly syndrome, necrotic hemorrhagic hepatomegalic hepatitis, hepatitis-liver hemorrhage syndrome, and chronic fulminating cholangiohepatitis (35, 38, 51). There are only a few reports of HS syndrome outbreaks in the United States (11, 13), even though avian HEV infection is widespread in chicken flocks worldwide (2, 14, 29, 35, 39). In Australia, BLS was considered an economically significant disease of broiler breeders causing a drop in egg production (35, 37).

In addition to chickens, strains of HEV have also been genetically identified in humans and a number of other animal species (31, 32, 34). Swine HEV from pigs infects humans (31, 33), and the HEV strains from rabbit, deer, and mongoose may be zoonotic as well (31, 32). However, human infections by avian HEV have not been reported (25, 35).

Etiology

The primary causative agent of HS syndrome or BLS is avian HEV (21, 37, 38). Attempts to link the cause of HS syndrome to toxins or bacterins were unsuccessful, and bacteria could not be routinely isolated from affected livers except in one outbreak in which *Campylobacter spp.* were isolated (35).

Classification

All HEV are classified in the family of *Hepeviridae* consisting of two genera (43): genus *Orthohepevirus* (all mammalian and avian HEV) and genus *Piscihepevirus* (cutthroat trout virus). There are four species within the genus *Orthohepevirus*: *Orthohepevirus A* (HEV isolates from human, pig, wild boar, deer, mongoose, rabbit, moose, and camel), *Orthohepevirus B* (avian HEV from chickens and wild birds), *Orthohepevirus C* (isolates from rat, greater bandicoot, Asian musk shrew, ferret, and mink), and *Orthohepevirus D* (isolates from bat).

At least four genetically distinct genotypes of avian HEV have now been identified from chickens worldwide (26, 29): genotype 1 from chickens in Australia (29, 37), genotype 2 from chickens in the United States (21), genotype 3 from chickens in Europe (29) and China (55), and a putative new genotype from Taiwan and Hungary (2, 22, 26). Recently, a divergent avian HEV strain most closely related to *Orthohepevirus C* was identified in Hungary from a common kestrel (*Falco tinnunculus*) and red-footed falcon (*F. vespertinus*) (40).

Morphology

Human HEV is a spherical, non-enveloped, symmetrical virus particle of approximately 32–34 nm in diameter with cup-shaped depressions on the surface, similar to caliciviruses. The avian HEV particles revealed by negative staining EM of bile samples from chickens with HS syndrome are similar in size and morphology to human HEV (21) (Figure 14.21).

Chemical Composition

The genome of avian HEV is a polyadenylated, single-stranded, positive sense RNA molecule of 6,654 bp in length excluding the poly (A) tail, which is approximately 600 bp shorter than that of mammalian HEVs (7, 25, 29). The avian HEV genome consists of a short 5' non-coding

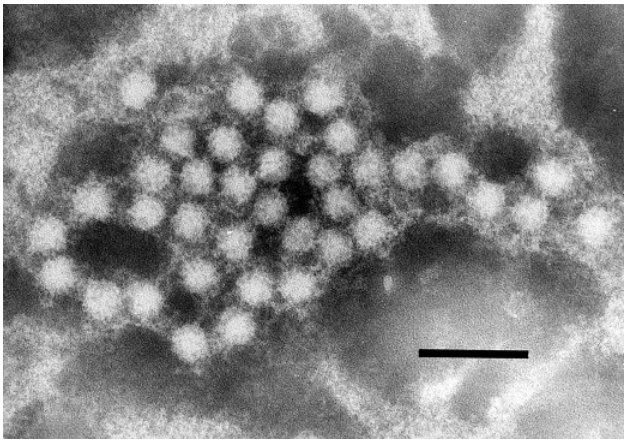


Figure 14.21 Electron micrograph of negatively stained 30–35 nm diameter avian hepatitis E virus particles in bile sample from a chicken with hepatitis-splenomegaly syndrome. Bar = 100 nm. Reproduced with permission from the Society for General Microbiology (21).

region (NCR) followed by three open reading frames (ORFs), and a 3' NCR. Open reading frame 1, located at the 5' end of the genome, encodes the nonstructural proteins. Open reading frame 2 encodes the immunogenic capsid protein (17). Open reading frame 3 encodes a small protein with unknown function.

Virus Replication

Avian HEV cannot be propagated in cell culture. In specific pathogen free (SPF) chickens experimentally infected with avian HEV, replicating viruses were detected in the liver as well as in several extrahepatic tissues including colon, cecum, jejunum, ileum, duodenum, and cecal tonsils (5), indicating that avian HEV replicates not only in the liver but in the gastrointestinal tissues as well. It is believed that avian HEV first replicates in the gastrointestinal tract following oral ingestion of the virus prior to reaching the liver (6). Avian HEV is excreted in large amount in feces (4, 5, 46).

Susceptibility to Chemical and Physical Agents

Liver suspensions containing avian HEV remained infectious after treatment with chloroform and ether (12) but lost infectivity after incubating at 56°C for 1 hour or 37°C for 6 hours. Avian HEV infectivity in liver suspensions was reduced 1000-fold after treatment with 0.05% Tween-20, 0.1% NP40, and 0.05% formalin (12, 35). The fecal–oral route of transmission indicates that avian HEV is resistant to inactivation by acidic and mild alkaline conditions in the intestinal tract.

Strain Classification

The nucleotide sequence identity among the genotypes 1, 2, and 3 avian HEV strains ranged from 82–83% over the entire genome, although the sequence identity among isolates within the same genotype is higher with approximately 90% among genotype 2 isolates (3). The putative genotype 4 of avian HEV from chickens in Hungary and Taiwan shared only approximately 82–87% nucleotide sequence identity with the three known genotypes (2, 22). The virus isolated from chickens with BLS in Australia is a genetically variant strain of avian HEV (30, 37) with approximately 80% nucleotide sequence identity with the genotype 2 avian HEV from the United States and Canada (1, 20, 21, 23, 25, 45). An apparently "avirulent strain" of avian HEV was identified from healthy chickens in Virginia (45), and unique genetic differences between the strains from chickens with HS syndrome and from healthy chickens were identified (6). Subsequent comparative pathogenesis studies in SPF chickens demonstrated that the avian HEV strain recovered from a healthy chicken is only slightly attenuated when compared to the strain recovered from a chicken with HS syndrome (5, 27), indicating that other cofactors are likely required for the manifestation of the full-spectrum of HS syndrome.

Laboratory Host Systems

Avian HEV can be propagated in chicken embryos only when the virus is inoculated intravenously but not by other conventional inoculation methods (8, 38). It has been demonstrated that Leghorn male hepatoma (LMH) chicken liver cells (ATCC CRL-2117), when transfected with RNA transcripts from infectious cDNA clones of avian HEV, supported avian HEV replication (24, 27). Viral antigens were detected in transfected LMH cells by immunofluorescence (IF) assay with avian HEV antiserum, and the fluorescent signals were mainly in the cytoplasm. However, the virus does not spread from cell to cell (24, 27).

Pathobiology and Epizootiology

Incidence and Distribution

First reported in western Canada in 1991 (42), HS syndrome has since been recognized in eastern Canada and the United States (1, 11, 13, 35). Avian HEV infection has now been reported in many countries worldwide (3, 8, 10, 19, 29, 36–38, 44, 49, 53, 55). Leghorn hens in cages are typically affected and HS syndrome frequently reoccurs on some farms (42). The disease has also been recognized in broiler breeder hens, and may be associated with sporadic mortality in dual-purpose hens and in small flocks kept on litter (35).

In the United States, avian HEV infection is enzootic in chicken flocks. Approximately 71% of chicken flocks and 30% of chickens in the United States were positive for antibodies to avian HEV (23). In another study, approximately 45% of the layer chickens were tested positive for anti-avian HEV IgY antibodies and 63% were positive for avian HEV RNA (14). In Spain, approximately 90% of the chicken flocks and 20–80% chickens were also tested seropositive for avian HEV antibodies (39).

Natural and Experimental Hosts

Under field conditions, in addition to chickens, genetically-divergent strains of avian HEV were also identified from a number of wild bird species including little egret, common kestrel, red-footed falcon, song thrush, little owl, feral pigeon, and common buzzard (40, 41, 52). However, the clinical significance of avian HEV infection in wild birds is unclear. Under experimental conditions, chickens of all ages are susceptible to avian HEV infection via both intravenous and oronasal routes of inoculation (4, 6, 24, 38, 46). Similarly, turkeys intravenously inoculated with an infectious stock of avian HEV also became infected (46). However, attempts to experimentally infect rhesus monkeys (25) and mice (Sun and Meng, unpublished data) with avian HEV were unsuccessful.

Transmission, Carriers, Vectors

Transmission within and between flocks appears to occur readily. In a prospective study of natural avian HEV infection in a chicken flock in Virginia (45), all 14 chickens monitored in the study were seronegative at 12 weeks of age. The first chicken seroconverted at 13 weeks of age, and by 21 weeks of age all 14 chickens in the flock had seroconverted (45). The transmission route for avian HEV is presumably fecal–oral, and experimental avian HEV infection has been successfully reproduced via oronasal route inoculation of SPF chickens (4). Feces are likely the main source of virus for transmission as large amounts of virus are shed in feces in experimentally infected chickens (4, 46). Avian HEV RNA was detected in day-old chicks, indicating vertical transmission (49). Egg whites from eggs of chickens experimentally infected with avian HEV contain infectious virus although experimental evidence of complete vertical transmission is lacking (15). Experimental aerosol transmission of avian HEV was unsuccessful (9, 12). It has been demonstrated that uninoculated chickens housed in the same room with avian HEV-inoculated chickens became infected through direct contacts (46). There is no known carrier or vector in the transmission of avian HEV.

Clinical Signs

The morbidity and mortality due to the disease in the field are low, and the majority of avian HEV infections are subclinical (23, 45). No clinical signs have been recognized in birds with HS syndrome prior to death (35). In some outbreaks, there has been a drop in egg production of up to 20%, but in other outbreaks egg production has not been affected (35). Under experimental conditions, 10–30% rate of decreased egg production over a 12-week period was observed in experimentally-infected laying hens (53). Hepatitis-splenomegaly syndrome is characterized by above-normal mortality in broiler breeder hens and laying hens of 30–72 weeks of age, with the highest incidence occurring between 40–50 weeks of age (35). Weekly mortality increases to approximately 0.3% for several weeks during the middle of the production period and may sometimes exceed 1.0% (13, 35, 42). In a Midwestern commercial layer farm, flocks had a 45% decrease in daily egg production from weeks 19 to 27, and 73% of the affected chickens were tested positive for avian HEV RNA (13). The clinical signs for BLS in Australia also vary from subclinical infection to egg drops that may reach 20% and are accompanied by up to 1% mortality per week over a period of 3–4 weeks (10, 19). Diseased birds may have pale combs and wattles, depression, anorexia, and soiled vent feathers or pasty droppings (9, 10, 19, 35). Small eggs with thin and poorly pigmented shells are produced in affected flocks, however the internal quality, fertility and hatchability of the eggs are unaffected (35).

Pathology

Under field conditions, dead chickens usually have regressive ovaries, red fluid in the abdomen, and enlarged liver and spleen (19, 35, 42). Prior to death, affected birds are usually in good condition, with pale combs and wattles, but some birds are also in poor condition (35, 42). Livers are enlarged with hemorrhage and/or clotted blood can be seen in the abdominal cavity (Figure 14.22). Livers can often be friable, mottled and stippled with red, yellow, and/or tan foci, and may have subcapsular hematomas and attached blood clots on the surface (35, 42). Spleens from affected birds are mild to severely enlarged (Figure 14.23), sometimes with white mottling, and some birds also have active ovaries (35).

Microscopically, liver lesions varied from multifocal hemorrhage to extensive areas of necrosis and hemorrhage and infiltration of heterophils and mononuclear inflammatory cells around portal triads. There is often segmental infiltration of lymphocytes and a few plasma cells in and around portal veins. Also, accumulation of homogenous eosinophilic material, amyloid in the interstitium of the liver and separation of hepatocytes are



Figure 14.22 Enlarged and hemorrhagic liver from a 63-week-old chicken with hepatitis-splenomegaly syndrome. Note the liver is not fatty.



Figure 14.23 Two enlarged and mottled white spleens from 56-week-old chickens with hepatitis-splenomegaly syndrome. The spleen on the left is of normal size.

common. In severe cases, discrete granulomas and possible thrombosis of portal veins were recognized. Lesions in spleens consisted of lymphoid depletion accompanied by an increase in the cells of mononuclear phagocyte system in later stages. There is accumulation of homogenous eosinophilic material, amyloid in the walls of small arteries and arterioles and in the interstitium. Eosinophilic material in both livers and spleens was identified as amyloid using Congo red stain (35) (Figure 14.24).

Under experimental conditions, gross lesions were observed primarily in the liver of SPF chickens experimentally infected with avian HEV (4). Subcapsular hemorrhages, and slightly enlarged right intermediate lobe of the liver (Figure 14.25) were observed in some of the infected chickens (4).

Microscopically, lymphocytic periphlebitis and phlebitis foci were observed in liver sections (Figure 26). The severity of liver lesions peaked at 10 days postinoculation (DPI) in the intravenous inoculated chickens. Other liver lesions such as foci of hepatocellular necrosis, amyloid in the interstitium, and subcapsular hemorrhages were also observed in some chickens (Figure 14.26). Microscopic lesions were also observed in spleen (mild lymphoid hyperplasia), thymus (mild cortical hypoplasia), kidney (occasional mild lymphocytic interstitial nephritis), and lung (mild lymphocytic and heterophilic parabronchial and interstitial inflammation) of SPF chickens infected with avian HEV (4, 5, 27).

Pathogenesis of the Infectious Process

The pathogenesis of avian HEV infection in chickens is largely unknown. Avian HEV is thought to enter the host through the fecal–oral route. After replication in the liver, avian HEV is released to the gallbladder from hepatocytes and then is excreted in feces (35). In addition to the liver, replicating avian HEV was also detected in colon, cecum, jejunum, ileum, duodenum tissues, and cecal tonsils of experimentally infected chickens (6). The gastrointestinal tissues appear to be the first site of avian HEV replication following oral inoculation, although the clinical and pathological significances of these extrahepatic sites of avian HEV replication remain unknown.

Immunity

The humoral antibody response in chickens infected with avian HEV appears at approximately 1 to 4 weeks PI (4, 46). The cell-mediated immunity in response to avian HEV infection in chickens is unknown. Avian HEV is not only genetically, but also antigenically, related to mammalian HEVs (16–18, 20, 21, 50). The capsid protein of avian HEV is immunogenic and induces protective immunity against avian HEV infection (17). Common as well as distinct antigenic epitopes in the capsid protein between avian HEV and mammalian HEV have been identified (18, 20, 50). A total of four putative antigenic domains (I, II, III, IV) have been identified in the avian HEV capsid protein. However, an animal challenge study showed that the immunodominant epitopes in the capsid protein are non-protective, suggesting that the protective neutralizing epitopes are likely not linear for avian HEV (16).

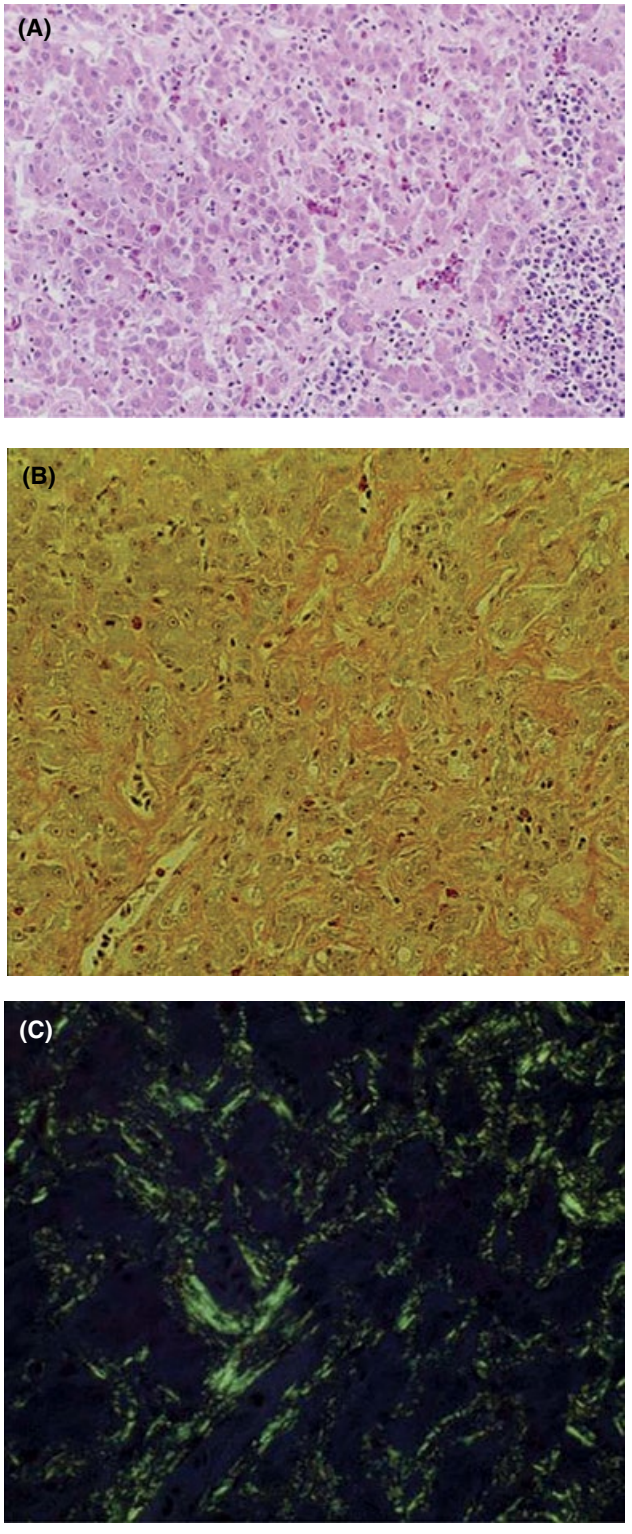


Figure 14.24 Photomicrographs of a liver from a chicken with spontaneous case of hepatitis-splenomegaly syndrome showing accumulation of homogeneous eosinophilic material, amyloid in the interstitium stained with H&E (A), congo red stain positive orange colored amyloid (B), and apple green birefringence property of amyloid under polarizing filter (C).

Diagnosis

A presumptive diagnosis of HS syndrome can be made on the basis of clinical signs and pathological lesions. However, HS syndrome needs to be differentiated from hemorrhagic fatty liver syndrome (HFLS) due to the presence of clotted blood in the abdominal cavity and hemorrhages in the liver with HS syndrome. The livers in HS syndrome are not fatty as in HFLS. Clotted or unclotted blood in the abdominal cavity or around the liver sometime can also be seen in cases of rodenticide (anticoagulants) toxicities. Due to the enlarged liver and spleen with HSS, the disease can be confused with leucosis but histopathology will help differentiate the two diseases.

Avian HEV does not replicate in cell culture. Although embryonic chicken eggs can be experimentally infected with avian HEV via intravenous inoculation (38), virus isolation with chicken embryos is not practical due to the technical difficulty and high mortality associated with the intravenous inoculation procedure (Haqshenas and Meng, unpublished data). Currently, the diagnosis of avian HEV infection is primarily based on detection of avian HEV RNA by RT-PCR or detection of antibodies by ELISA which indicates prior infection with avian HEV (23, 28, 47, 54). Avian HEV-specific RT-PCR and real-time quantitative PCR assays have been developed (23, 45, 46, 48, 54). However, the specificity of these PCR-based assays in detecting avian HEV strains in chickens from different geographic regions is not known, since at least four distinct genotypes of avian HEV exist worldwide (26).

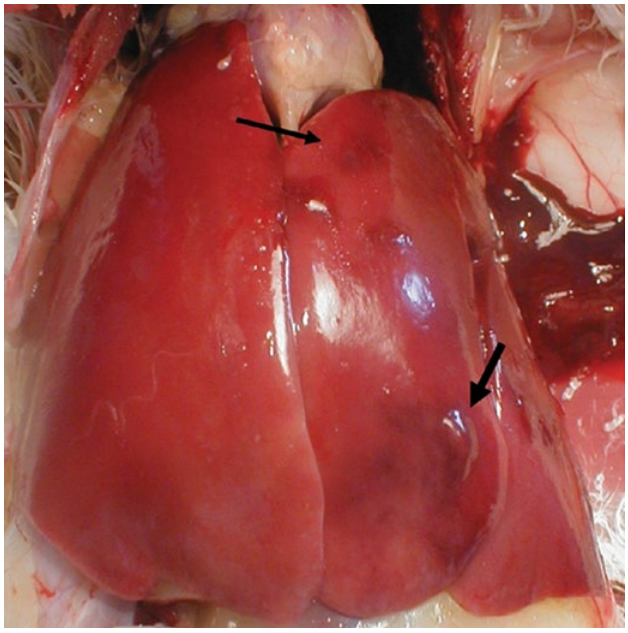


Figure 14.25 Gross lesion of a liver from a specific pathogen free chicken experimentally infected with avian hepatitis E virus (HEV): showing subcapsular hemorrhages (arrows). Reproduced with permission from American Society for Microbiology (4).

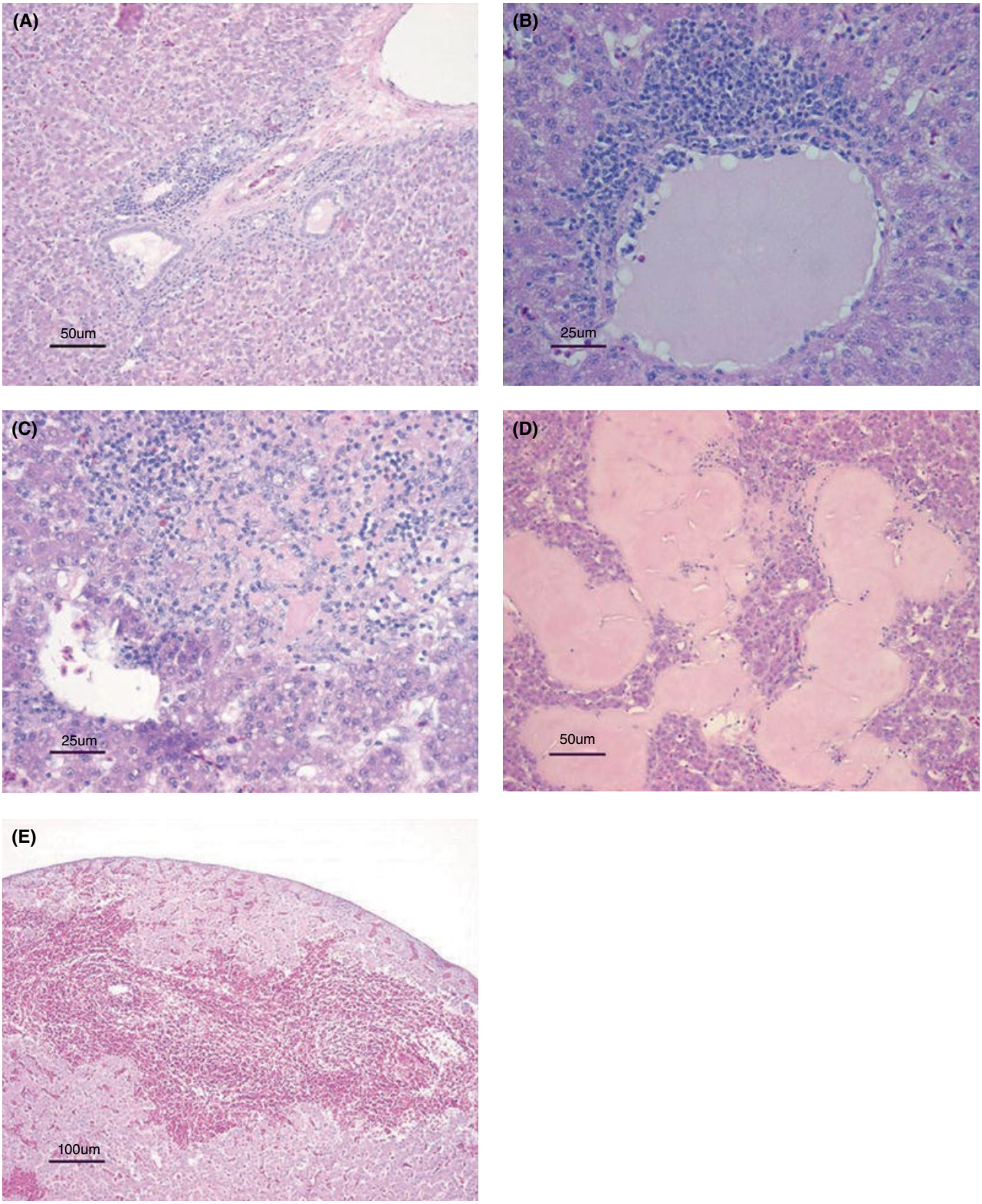


Figure 14.26 Microscopic lesions of the liver from chickens experimentally infected with avian hepatitis E virus (HEV). (A) A liver section from an oronasally-inoculated chicken, showing lymphocytic and scattered heterophilic portal vein periphlebitis. (B) A liver section from an intravenously (IV)-inoculated chicken showing focally intense lymphocytic venous phlebitis and periphlebitis. (C) A liver section from an IV-inoculated chicken showing locally extensive hepatocellular necrosis with lymphocytic inflammatory cell infiltration. (D) A liver section from an IV-inoculated chicken. Note architectural disruption and coalescing deposition of hypocellular homogenous eosinophilic matrix with displacement of hepatocellular cords. (E) A liver section from an oronasally-inoculated chicken. Note large focus of acute hemorrhage with local architectural disruption of hepatocellular cords and hepatic sinusoids. H&E staining. Reproduced with permission by American Society for Microbiology Press from (4).

Intervention Strategies

A vaccine against avian HEV is not yet available. Currently there is no treatment for avian HEV infection. Implementation of strict biosecurity in chicken farms may limit the spread of virus.

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15

Neoplastic Diseases

Introduction

Venugopal Nair

Summary

Agents, Infections, and Disease. This chapter refers to all the diseases that have neoplasm or cancer as a common feature. The majority of these diseases are caused by herpesviruses or retroviruses, designated as Marek's disease (MD), avian leukosis, and reticuloendotheliosis. The chapter also describes a set of tumors of a non-infectious etiology.

Diagnosis. Most of the neoplastic diseases can be diagnosed by the characteristic pathological changes in the affected tissues, but confirmed by virological, serological, and molecular methods of diagnosis.

Intervention. While diseases such as MD is controlled mainly by the widespread use of vaccines, most of the retroviral diseases are controlled by eradication of the virus from the infected flocks, supplemented by selection for genetic resistance.

Neoplastic diseases of poultry comprise a variety of conditions possessing a single common denominator: neoplasia involving one more of the cell types. Unlike in human medicine where the vast majority of cancers are of non-infectious origin (1), most of the neoplastic diseases affecting avian health have a viral etiology. Indeed, studies on avian oncogenic viruses have contributed immensely to our current understanding of a number of molecular mechanisms of cancer (2). This chapter deals primarily with the three most economically important virus-induced transmissible neoplastic diseases of poultry, namely: (1) the herpesvirus-induced Marek's disease (MD); (2) retrovirus-induced avian leukosis/sarcoma, and (3) reticuloendotheliosis. An additional final section covering tumors of unknown etiology is also included. Each of these neoplastic diseases or disease complexes are described in a separate section because of its etiologic distinctness.

The first section describes MD, a T-cell lymphoma induced in chickens by the highly cell-associated Marek's disease virus (MDV). Marek's disease lesions consist of CD4+ T-cell lymphomas affecting a number visceral organs and tissues, together with lymphoid cell infiltration into the peripheral nerves resulting in paralytic symptoms. Recent advances in MD research have provided significant insights into the molecular mechanisms of the disease, further strengthening its significance as an excellent biomedical model for T-cell lymphomas (3–6). Marek's disease has been controlled since the early 1970s by use of conventional live attenuated antigenically related vaccines. During the last four-and-a-half decades, research on MD has provided a better understanding of the viral gene functions, molecular mechanisms of the disease and factors affecting host genetic resistance (7). However, despite widespread use of vaccines and development of new methods of vaccination, MD still remains a major challenge to poultry health, particularly from the continuing increase in virulence of MDV strains (8, 9), possibly contributed by the vaccines themselves (10). Furthermore, the incidence of MD in other avian species such as turkeys and geese (11, 12) demonstrate the increasing host range and economic significance. Clearly, in the absence of control measures including vaccine failures, MD is capable of causing devastating losses in poultry (13, 14). As a disease occurring worldwide, with reports of vaccination breaks and probable emergence of more virulent pathotypes, MD continues to pose severe threats to the poultry industry, and developing strategies for its control remains one of the great challenges today (7). This section gives an up to date account of the scientific understanding of MD and its control strategies.

A second section describes a group of leukoses, sarcomas, and related neoplasms induced by a number of closely related groups of avian retroviruses termed the

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leukosis/sarcoma (L/S) viruses. The contributions of these retroviruses to tumor virology, such as the landmark study on transmissible tumors, the discovery of the *oncogenes* and the *reverse transcriptase*, have been hugely significant. The term leukosis is used because a leukemic blood picture is not always present during the course of leukemia-like proliferative diseases of the hemopoietic system (15–17). The various forms of hemopoietic system neoplasia induced by the L/S group of avian retroviruses include the lymphoid leukosis (LL), myeloid leukosis (ML), and erythroid leukosis (EL) caused by different subgroups of the virus. Primarily affecting the bursa of Fabricius and visceral organs, LL is the most common form of leukosis although efforts in the past have successfully eradicated these viruses from many of the commercial breeding flocks (15, 18). Other neoplasms of hematopoietic origin that can also be seen in avian leukosis virus (ALV)-infected chickens, albeit infrequently, include erythroblastosis, myeloblastosis, myelocytomatosis, and certain related neoplasms such as nephroblastoma and osteopetrosis (19). With the emergence of a new ALV subgroup J in the late 1980s, myelocytomatosis, became a major neoplasia particularly in meat-type chickens (16, 20). The incidence of ALV-J has been significantly reduced in Europe and North America through the successful implementation of eradication programs. However, it is alarming that significant losses continue to occur in countries such as China, where in addition to the commercial meat-type chickens, commercial layers and native breeds of chickens are affected (21–23). The section on leukoses/sarcoma viruses gives a comprehensive coverage of the recent advances in our understanding of the pathogenic mechanisms, diagnosis, and eradication methods. Technological advances in genome editing and the potential for inducing genetic resistance as a tool for disease control are also mentioned (24).

The third section describes reticuloendotheliosis (RE), a group of disease syndromes caused by reticuloendotheliosis virus (REV), unrelated to the leukoses/sarcoma group of viruses (25). The REV group includes different viruses associated with a number of disease syndromes. These consist of defective REV-T strain that induces rapid neoplastic transformation by virtue of the very potent virus-encoded oncogene v-*Rel*, an NFkB homolog (26). The most common clinical diseases induced by REV include chronic lymphoma as well as immunosuppressive runting disease. Reticuloendotheliosis virus infects chickens, turkeys, ducks, geese, pheasants, quail, and probably many other avian species. However, a major economic concern of REV arises from its potential as contaminants of live vaccines produced in chicken embryo cells or tissues. Reticuloendotheliosis virus could also be a barrier for the export of breeding stock to certain countries. The section gives very detailed account of some of pathogenic mechanisms of REV, and the recent developments in diagnosis and control.

Table 15.1 Transmissible neoplasms.

Virus type	Nucleic acid type	Virus classification of etiological agent	Neoplastic diseases
Retrovirus	RNA	Leukosis/sarcoma group	Leukoses
			Lymphoid leukosis
			Erythroblastosis
			Myeoloblastosis
			Sarcomas and other connective tissue tumors
			Fibrosarcoma, fibroma
			Myxosarcoma, myxoma
			Osteogenic sarcoma, osteoma
			Histiocytic sarcoma
			Related neoplasms
			Hemangioma
			Nephroblastoma
			Hepatocarcinoma
			Osteopetrosis
		Reticuloendotheliosis group	Reticuloendotheliosis
Herpesvirus	DNA	Marek's disease virus	Marek's disease

The fourth and final section describes tumors of unknown etiology on the basis of morphologic characteristics. Included are a wide variety of benign and malignant neoplasms derived from muscle, epithelial, and nerve tissues; serous membranes; and pigmented cells.

Lymphoproliferative disease (LPD), another neoplastic disease of turkeys reported in Europe and Israel is also induced by another retrovirus (27). The incidence of LPD of turkeys has always been sporadic and hence not included in this chapter.

Because many of the avian tumor viruses appear to have multipotent characteristics, that is, they can sometimes induce a variety of neoplasms, classification and nomenclature of virus-induced neoplasms present a problem. The dilemma is largely due to the fact that certain strains of these viruses induce some pathologic lesions difficult to distinguish from those induced by another unrelated virus. The two major lymphoid neoplastic diseases MD and lymphoid leukosis are particularly confusing. Although REV-induced lymphomas are infrequent and generally arise from contaminated vaccines, it could also add to the problems in differential diagnosis. A practical and useful strategy for the differential diagnosis of viral lymphomas has been proposed (28). The choice of terminology for the neoplasia used in this chapter (see Table 15.1) is based on that originally adopted by the World Veterinary Poultry Association (29) with suitable modifications.

Marek's Disease

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Summary

Agent and Disease. Marek's disease (MD) is widespread disease of chickens characterized by rapid-onset lymphoid tumors, immunosuppression, and paralysis caused by the highly contagious Marek's disease virus, an alphaherpesvirus belonging to *Mardivirus* genus.

Diagnosis. Marek's disease virus is ubiquitous and detecting the virus alone is not sufficient to make a confirmatory diagnosis, unless it is associated with characteristic clinical signs and lesions including visceral tumors and peripheral nerve lesions. A number of molecular diagnostic tests based on the detection of viral nucleic acids and viral proteins.

Intervention. Control of the disease is a challenge because of the ubiquitous nature of the virus, latent infection, and continuous shedding of the virus from the infected birds and long-term persistence of the virus outside the host in the poultry environment. Vaccination is the cornerstone of the control of MD along with improved biosecurity measures.

Introduction

Marek's disease (MD) is a common lymphoproliferative disease of chickens, usually characterized by mononuclear cellular infiltrates in peripheral nerves and various other organs and tissues including iris and skin. The disease is caused by a herpesvirus, is transmissible, and can be distinguished etiologically from other lymphoid neoplasms of birds.

Because the literature on MD has greatly expanded, it is no longer feasible to cite all relevant publications that provide the scientific basis for our current knowledge of the disease. In this chapter, literature is cited selectively, and reviews are often substituted for original papers. Readers are advised to refer to the chapter on MD from the previous editions of this book for more details from previous years. Useful recent books on MD are *Marek's Disease* (259) and *Marek's disease: An evolving problem* (158) and proceedings from the international symposia on MD (1).

Definition and Synonyms

The seminal description by Jozsef Marek (360) identified the disease as *polyneuritis*. Other common synonyms included *neuritis*, *neurolymphomatosis gallinarum*, and *range paralysis*. Jungherr and colleagues (294) proposed that the term *lymphomatosis* be subdivided into *visceral*, *neural*, and *ocular* forms. In 1961, Biggs (44, 48) proposed the term *Marek's disease* to distinguish the condition clearly from etiologically different lymphoproliferative diseases.

Marek's disease has also been subdivided into *acute* and *classical* forms, where the latter term designates forms of the disease prevalent prior to the 1950s (44). Marek's disease virus (MDV) can also induce other clinically distinct disease syndromes such as *transient paralysis*, *early mortality syndrome*, *cytolytic infection*, *atherosclerosis*, and *persistent neurological disease*.

Economic Significance

Prior to use of vaccines, MD constituted a serious economic threat to the poultry industry causing up to 60%

mortality in layer flocks and 10% condemnations in broiler flocks. Because vaccines are not 100% effective, sporadic losses still occur, but they are no longer as serious a problem. In 2004, the worldwide annual losses from MD were in the range of US$1 to 2 billion, although the authors have indicated that these figures are impossible to verify (381). The disease remains a major concern for the poultry industry due to the unpredictability of outbreaks and the possibility that vaccines may ultimately fail as a consequence of the evolution of more virulent strains of MDV.

Public Health Significance

Although there are several reports suggesting a role for MDV in the etiology of multiple sclerosis, there has been no evidence to suggest the MDV is infectious to humans (496, 562) or associated with human cancer (455).

Scientific Significance

Research on MD has contributed greatly to veterinary medicine, basic science, and comparative oncology. The disease is uncommonly complex, featuring an interplay of neoplasia and inflammation expressed as several distinct clinical syndromes, each modified in important ways by host genetic influences. Marek's disease virus, an alphaherpesvirus with lymphotropic properties of gammaherpesviruses, is highly cell-associated but readily transmitted, and its virulence varies and evolves. It has two unique sister viruses, both nononcogenic, that naturally infect chickens and turkeys. Infection induces complex immune responses usually resulting in high levels of protection. Vaccination for MD constitutes an outstanding example of successful disease control in veterinary medicine, and MD vaccines are the first effective vaccines against cancer in any species. Evolution of MDV virulence has also been shown as an example of a biological arms race of pathogens in the face of vaccination (57).

History

The seminal report by József Marek, published in 1907 (360), of paresis in four roosters is the first account of the disease named after him. A detailed history of MD research can be found in the previous edition of *Diseases of Poultry* (499), a paper by Professor Biggs (47), the videotape *Legacy of the 1960s*, and the historical archives of the American Association of Avian Pathologists. A long view on the last 40 years of MD research has recently been published as part of the 40th Anniversary of *Avian Pathology* (51).

Etiology

Classification

Marek's disease virus is a cell-associated herpesvirus (citations in [484]) with lymphotropic properties similar to gammaherpesviruses. However, its molecular structure and genomic organization are similar to alphaherpesviruses (68, 335, 567). As per the recent classification by the International Committee on Taxonomy of Viruses (ICTV) all MDV serotypes are grouped together in the genus *Mardivirus* (314). Members of the genus *Mardivirus*, described previously as three serotypes are now grouped as three species: Gallid alphaherpesvirus 2 (serotype 1), Gallid alphaherpesvirus 3 (serotype 2), and Meleagrid alphaherpesvirus 1 (herpesvirus of turkey [HVT], serotype 3). Serotype 1 MDV is the prototype virus for this group of avian viruses, and except where otherwise indicated MDV refers to serotype 1 virus. On the basis of their virulence, serotype 1 strains are further divided into pathotypes, which are often referred to as mild (m)MDV, virulent (v)MDV, very virulent (vv)MDV, and very virulent plus (vv+)MDV strains (597, 604). Nononcogenic Gallid herpesvirus 2 isolated from chickens (50, 125, 490) and HVT (307, 613) belonging to genus *Mardivirus* are also included in this chapter.

Morphology

The morphology and morphogenesis of MDV have been reviewed (142, 163, 164). Hexagonal nucleocapsids 85–100 nm in diameter and enveloped particles 150–160 nm in diameter may be seen in thin sections of infected cell cultures. Keratinocytes derived from chicken embryonic stem-cells showed capsids/virions although extracellular viruses could not be demonstrated (141). Enveloped virus particles appearing as irregular amorphous structures and measuring 273–400 nm have been demonstrated in negatively stained preparations of feather follicle epithelium (FFE) (92). Thin-section preparations of the FFE revealed large numbers of cytoplasmic enveloped herpesvirus particles in keratinizing cells. The morphology of MD virions in cell cultures and FFE is shown in Figure 15.1A.

The morphology of serotype 2 and 3 strains resembles that of MDV serotype 1 (393, 427, 490). In thin sections, however, nucleocapsids of HVT commonly show a unique crossed appearance (393).

Chemical Composition

Viral DNA

Physical Properties. The complete sequence of several strains belonging to the three serotypes confirmed that the genomes are very similar consisting of linear,

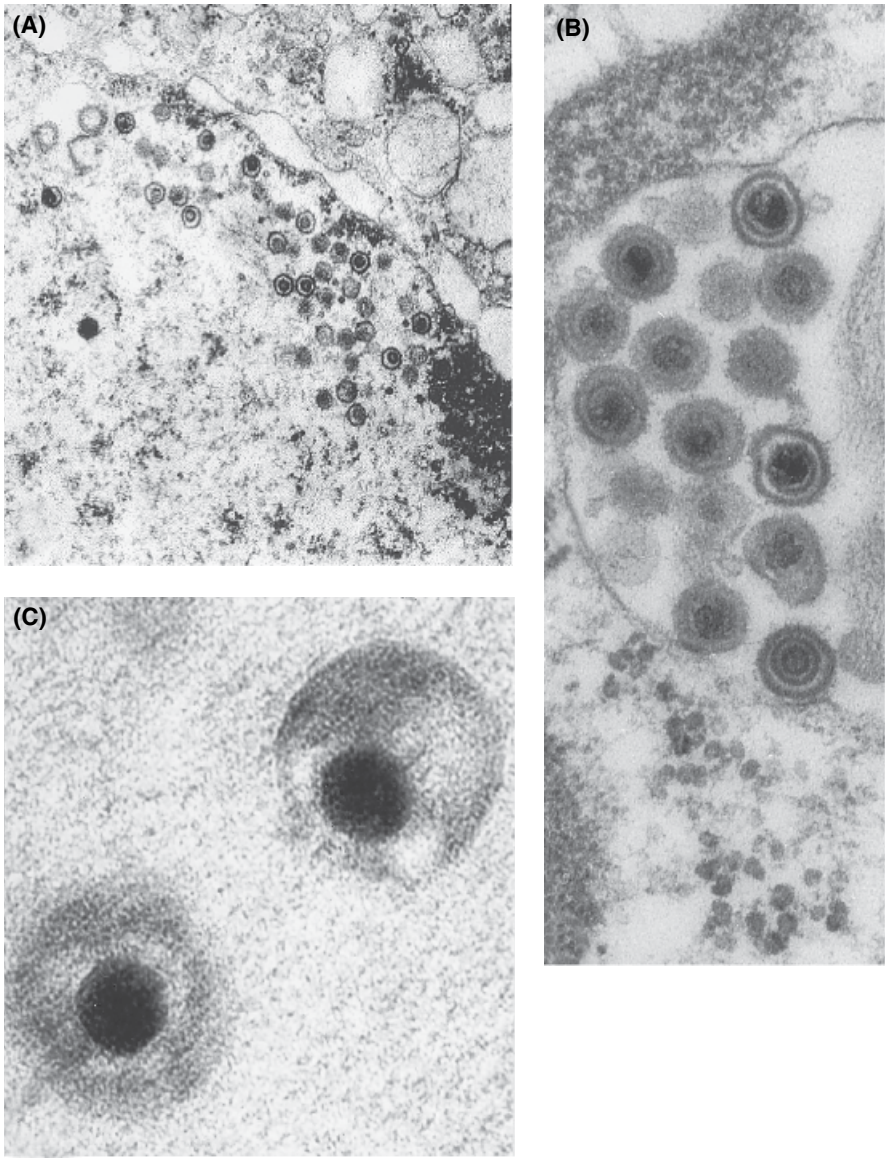


Figure 15.1 Electron micrographs of Marek's disease virus (MDV). (A) Thin section of cultured duck embryo fibroblasts infected with MDV showing scattered virions in nucleus. ×38,400. (B) Thin section of cultured duck embryo fibroblast infected with MDV, showing enveloped virions in a nuclear vesicle. ×360,000. (C) Thin section of feather follicle epithelium (FFE) of chicken infected with MDV showing enveloped virions within the cytoplasmic inclusions. Note difference in morphology compared with (B). ×70,000. (413). (Nazerian)

double-stranded DNA molecules of approximately 160–180 kb with a buoyant density in neutral CsCl of 1.706 g/mL for serotype 1 (158, 499). It is difficult to separate viral DNA from host cell DNA because its density is close to that of chicken DNA. Cloning of the complete MDV genome as bacterial artificial chromosomes (BAC) has greatly facilitated the production of MDV DNA (506, 645). Infectious BAC clones of a number of strains have been constructed allowing rapid manipulation of MDV genomes to identify various determinants associated with MD biology (118, 313, 541). Similarly, the rescue of infectious MDV from overlapping cosmid clones of the Md5 strain of MDV has also been reported (463).

Structural Organization. The genomic structure of the three serotypes is typical for alphaherpesviruses as previously suggested (114) with a unique long (UL) and a unique short (US) sequence. These unique sequences are flanked by sets of inverted repeat sequences: the terminal repeat long (TRL), internal repeat long (IRL), internal repeat short (IRS), and terminal repeat short (TRS), respectively. Alpha (a)-like sequences, believed to be important for the cleavage and packaging of viral DNA into virions, are located at the terminal ends of the TRL and IRL and between the IRL and IRS regions (11, 282, 335, 540, 567).

The complete genome sequences of a number of serotype 1 MDV strains as well as other vaccine serotypes

have now been determined (499). Comprehensive analysis of the sequence of the TRL/IRL regions in the genomes of 13 strains of varying virulence has identified several single nucleotide polymorphisms (SNPs) which loosely partition between attenuated and non-attenuated strains (537, 539).

DNA Structure in Infected Cells. The structure of viral DNA in infected cells is dependent on the virus–cell interaction. Linear viral DNA can be found in nuclei of cells undergoing virus replication (114). It remains unknown how viral DNA is maintained in latently infected, nontransformed cells (80, 376), although there was some evidence on the role of epigenetic regulation of the viral genome (63). The status of DNA in transformed cells has been difficult to determine in part because a variable percentage of the transformed cells may undergo viral replication at any point in time, in which case linear DNA can be detected. Initial investigations into the status of the MDV genome have indicated an absence of integration (553), MDV genome is now thought to exist as a mixture of integrated and episomal DNA (499). Marek's disease virus can be found integrated at multiple sites in the chromosomes of cells derived from MDV-related T-cell lymphomas, suggesting a correlation between integration and oncogenicity. The integration sites are preferentially located near the ends of the chromosomes within the telomeric region. The telomere-like sequences located at the terminal ends of the MDV genome are thought to assist in the preferential integration of the viral DNA (312). Interestingly, MDV encodes an RNA telomerase subunit (viral TR [vTR]) that shares 88% sequence identity with the chicken TR (cTR) gene (201). The role of telomeres and telomerases in MDV integration, pathogenesis and oncogenesis has been recently reviewed (312).

Structural Changes by Recombination and/or Mutation. Serotype 1 strains quickly develop altered biologic characteristics upon serial passage *in vitro*, such as loss of oncogenicity, reduced expression of glycoprotein C (gC) (131), and decreased replication *in vivo* (494) indicating that spontaneous mutations may have occurred. Accumulations of such mutations after continuous cell culture passages and generations of mixed population of viruses have been recently demonstrated by sequence analysis (537, 539). The gradual evolution of pathotypes toward greater virulence and the changes in biologic properties of MDV during *in vivo* backpassage (591) further support the mutability of MDV.

These biological changes are accompanied by several molecular changes, although it is not clear which molecular change correlates with a specific biological change. An expansion was found within the Bam-HI D and H fragments that are commonly associated with cell culture passage and attenuation in serotype 1 strains (204, 260, 528). This expansion was caused by a tandem amplification of direct 132 bp repeat (266, 359, 476). Other changes have also been described including the deletion of 400 bp in the Bam-HI A fragment of CVI988 clone C (988C) and 988 C/R6 (266) and a deletion of 200 bp in the BamH1 L fragment of the vvMDV strain Md11 (587). Additional changes have been reported for CVI988 in the meq gene (335, 538) and the ICP4 promoter/enhancer region of CVI988 (299). It is not clear whether these differences are a consequence of cell culture passage or reflect strain differences.

Viral Genes and Proteins

Over the last 25 years, a number of individual genes of MDV-1 have been identified and sequenced, and the proteins have been characterized (478, 498). Comprehensive reviews based on the complete sequences for the three serotypes have been published including lists of open reading frames (ORFs) and their putative products (499). Table 15.2 summarizes the location of the ORFs and indicates the number of ORFs with homologues to HSV, the number of ORFs with homologues shared among the three serotypes, and the number of unique genes for each serotype. Many of the genes in the U_L and the U_S regions have homologues with HSV and equine herpesvirus-1 and 4-, and the genome organization is similar to these two alphaherpesviruses (354). For this chapter, the MDV genes are grouped into two general categories: genes with homologues in alphaherpesviruses and genes unique for MDV. Only the genes that are important for the pathogenesis and immune responses will be reviewed briefly. The reader is referred to the contemporary literature for additional information (158, 499).

Genes with Homologs in Alphaherpesviruses. This broad category of genes can be divided into immediate early (IE), early, and late genes, which are with few exceptions important for virus replication.

Immediate Early and Early Genes with Homology to HSV. The IE genes are important transcriptional regulators. Four IE genes have been identified: intracellular protein (ICP)4, ICP0, ICP22, and ICP27. Anderson *et al.* (15) identified ICP4 as a 4245 bp ORF, but sequence data indicated the presence of an ORF of 6969 bp. This agrees with the finding that two functional promoter/enhancer regions are located upstream of the larger ORF and that the putative promoter/enhancer region for the short ORF was nonfunctional *in vitro* (299). Proof that ICP4 protein is a transactivator was provided by transfection of the MD cell line (MDCC) MSB-1 with the short form of ICP4, showing increased transcription of the endogenous ICP4, pp38, and pp24 genes (185, 447).

Table 15.2 Number of tentative genes in the three serotypes of Marek's disease virus (MDV) in relation to other alphaherpesviruses.[a]

		Location of expected functional open reading frames (ORFs)[b]						
Serotype	**Gene classification**	**TRL (R-LORF)**	**UL (L-ORF)**	**IRL (R-LORF)**	**IRS (RS)**	**US (S-ORF)**	**TRS(RS)**	**Total[c]**
1	HSV Homolog[d]	0	57	0	1	7	1	*65*,**66**
	MDV-specific[e]	1	4	1	0	1	0	*6*,**7**
	Serotype-specific	13[h]	8	13	2	3[H]	2	*26*,**41**
	Total	14	69	14	3	11	3	*97*,**114**
2	HSV Homolog	0	59	0	1	7	1	*67*,**68**
	MDV-specific[f]	1	4	1	0	1	0	*6*,**7**
	Serotype-specific	9	4	9	1	4	1	*17*,**27**
	Total	10	66	10	2	12	2	*90*,**102**
3	HSV Homolog	0	59	0	1	8[i]	1	*68*,**69**
	MDV-specific[g]	0	6	0	0	1	0	*7*,**7**
	Serotype-specific	4	2	4	6	1	6	*13*,**23**
	Total	4	67	4	7	10[i]	7	*88*,**99**

[a] For reference see (15, 279, 344, 373).
[b] Based on the location of the start codon.
[c] The italic numbers indicate the number of single genes for each serotype; the bold figures give the total number of genes including the duplications in the repeat regions.
[d] Based on the sequence of the GA strain, nomenclature adapted from (340).
[e] Serotype-specific genes with homologues present in serotype 2 or 3.
[f] Serotype-specific genes with homologues present in serotype 1 or 3.
[g] Serotype-specific genes with homologues present in serotype 1 or 2.
[h] The sequence for Md5 has minor differences compared with GA.
[i] Includes 2 copies of US8.

The MDV ICP27 phosphoprotein (465) localizes in the nucleus, can transactivate pp38 and pp14 independently of ICP4, and represses the early thymidine kinase gene (466). Additionally, MDV ICP27 also interacts with SR proteins and inhibits splicing of cellular telomerase chTERT and viral vIL8 transcripts (14). ICP0 (LORF1) has been identified as an ORF in the TR_L and IR_L, and a recent study using proteomic approaches has demonstrated that the ICP0 gene product is expressed in MDV-infected CEF (348).

Late Genes. The late gene products include the nucleocapsid proteins, the tegument proteins, including VP16, and the glycoproteins (354). The glycoproteins (gB, gC, gD, gE, gH, gI, gK, gL, and gM) are presumed to be important for infection of cells, transfer of virus from cell to cell, and immune responses.

gB, encoded by UL27, consists of a complex of three glycoproteins with molecular weights of 100, 60, and 49 kDa and is important for cell attachment and/or penetration based on the production of gB-specific virus-neutralizing (VN) antibodies (reviewed by [354]). Deletion of gB from MDV prevented the cell to cell spread demonstrating the essential nature of this protein for MDV replication (506). In a recent study adenovirus-based expression of gB was shown to be sufficient in inducing protection against virulent MDV challenge (28). Similarly, some of the peptides derived from gB showed antiviral properties providing some insights into MDV entry into cells (583).

The UL44 gene encodes gC, a 57–65 kDa glycoprotein identified in some early references as gA, which is extensively synthesized in productively infected cells and is expressed on the cell surface. In addition, gC is actively secreted by infected cells (139, 272–274) and is one of the major antigens to which the chicken immune system mounts a substantial serological response. More recently, gC and UL13 were shown to be important in horizontal transmission (286).

The importance of gD, coded by US6, is poorly understood. It is nonessential for horizontal transmission (16) and is expressed poorly (406, 552) *in vitro* probably as a consequence of no or limited transcription. Limited expression of gD compared to pp38 and gB has been described in FFE (398), suggesting that specific transcription factors in the FFE may be needed for the production of gD.

The functions of the other glycoproteins have not been studied in detail. The gI and gE proteins interact with each other based on immunoprecipitation assays (552). Associations of mutations in the gL with MDV pathogenesis

and reduced immune responses have been reported (479, 555). Mutants constructed in a BAC clone carrying deletions in the gM, gI, or gE gene indicate that the encoded glycoproteins are essential for virus replication, because the deletion mutants are unable to transfer infectivity from infected to uninfected cells (507, 564).

Genes Unique for MDV. Several genes have been identified that are unique for MDV strains (Table 15.2). Some of these genes are present only in serotype 1, and others may have homologues in MDV serotype 2 and/or HVT.

Latency Associated Transcripts (LATs). The LATs are a group of transcripts antisense to ICP4 and have been reviewed in detail (80, 376). These include a large 10 kb transcript as well as several spliced transcripts referred to as MSR (MDV small RNA) or SAR (small antisense RNA). The importance of LATs for latency or transformation is unclear. Latency associated transcripts are expressed in both lytically infected and transformed cells, including the MDV positive QT35 cell line (633).

Meq (Marek's EcoQ). The molecular biology of Meq (R-LORF7) has been reviewed (324, 376, 388, 416, 551). The Meq protein of 339 amino acids contains a basic leucine zipper (bZIP) domain at the N terminal closely resembling the jun/fos oncogene family. Several studies, inclusion deletion analysis have clearly demonstrated the critical role of Meq in oncogenesis (352). Meq gene also shows diversity among different isolates and it has been suggested that the positive selection may be driving evolution (410, 626). Variations in the sequence of the proline-rich domains also showed association with virulence (512).

Meq shows differential binding to different promoters depending on its dimerization status. As homodimers or heterodimers with leucine zipper proteins, Meq can transactivate different promoters (64, 546, 547) resulting in the upregulation of a number of genes including interleukin (IL)-2 and CD30, a member of the tumor necrosis factor receptor II (TNFR-II) family (78, 85). A number of subsequent studies have demonstrated efficacy of Meq-deleted viruses as efficient vaccines (331, 336, 526). The mechanism behind such improved immunogenicity is not fully understood, however gene expression changes in Meq-deleted viruses (336) and reduced immunosuppressive effects (344) are thought to contribute to this effect.

vIL-8. The vIL-8 gene (R-LORF2) is located in the long repeat region and originally was identified as a spliced meq variant (432, 433). The gene consists of three exons and is expressed late during cytolytic infection. IL-8 attracts T cells, especially after IL-8 receptors are upregulated by interferon-γ (IFN-γ) suggesting a role in the switch of infection from B to T lymphocytes (502). Subsequent studies have also shown that vIL-8 promotes lymphoma formation through targeted recruitment of B cells and CD4+ CD25+ T cells (186).

Viral Lipase. All the three serotypes of MDV encode the viral lipase gene (vLIP) (11, 282, 316, 335, 567). vLIP, a soluble, glycosylated protein, is encoded by the LORF-2 gene consisting of two exons. The first exon codes for the signal peptide, and the second exon codes for the lipase activity. vLIP is probably an IE or early protein (296). The glycosylated protein is required for the efficient lytic replication in birds (297).

pp38/pp24. The MDV phosphorylated protein complex, often referred to as pp38/pp24, is coded by two genes located at opposite ends of the UL region (654). Details of the previous research to understand the role of pp38/pp24 have been reviewed in previous editions of the book (499). Homologs for pp38 have been identified in serotype 2 strains (282, 407) and HVT (11, 530), but their functional relationships are not known.

The function of the pp24/pp38 complex has not been elucidated. Originally, it had been linked to oncogenicity because pp38 is expressed in the cytoplasm of a variable proportion of MDV-transformed, latently infected lymphocytes (146, 271). Expression of pp24/pp38 can be enhanced by activation (447, 633) suggesting that pp38 may play a role during reactivation and subsequent virus replication. pp38 is essential for cytolytic infection of B cells and maintenance of transformed state (463), although deletion did not affect the ability of the virus to spread horizontally (228). CVI988 also expresses pp38, but shows an amino acid mutation in an epitope defined by monoclonal antibody (mAb) H19 (148). The demonstration that vvMDV5 strain expressing the pp38 protein from CVI988 remains oncogenic indicates that the attenuation of CVI988 is not associated with pp38 (329).

The 1.8 kb Gene Family. The promoter/enhancer regions of pp38 and pp24 are part of a bidirectional promoter complex regulating the transcription of pp38/pp24, the 1.8 kb gene family and the origin of replication (165, 522). Please refer to the chapter in the previous edition of this book for detailed descriptions of the previous research on this gene family (499). Several IE transcripts originate from the 1.8 kb gene family containing three exons (376, 478). These transcripts are truncated in attenuated strains due to an expansion of a tandem 132 bp direct repeat (DR) (132 bp DR) (58, 476). The difference in the numbers of copies of these repeats between virulent and vaccine strains forms the basis of their differentiation by polymerase chain reaction (PCR) assays (34, 525, 655), although these are not directly linked to the oncogenicity (527).

Telomerase RNA (vTR). The existence of a unique gene encoding the RNA telomerase subunit (vTR) was identified

in the IR_L/TR_L region of the MDV genome (201). Marek's disease virus vTR showed nearly 88% sequence identity to the chicken telomerase RNA (ChTR) indicating its transduction from the host genome. vTR, regulated also by the action of c-myc (523), can constitute telomerase activity by interacting with chicken telomerase reverse transcriptase (ChTERT) more efficiently than ChTR (201, 202). The direct association between MDV oncogenicity and vTR was demonstrated using MDV lacking either one or both copies of vTR (119, 565) that resulted in significantly impaired ability to induce lymphomas with reduced ability for dissemination (303–305). The role of telomeres and telomerase in MD pathogenesis has recently been reviewed (312).

MDV-encoded microRNAs. MicroRNAs (miRNAs) are a distinct class of small regulatory molecules of approximately 22 nucleotides affecting gene expression in various cell types (635). These have been identified in a large range of organisms including several herpesviruses (389). A number of miRNAs have been identified in MDV many of which are expressed at very high levels (138, 561, 636, 639). Some of these miRNAs have been shown to have direct roles in pathogenesis (556, 652, 653, 656).

Other Unique Genes. Proteins have not been identified for several unique ORFs that are transcribed in tumor cells. Most of these have not been further studied with a few exceptions. RLOR5a (400) is expressed in tumor cell lines, although its function remains unknown. Similarly, RLORF4 has also been shown to be associated with tumor development (289). Marek's disease virus also encodes the ubiquitin-specific protease domain within the major tegument protein-coding gene UL36 (285, 570). Expressed at high levels in the FFE cells, it may have a role in virus morphogenesis in the feather (286).

Viral Vectors. Several nonessential sites in the three serotypes of MDV can be used for the insertion and expression of foreign and specific MDV genes (reviewed in [261, 349, 412]). The anticipated advantages of MDV-vectored vaccines are that these vaccines will protect simultaneously against MD and other pathogens, and reactivation from latency will reinforce immune responses against MD and the other pathogens (18, 67, 584).

Virus Replication

Replication of the three serotypes is typical of other cell-associated herpesviruses and has been reviewed extensively (47, 301, 473, 484, 498). For initial infection of cultures or chickens by cell-free virus, enveloped virions bind to cellular receptors probably by gB perhaps in combination with other glycoproteins. Heparan sulfate, a member of the glycosaminoglycans, has been identified as one of the cellular receptor molecules (338). Recent data demonstrate the role of the US3-encoded kinase in the morphogenesis as well as cell to cell spread of virions through the effect on stress fiber breakdown and polymerization of actin (509). The glycoproteins gE, gI, and gM play a role in the transfer of virus from infected to uninfected cells (507, 564). Replication rates vary with serotype, passage level of the virus strain, cell type, and temperature of incubation.

The spread of virus *in vivo* from cell to cell will require intimate contact between infected and uninfected cells, which are most often lymphocytes, although epithelial cells also can be involved in this process. The precise interaction between these cells remains one of the important unsolved issues, although recent demonstration of epithelial-specific expression of the UL47 tegument protein (287) suggest distinct virus–host interactions in these cell types.

Virus-cell interactions

Three general types of virus–cell interactions are recognized: productive, latent, and transforming.

Productive Infection. During productive infection, replication of viral DNA occurs; proteins are synthesized, and in some cases, virus particles are produced. Replication is correlated with virulence (172), but with all serotypes the number of genome copies per cell can increase 100-fold and exceed 1,200 in the case of HVT (300). Two types of productive infection exist. Fully productive infection in the FFE of chickens results in development of large numbers of enveloped, fully infectious virions (92). In productive-restrictive infection, most of the virions are nonenveloped and noninfectious. A variable number of the virions in cultured cells may be enveloped, which can be recovered as cell-free, infectious virus by disruption of cells. In all susceptible cells, productive infection leads to intranuclear inclusion body formation and lysis of the cell. A gene for the viral host shut-off protein has been identified, UL41 (354), that is probably responsible for the initiation of the lytic process. Lytic infection *in vivo* can cause frank necrobiotic lesion formation. Because of this, productive infection has been termed cytolytic, and the terms are used synonymously (89).

Latent Infection. Latency and tumorigenesis in MD have been reviewed (387). Latent herpesvirus infections have been defined as the presence of viral DNA in the absence of viral transcripts and proteins, although LATs have been described for many herpesviruses. This definition is appropriate for the nontransforming serotype 2 and 3 strains. For serotype 1 strains, the distinction between latency and transformation is often problematic. In both cases, the viral genome is present, but no information is available on differences in transcriptional regulation

between latently infected and transformed cells, because it is impossible to separate latently infected, nontransformed cells from noninfected cells. As a consequence, studies on latency have often been done in MD transformed cell lines.

Marek's disease virus latency mostly is associated with CD4+ T cells, although CD8+ T cells and B cells can also be latently infected (105, 337, 386). Fewer than five copies of the viral genome are present in latently infected cells (473). Marek's disease virus latency is maintained through various mechanisms including non-random *de novo* DNA methylation and histone modifications, with different genomic regions separated by chromatin boundary elements (63, 351). The MDV genome can be reactivated from latently infected cells and tumor cells by inoculation of susceptible chickens, co-cultivation with permissive cells, and *in vitro* cultivation of latently infected lymphocytes. The latter approach can be used to estimate the number of latently infected cells by enumeration of antigen-positive cells at 0 hours and 48 hours in culture (107).

Transforming Infection. Transforming infections occur only in cells infected with serotype 1 MDV. Selection of transformed cells from the background of immunologically committed and noncommitted cells (431) would facilitate comparative studies on transformed cells in tumors and tumor cell lines. The search for specific surface markers associated with tumors has identified antigens, broadly referred to as MD tumor-associated surface antigen (MATSA), detected on cells from MD lymphomas and lymphoblastoid cell lines but not on the surface of productively infected cells (443, 623). Recent studies confirm that $CD30^{hi}$ expression is characteristic of MD lymphomas suggesting that CD30 is a component of a critical intracellular signaling pathway perturbed in neoplastic transformation (78, 511). Both MATSA and CD30 can be used to enrich for transformed cells in tumor cell suspensions.

Virus Stock Production and Stability. Productively infected cell cultures are a common source of cell-associated virus stocks for all three viral serotypes and for cell-free HVT stocks. Techniques for the production and cryopreservation of cell-free and cell-associated virus stocks have been described (reviewed in 130). Cell-associated stocks of MDV or HVT are routinely stored at –196°C. The infectivity of such stocks, however, is directly related to viability of the cells contained in these preparations and depends also on optimal freezing and thawing techniques. Under ideal conditions, the half-life of diluted, cell-associated virus stocks or vaccines should be at least 2–6 hours (559).

Cell-free serotypes 1 and 2 virus stocks are best obtained from FFE (low-passage virus) or infected cell cultures (high-passage virus). Small quantities of low-passage virus can be obtained from infected cell cultures by lysing cells in SPGA (sucrose–phosphate–glutamate–albumin) buffer (98). The production of cell-free HVT is best achieved by lysing heavily infected cell cultures. Cell-free MDV and HVT can be stored at –70°C or lyophilized (98). Potency of both cell-associated and cell-free vaccines can be affected adversely by storage temperature, reconstitution technique, choice of diluent, and holding time and temperature after reconstitution (239, 423).

Susceptibility to Chemical and Physical Agents

The stability of cell-associated MDV serotype 1 and 2 strains is completely dependent on the viability of the cells. Any treatment affecting cell viability will impact directly the infectivity of virus stocks.

Cell-free MDV obtained from the skin of infected chickens was inactivated when treated for 10 minutes at pH 3 or 11 and stored for 2 weeks at 4°C, 4 days at 25°C, 18 hours at 37°C, 30 minutes at 56°C, or 10 minutes at 60°C (91). Dander, litter, and feathers from infected chickens are infectious and presumably contain cell-free virus from the FFE bound to cellular debris. The infectivity of such materials was retained for 4–8 months at room temperature (263, 602) and for at least 10 years at 4°C (87). Virus infectivity was inactivated by a variety of common chemical disinfectants within a 10-minute treatment period (97, 262). Survival of virus in litter may be affected adversely by increased humidity (602).

Strain Classification

Serotypes

Following full genome sequencing, the three MDV serotypes have now been designated as three distinct species: Gallid alphaherpesvirus 2 (serotype 1), Gallid alphaherpesvirus 3 (serotype 2), and HVT (serotype 3). Von Bülow and Biggs (576, 577) originally classified the MDV herpesvirus group into three distinct serotypes that correlated with biologic properties. Type-specific mAbs (270, 332) usually are used to determine virus serotype.

A number of biological characteristics are associated with viral serotypes (50, 484). Low-passage serotype 1 viruses grow best in duck embryo fibroblast (DEF) or chicken kidney cell (CKC) cultures, grow slowly, and produce small plaques. Serotype 2 viruses grow best in chicken embryo fibroblasts (CEF), grow slowly, and produce medium plaques with some large syncytia. Herpesviruses of turkey grow best in CEF, grow rapidly, and produce large plaques. More infectious virus can be extracted from HVT-infected cells than from cells infected with serotype 1 or 2 viruses.

Pathotypes

Virulence or oncogenicity is only associated with serotype 1 MDVs. Within this group, however, a wide variation in pathogenic potential is recognized and undoubtedly represents a continuum from nearly avirulent to maximally virulent. Pathotype classification schemes have evolved over the last 30 years with the continued increase in virulence. Current classification schemes recognize four groups of viruses. These groups are designated as mMDV, vMDV, vvMDV, and vv+MDV (597, 604). Pathotyping of virus isolates involves comparative pathogenicity tests in vaccinated and unvaccinated maternal antibody positive chickens with prototype viruses as controls (592, 601). Detailed reviews on the increasing virulence and the details of prototype viruses have been published (211, 498, 598, 599, 601). The evolution in the virulence of MDV strains is recognized, but the molecular basis for this evolution has not been elucidated, as genome sequence analyses of a number of these strains have not provided definite markers associated with the virulence phenotypes.

Certain biologic characteristics are associated with pathotypes of serotype 1 MDVs but are most pronounced between low-passage and high-passage (attenuated) strains. Serial passage *in vitro* (30–70 passages usually are required) results in attenuation of virulent isolates (470, 494, 589). Attenuated strains grow more readily *in vitro* but produce lower viremia titers *in vivo* (609), which may be associated with a marked decrease in their ability to infect and/or replicate in lymphocytes and spread between birds (160, 494, 591). There could also be incomplete attenuation resulting in minor lesions in susceptible chickens (439, 575) or over-attenuation resulting in low level *in vivo* replication and poor immune response (318, 614). The *in vivo* growth potential of attenuated serotype 1 isolates can be increased by backpassage in chickens (161, 591). A recent study on the molecular basis of attenuation identified several *de novo* mutations associated with reduced virulence (258).

Laboratory Host Systems

Marek's disease virus usually is propagated and assayed in tissue cultures, newly hatched chicks, and embryonated eggs. Lymphoblastoid cell lines from MD lymphomas are also an important laboratory host system.

Cell Cultures

The propagation of MDV serotypes *in vitro* has been reviewed (484, 487, 498). Isolation of low-passage MDV in CEF or embryonal CKC cultures is far less efficient than in CKC or DEF (487) as propagation in CEF leads to accelerated attenuation (494). In embryonal CKC, replication of serotype 1 MDV (but not HVT) is abortive, leading to loss of infectivity within two to three passages. Attenuated MDV and serotype 2 and 3 viruses can be isolated readily and propagated in CEF (490). Infected cultures usually develop discrete focal lesions, called foci or plaques, which consist of clusters of rounded, refractile degenerating cells when mature (Figure 15.2). Plaques are usually less than 1 mm in diameter and of variable cell density, although plaque size varies with viral strain, time, and other factors. Polykaryocytosis is seen in cultured fibroblasts and is a major component of the viral plaques or foci frequently used as a marker in virus assays. Affected cells may contain two to several hundred nuclei, and type A intranuclear inclusion bodies are commonly seen. Despite release of rounded cells into the medium as plaques mature, large areas of cell lysis are not seen.

Serotype 1 plaques develop in 5–14 days on primary isolation and in 3–7 days after adaptation to culture and usually are enumerated by microscopic examination, but different staining techniques have been developed, allowing enumeration at a later time. Differences in development and morphology of serotype 1 plaques in chick and duck cells and in plaques induced by the three viral serotypes (484) have been described. Other cell culture systems such as chick embryo skin (445), tracheal explants (460), and embryo fibroblasts from several avian species including Japanese quail (453) also have been used.

A few avian cell lines such as OU2 (8, 9), quail muscle cell line QM7 constitutively expressing MDV-1gE (471, 508), JBJ-1 (206), chicken embryo liver (327), and lines derived from Muscovy duck retinal tissue (293) have also been used for the propagation of MDV strains with varying success. Quail cell lines free of MDV can be used to propagate serotype 1 MDV and HVT, but SB-1 did not replicate efficiently (343). Recently developed keratinocyte cell lines support MDV replication, producing cell-associated viral progeny (140).

Serotype 1, but not serotype 2, MDVs can also be grown in chicken splenic lymphocytes *in vitro* (106). Passages are made by the addition of fresh spleen cells to the suspension cell cultures every two days, and infection is monitored by IF. Herpesvirus of turkey may be similarly grown in turkey spleen cell cultures, but viral antigen is rarely seen, if at all.

Chickens

Newly hatched chicks inoculated with virulent, serotype 1 MDV develop gross lesions or lesions that can be detected histologically in ganglia, nerves, and certain viscera after 2–4 weeks. Response is greatly dependent on genetic susceptibility of the chicken and virulence of the MDV isolate. Presence of virus or antibody, which can be detected by *in vitro* tests, or the presence of virus-associated antigen detected by FA tests on tissues, are also specific host responses of inoculated chickens to

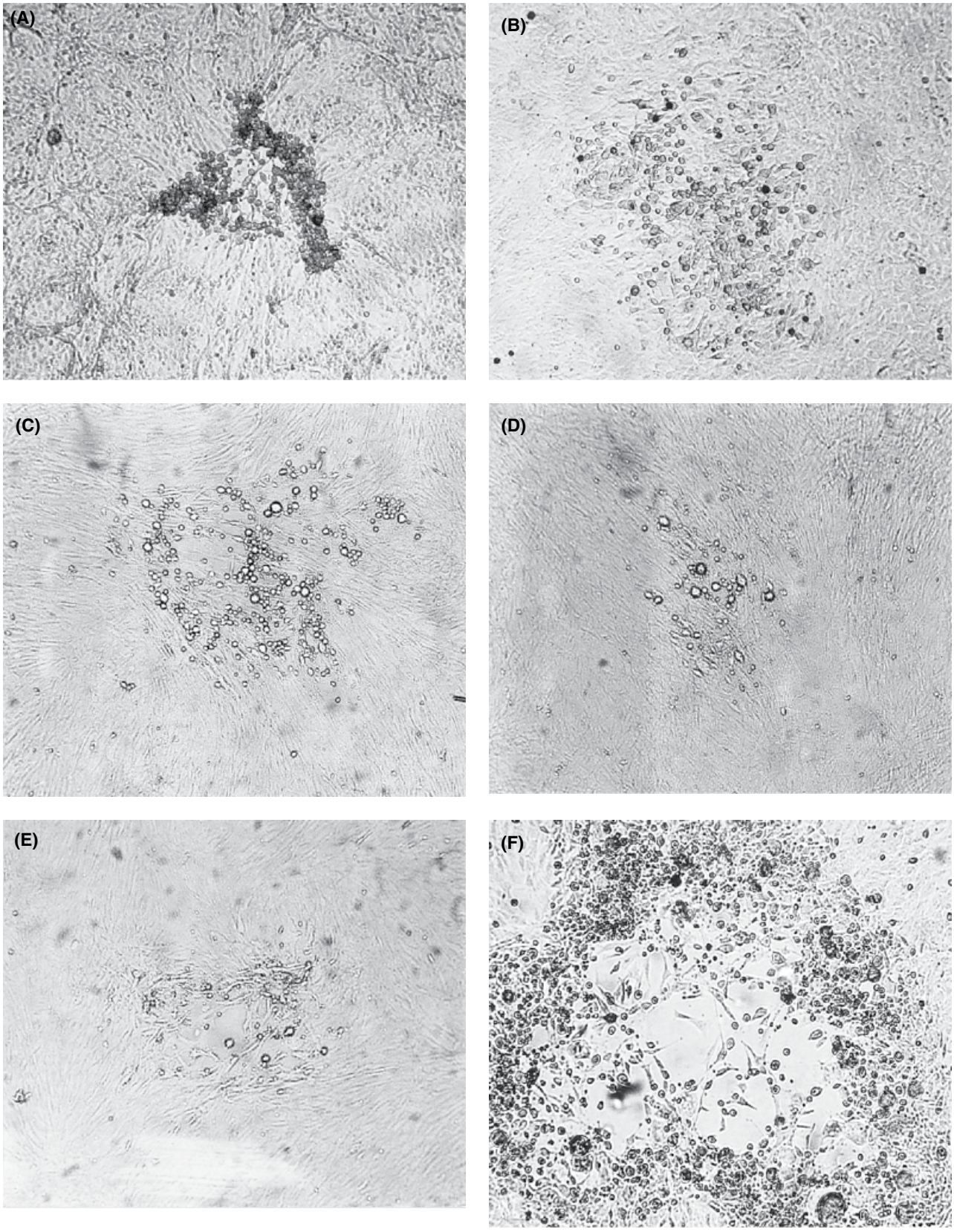


Figure 15.2 Focal lesions in cultured cells infected with various Marek's disease virus (MDV) serotypes. (A) Low-passage serotype 1 MDV in chicken kidney cells cultured from an infected chicken, 9 days. (B) Low-passage serotype 1 MDV in duck embryo fibroblasts (DEF), 5 days. (C) High-passage, attenuated serotype 1 MDV in chick embryo fibroblasts (CEF), 5 days. (D) Low-passage serotype 2 MDV in CEF, 8 days. (E) Low-passage HVT (serotype 3) in CEF, 4 days. (F) Low-passage turkey herpesviruses (HVT) in DEF, 12 days. All photos unstained, about ×40. (Witter)

MD infection. All these responses are markedly enhanced in chicks lacking maternal antibodies against MDV (86). The induction of virus-specific lesions in the wing web (99) or the feather pulp (377) constitutes alternate approaches that provide direct access to the site of lesion development.

Embryos

Virus pocks develop on the chorioallantoic membrane (CAM) of chicken embryos following yolk sac inoculation with cellular MDV preparations (49, 574). Embryos have also been used for MD vaccine evaluation, because *in ovo* vaccination is becoming increasingly common in the field. Embryos may also be used to isolate MDV viruses that cannot be isolated directly in cell culture for unknown reasons. Yamaguchi et al. (633) reported the isolation of MDV from the QT35 cell line by using kidney cell cultures prepared from 4- to 7-day-old chicks that had been inoculated at embryonic day (ED) 8 with QT35 cells.

Lymphoblastoid Cell Lines

Lymphoblastoid cell lines developed from MD lymphomas grow continuously in cell culture without attachment to the culture vessel. Success rates for establishing cell lines from MD lymphomas have improved because of better methodology (108, 428). Many cell lines are now available including several from MD lymphomas in turkeys (392). The majority of the chicken cell lines established from lymphomas are CD4+/CD8- T cells expressing major histocompatibility complex (MHC) class II and T cell receptor (TCR) 2 or 3 (386, 417, 495). Lymphoblastoid cell lines can also be established from lymphocytes harvested from early (4–6 days postinfection) lesions induced in the wing web or pectoral muscle by injection of a mixture of MDV and allogeneic kidney cells. The cell lines from early lesions may be CD4+/CD8–, CD4–/CD8+, or CD4–/CD8– (495). Cells of the MDCC-RP1 line are illustrated in Figure 15.3.

Some transformed cells contain about 5–15 copies of viral genome, although the mean number may be considerably higher in different cell lines, perhaps in relation to the proportion of productively infected cells in the population (376, 473). Most cell lines can be termed "producer" lines, because a small proportion (1–2%) of the cells enter into productive infection (108, 443). Viral DNA can be highly methylated in cell lines although methylation is not essential for maintaining the transformed state (298, 417). Methylation profiles of the viral genome in the cell lines are not very different from those in the primary tumors (63).

Marek's disease tumor-derived cell lines have been used to analyze the potential interaction with tumor suppressor genes and cellular oncogenes, such as Meq. The data generated from the systematic analysis of the gene expression from a number of MDV-induced tumors and transformed cell lines (358, 531, 559) will help to identify the molecular and biochemical pathways of transformation and the maintenance of the transformed phenotype in these cells.

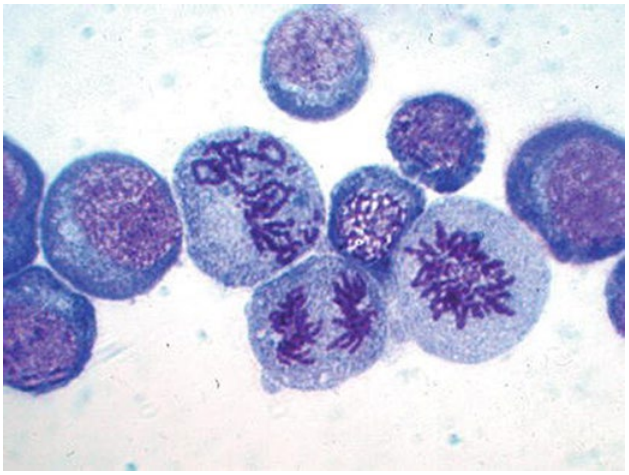


Figure 15.3 Smear from the MDCC-RP1 cell line. Note the characteristic lymphoblastoid morphology and the mitotic figures. Giemsa, ×1500. (Nazerian)

Pathobiology and Epizootiology

Multiple Syndromes

Marek's disease consists of several distinct pathologic syndromes (90) with apparent differences among these syndromes typically seen in commercial flocks, and those induced in the laboratory. Lymphoproliferative lesions, especially lymphomas, are most frequently associated with MD and have the most practical importance (Table 15.3A). However, skin leukosis in broilers, fowl paralysis, persistent neurological disease, and ocular lesions are additional clinical manifestations with lymphoproliferative components. Some of the lymphoproliferative syndromes may also have degenerative components. Several additional clinical syndromes characterized solely by degenerative and inflammatory lesions, often with accompanying immunosuppression, are induced by experimental infection (Table 15.3B). Non-neoplastic brain pathology, mainly vasogenic edema, is responsible for transient paralysis (231). Vascular lesions are manifested as atherosclerosis (192). Under laboratory conditions, young chicks inoculated with tumor cells may develop transplantable tumors (267, 544, 558). Inoculation of MDV-infected, allogeneic CKC in the wing web may induce local tumor lesions (99). Some of the syndromes induced under laboratory conditions are rare or nonexistent in the field, probably because most

Table 15.3A Clinical and pathologic syndromes associated with Marek's disease virus (MDV) (part A).

	Lymphoproliferative syndromes[ab] (Marek's disease)			
Situation in which syndrome observed	**Lymphomas and nerve lesions**	**Fowl paralysis (nerve lesions)**	**Skin leukosis (integument)**	**Blindness and ocular lesions**
Experimental chickens (laboratory)				
Clinical signs	Depression, death, stunting, paralysis	Paralysis	Swollen feather follicles	Blindness, ocular lesions
Mortality	0–100%[c]	0–30%[cd]	None	Rare or none[c]
Age	Onset 2–8 weeks PI	Growing birds	Young birds[E]	4–8 weeks PI
Organ	Visceral organs+peripheral nerves	Mostly peripheral nerves	Skin	Eye (iris, cornea)
Layer/breeder flocks (field)				
Clinical signs	Depression, death, paralysis	Paralysis, death	Swollen feather follicles	Blindness, gray eye
Prevalence	Common	Occasional[d]	Rare or none[e]	Rare
Mortality	0–60%	0–20%	None	None
Age	4–90 weeks	8–20 weeks	4–8 weeks PI	>10 weeks
Broiler flocks (field)				
Clinical signs	Depression, death, paralysis	Paralysis, death	Swollen feather follicles, red leg	Blindness, gray eye
Prevalence	Common	Rare or none[d]	Common[e]	Rare or none
Mortality	Minor	—	None	None
Age	At processing	—	At processing	—

[a] Neoplastic lesions may include inflammatory components.
[b] Severity of syndrome usually less in vaccinated flocks.
[c] Depends on experimental conditions (virus strain, dose, chicken genotype, maternal antibody status, prior vaccination, etc.).
[d] Rarely induced by contemporary MDV strains, except in conjunction with visceral neoplastic lesions.
[e] Not usually recognized except at broiler processing or after feather removal.

commercial chickens are maternal antibody positive and vaccinated.

Subclinical syndromes may also occur but are more difficult to define. Vaccinated flocks produced more eggs than nonvaccinated flocks, indicating that MDV may depress productivity in otherwise normal-appearing, nonvaccinated chickens (454).

Incidence and Distribution

Marek's disease exists in all poultry-producing countries. Marek's disease is generally considered ubiquitous among vaccinated commercial flocks, although recent evidence suggests not all flocks are necessarily infected (56, 581). Reporting systems vary, however, and it is difficult to determine the true MD incidence (174). Even in susceptible chickens, infection does not always induce clinical disease and, in genetically resistant or vaccinated chickens, infection may rarely cause overt disease.

Since 1961, the United States Department of Agriculture (USDA) Food Safety and Inspection Service has collected condemnation data from processing plants, including young broiler chickens condemned for leukosis, which almost exclusively refers to MD. This data, analyzed and reported from the USDA National Agricultural Statistics Service, shows a gradual decrease in leukosis condemnations of young broiler chickens since the early 1970s, reaching an all-time low of 0.00069% during 2016 (Figure 15.4). Regional differences are striking, as illustrated by annual leukosis condemnation rates in Delaware versus Georgia. The data has also revealed a biphasic pattern with maximum condemnation rates around April and minimum frequencies around August, although this seasonal trend has steadily decreased since about 1990 (309, 498, 596).

Sporadic outbreaks of MD occur on individual farms or regions. Recent industry-wide surveillance study in Pennsylvania demonstrated the persistence of the virus with fluctuation in virus loads at different farms (309). Several reports lend credence to the implication of exceptionally virulent MDV isolates in vaccine failures (e.g., 35, 597), but it is important to exclude CIAV as a

Table 15.3B Clinical and pathologic syndromes associated with Marek's disease virus (MDV) (part B).

	Lymphodegenerative syndromes	CNS syndromes	Vascular syndromes	Other syndromes
Situation in which syndrome observed	**Early mortality syndrome, cytolytic infection, immunodepression**	**Transient paralysis and persistent neurological diseases**	**Atherosclerosis**	**Local lesions; transplants (transpl.)**
Experimental chickens (laboratory)				
Clinical signs	Depression, stunting, death, increased disease susceptibility	Transient paralysis, tics, torticollis, death	None	Swelling at inoculation site
Mortality	0–100%[ab]	0–100%[ab]	None	Yes (transpl.)
Age	9–20 days PI	9–28 days PI	Adult birds	Young birds
Organ	Bursa, thymus, spleen	Brain	Blood vessels	Web–local and many–transpl.
Layer/breeder flocks (field)				
Clinical signs	Increased disease susceptibility	Transient paralysis, tics, torticollis	—	N/A: Only experimental
Prevalence	Rare[a]	Rare[a]	Rare or none	—
Mortality	—	Rare	—	—
Age	—	5–12 weeks	—	—
Broiler flocks (field)				
Clinical signs	Increased disease susceptibility	Transient paralysis, tics, torticollis	—	N/A: Only experimental
Prevalence	Rare[a]	Occasional[a]	None	—
Mortality	—	Rare	—	—
Age	—	5–7 weeks	—	—

[a] Not normally observed in chickens vaccinated for MD.
[b] Depends on experimental conditions (virus strain, dose, chicken genotype, maternal antibody status, prior vaccination, etc.).

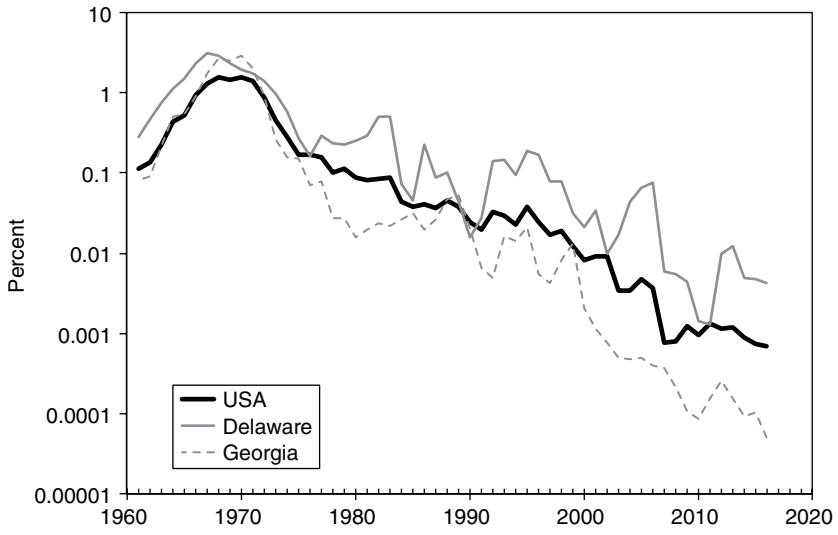


Figure 15.4 Marek's disease condemnations in young broilers for the period 1961–2016 (data from National Agricultural Statistics Service). (Dunn)

cofactor (370). It is usually difficult to associate increased virulence of MDV isolates with regional fluctuations in MD frequency, but layer outbreaks in Ohio in 1995 yielded isolates of unusual virulence (95, 597).

In recent years, MD has been reported as the most commonly diagnosed infectious disease among backyard chickens in multiple countries (369, 438).

Natural and Experimental Hosts

Virtually all chickens including game fowl are susceptible to MDV infection and tumor development (498) and are by far the most important natural host. Quail, turkeys, partridges, pheasants, and some species of ducks and geese are also susceptible to infection and disease (1, 499). Outbreaks in Japanese quail are most commonly reported among quail. In turkeys, occasional outbreaks have recently been reported (54). Vaccination with CVI988 appears to offer protection (137), whereas HVT, interestingly, does not (182). Marek's disease virus can be re-isolated from experimentally infected ducks (39), but development of MD has not been demonstrated. Most other avian species including sparrows, pigeons, and peafowl are probably refractory (reviewed in [499]). Recently a herpesvirus was isolated from three species of endangered pheasants causing hepatocellular necrosis. Based on sequence analysis of two genes, this virus is related to HVT and is proposed to be a new member of the genus *Mardivirus* (510). Marek's disease-like lymphoid tumors in passerines are frequently cited, but need to exclude lymphoma-like lesions caused by infection with *Isospora*, especially in captive populations (150). Mammals, including several species of primates, are refractory to experimental inoculation (reviewed in [499]).

Transmission, Carriers, Vectors

Marek's disease virus is transmitted readily by direct or indirect contact between chickens by the airborne route (citations in 46, 499). The FFE produces fully infectious virus (92). These cells present in feathers and dander form the major source of contamination of the environment and infection of chickens (41, 110). Contaminated poultry house dust remains infectious for at least several months at 20–25°C and for years at 4°C (citations in 89, 499). Under commercial conditions, young chickens are most commonly exposed to MDV by contact with residual dust and dander in the growing house or by aerosolized dust from adjacent chicken houses, fomites, or personnel. After the virus is introduced into a chicken flock, regardless of vaccination status or genetic resistance, infection spreads quickly from bird to bird. Early studies, based on contact infection, demonstrated that virus excretion begins about two weeks postinfection (310) and continued indefinitely (622). The development of qPCR assays for the three serotypes has provided measurements of virus load in feather pulp and dust (2, 31, 276, 469). These assays may measure cell-associated virus in the feather pulp and therefore do not necessarily provide a measure for the amount of infectious virus shed into the environment. The latter question is relevant because dust collected from poultry houses containing HVT-vaccinated flocks is positive for HVT DNA. It is possible that this DNA is derived from cell-associated HVT orginally present in feather pulp cells rather than true cell-free infectious virus. Older literature had suggested that horizontal spread of HVT from vaccinated chickens is limited at best (498). Feather dust has recently been used as a direct source for MDV whole genome sequencing for representing shed virus from a flock versus a point source in one animal (413).

Vertical transmission of MDV does not occur (470, 535, 536). Transmission from dam to progeny as the result of external egg contamination is also unlikely because of poor virus survival at temperature and humidity levels used during incubation (97). Passive transmission by darkling beetles (*Alphitobius diaperinus*) has been reported, but free-living litter mites, mosquitoes, and coccidial oocysts do not transmit MDV (40, 59, 60, 179).

Experimental transmission is commonly accomplished by parenteral inoculation of susceptible chickens with cell-associated virus from cell cultures, tumor cell suspensions, or blood cells from infected chickens. Exposure by direct or indirect contact with infected chickens is also effective. Cell-free but not cell-associated virus can be used for intratracheal instillation or inhalation exposure. An aerosol-based exposure model was described (6), which may facilitate studies on the early events in the pathogenesis.

Incubation Period

The incubation period for experimentally induced MD is well established (see reviews 27, 90). Mononuclear infiltrations containing $AV37^{+}CD4^{+}$ lymphocytes can be detected in nerves of maternal antibody-negative, genetically susceptible chickens as early as 5 days PI (75). Monoclonal antibody AV37 recognizes chicken $CD30^{hi}$, which is considered a marker for transformed lymphocytes (78). Clinical signs and gross lesions generally do not appear until between the third and fourth weeks.

The incubation periods can be short for several nonlymphomatous syndromes associated with MDV infection. Cytolytic infections occur at 3–6 days PI and are followed by degenerative lesions (atrophy) of the thymus and bursa of Fabricius within 6–8 days PI (74). The early mortality syndrome (EMS) is characterized by deaths at 8–14 days PI (615). The clinical expression of both acute and classical forms of transient paralysis usually occurs from 8–18 days PI (311, 607) and can occur when SPF birds are challenged with vv+MDV between 30 and 102

weeks of age (605). Field cases of transient paralysis are seen mainly between 6–12 weeks of age, probably reflecting MDV exposure 8–10 days prior to the onset of symptoms. Development of atherosclerosis requires 3–7 months (191). Induction of tumors within 10–14 days after inoculation of cellular material is suggestive of a transplantation response (544). Local lesions in the wing web are visible 3–4 days PI with allogeneic MDV-infected CKC. Cell lines have been established from these lesions suggesting that transformed cells are present (99).

Under field conditions, MD outbreaks sometimes occur in unvaccinated layer chickens as young as 3–4 weeks. Most of the serious cases begin after 8–9 weeks but sometimes commence well after the onset of egg production, especially in broiler breeders (381) or subsequent to molting (600). Witter (600) differentiated the outbreaks in commercial, vaccinated chickens as "early" or "late" breaks. Witter and Gimeno (605) suggested that late breaks were not likely the result of recent infections alone and that additional factors are needed to cause the late breaks. The different manifestations of MD, including EMS (113), may occur in backyard flocks, which are often not vaccinated.

Clinical Signs

Signs associated with MD vary according to the specific syndrome (Tables 15.3A and 15.3B). Chickens with MD lymphoma or fowl paralysis syndromes may exhibit signs, but few are specific to MD (45). In general, signs related to peripheral nerve dysfunction are those associated with asymmetric progressive paresis and, later, complete spastic paralysis of one or more of the extremities. Involvement of the vagus nerve can result in paralysis and dilation of the crop and/or gasping. Because locomotory disturbances are easily recognized, incoordination or stilted gait may be the first observed sign. A particularly characteristic clinical presentation is a bird with one leg stretched forward and the other back as a result of unilateral paresis or paralysis of the leg (Figure 15.5). However, chickens with MD lymphomas may appear clinically normal but have extensive neoplastic involvement when euthanized, while other birds may become depressed and comatose prior to death. Nonspecific signs such as weight loss, paleness, anorexia, and diarrhea may be observed, especially in birds in which the course is prolonged. Under commercial conditions, death often results from starvation and dehydration because of the inability to reach food and water or from trampling by flockmates. Some birds develop nervous tics or torticollis 18–26 days PI, often after recovery from classical transient paralysis. This syndrome, termed persistent neurological disease (231), can be induced by partially attenuated MDVs that no longer induce transient paralysis (229). However, the central nervous system (CNS) signs are difficult to distinguish from those associated with MD nerve lesions.



Figure 15.5 Fowl paralysis. Spastic paralysis of limbs associated with peripheral nerve involvement in Marek's disease. (Witter)

Birds with ocular involvement may show evidence of blindness (198, 415, 543). Ficken et al.(198) isolated two MD strains from commercial flocks with greater than 90% blindness. The blindness was reproduced in experimentally-infected commercial chickens. Gross ocular lesions were not always present in blind birds. The blindness can be unilateral or bilateral, although recognition of clinical blindness requires careful observation. Affected eyes gradually lose their ability to accommodate to light intensity.

Early mortality syndrome results in high mortality 8–16 days PI of young chickens with virulent MDV strains (571, 615). Chickens become depressed and comatose prior to death, which occurs within 48 hours of the onset of signs. Some affected chickens may also exhibit flaccid neck paralysis prior to death (607). Chickens undergoing acute cytolytic infection at 3–6 days PI may be depressed but rarely die during this period, although some may die later from EMS. Immunosuppressed chickens may succumb to ancillary infections, but some chickens die 20–40 days PI with few signs.

Classical and acute transient paralysis syndromes have been described in field flocks (643) and is associated with MDV infection (311). It has been observed infrequently in the field since vaccination for MD has become widespread, but may be encountered in non-vaccinated backyard flocks. In the classical form, affected chickens display varying degrees of ataxia and flaccid paralysis of the neck or limbs beginning 8–12 days PI (Figure 15.6). Signs typically last 1–2 days followed by a rapid and complete recovery, although recovered chickens may succumb a few weeks later with MD lymphomas. The acute (fatal) form results in death within 24–72 hours following the onset of paralytic signs (607).



Figure 15.6 Transient paralysis. Flaccid paralysis of neck of young chicken nine days after inoculation with Marek's disease virus. (Courtesy of Avian Diseases.) (Witter)

Morbidity and Mortality

The incidence of MD is quite variable in commercial flocks and in general low since the worldwide introduction of vaccines, although problems are sometimes reported (174, 381). Most birds developing clinical disease die. A few birds may apparently recover from the clinical disease (52, 75), but the recovery is rarely permanent. Prior to the use of vaccines, losses in affected flocks were estimated to range from a few birds to 30% and occasionally as high as 60% (381). In broilers, MD condemnations averaged 1.0% in 1970 with 10% or higher in individual flocks (452). The average condemnation rate for MD in the United States has decreased dramatically (see Incidence and Distribution).

Some flocks experience significant disease outbreaks despite vaccination. After the disease appears, mortality builds gradually and generally persists for 4–10 weeks. Outbreaks occur and abate spontaneously in isolated flocks or occasionally in several flocks in a region or in succeeding flocks on a farm. The reasons for these fluctuations are poorly understood.

Response rates or mortality approaching 100% for lymphomas, EMS, acute cytolytic infection, or transient paralysis can be achieved following inoculation or exposure of unvaccinated, susceptible chickens to MDV. Because the response frequency is influenced by many factors (see Factors that Influence Mortality and Lesions), laboratory experiments can be designed to produce a wide range of specific clinical and pathologic responses.

Factors that Influence Mortality and Lesions

Virus Strain. The virulence of MDV strains varies widely and appears to have increased over time (597) and is reviewed in (159, 489). Compared to the milder forms of the disease, which caused mainly peripheral nerve lesions, very virulent (vv) and vv+ pathotypes frequently induce higher mortality and more visceral lymphomas, and have the tendency to more frequently break through genetic host resistance or immunity induced by vaccination (408, 604). The extent of disease induced by a given strain depends in part on the genetic constitution of the host (493).

Virus Dose and Route. Dosage may influence disease frequency under natural conditions, although the MD response in genetically susceptible birds given virulent virus was found to be maximal even when a limiting dilution of virus was inoculated (532). Infection with cell-free MDV through intratracheal inoculation or by aerosol may enhance early virus replication and increase the development of lymphomas compared to parenteral inoculation (6, 19, 84).

Host Gender. Several studies indicated that females died earlier and experienced higher losses than males (357, 427), but the opposite has also been reported (363). The differences were apparently not due to sex hormones, varied with the genetic strain, and were most pronounced with genetically susceptible chickens and with viruses of higher virulence. In practice, the influence of gender is probably less important.

Maternal Antibodies. Maternal antibodies reduce and delay MD mortality (129), EMS (615), and transient paralysis (311), probably by limiting, but not preventing, the spread of virus in tissues during the first few days post exposure (86, 429). Thus maternal antibodies do not provide a sterilizing immunity. Breeder stocks are vaccinated uniformly and exposed to virulent MDV and virtually all chickens are hatched with maternal antibodies against multiple serotypes. Specific pathogen free flocks are a source of antibody-free chicks for laboratory studies.

Host Genetics and Age at Exposure. Genetic factors (see reviews [20, 71]) and age at initial exposure are important determinants of MD susceptibility (see Chapter 2).

Age-related resistance is an expression of genetic resistance and develops after hatching paralleling the development of immune competence. Newly hatched chicks and older chickens are both susceptible to infection and cytolytic infection (90), but cytolytic infections are resolved more rapidly in older birds (82) and virus load is somewhat lower (156). The frequency of lymphomas is variable and often markedly reduced in older chickens compared to newly hatched chicks, especially in genetically resistant lines (498). However, non-vaccinated, SPF, older chickens may develop high rates of lymphomas and transient paralysis following challenge with vv and vv+ strains (269, 605). Age-related resistance can be abrogated by neonatal thymectomy (520) suggesting that other immunosuppressive factors, for example, CIAV (501), may increase the susceptibility of older chickens to disease. Lesion regression has been linked to age-related resistance (75, 519).

Prior Infection. Early studies had shown that mild, serotype 1 strains can induce protective immune responses against challenge (533). Dunn et al. (176) demonstrated superinfection following a short interval, but if the second challenge was given after 14 days, regardless of virulence, superinfection was significantly reduced.

Environmental Factors and Stress. Various environmental factors and intercurrent infections appear to affect the incidence of MD, probably through interference with immune responses. Gross (235) observed increased incidence among chickens selected for high concentrations of plasma corticosterone or subjected to a high degree of social stress. The administration of corticosteroids to latently infected chickens precipitated the appearance of clinical MD (441). Feeding of corticosteroid inhibitors tended to increase resistance to MD (134). Restricted feed intake delayed and reduced incidence of MD (240) whereas high-protein diets (450) or the selection for fast growth rate were associated with increased susceptibility to MD (240).

Because MDV infection may depress host immune responses in its own right (see Immunosuppression), concurrent infections are often exacerbated. However, when the concurrent infection is itself immunosuppressive, the resulting immunosuppression usually will exacerbate both disease processes. Examples include IBDV, REV, and CIAV (291, 578, 610, 648). Problems with CIAV contamination invariably interfere with the evaluation of MDV stocks for relative virulence (371).

Pathology

Gross Pathology

Pathologic changes in MD have been reviewed (425, 427) and consist mainly of nerve lesions and visceral lymphomas. Macroscopic changes are not seen in the brain, but gross enlargements can be found in spinal ganglia.

Nerves. Severely affected peripheral nerves may show loss of cross-striations, gray or yellow discoloration, and sometimes an edematous appearance. Usually, plexi of the sciatic and brachial nerves are more enlarged than the respective trunks. Localized or diffuse enlargement causes the affected portion to be 2–3 times normal size, in some cases much more. Goodchild (233) reported that autonomic nerves and especially the celiac plexus are affected at a higher frequency than peripheral nerves, for example, the sciatic and brachial nerves. Witter (595) found the cervical vagus to be of particular diagnostic importance. Because unilateral and or minimal enlargements may be important indicators of disease, it is helpful to examine opposite nerves and, in experimental infections, to compare with age-matched normal controls to detect changes. Careful examination of the various nerve ramifications may be necessary to expose gross lesions in some birds, because enlargements can vary in both presence and degree from one portion of an affected nerve to another. Figure 15.7 illustrates unilateral gross enlargements in the sciatic plexus.

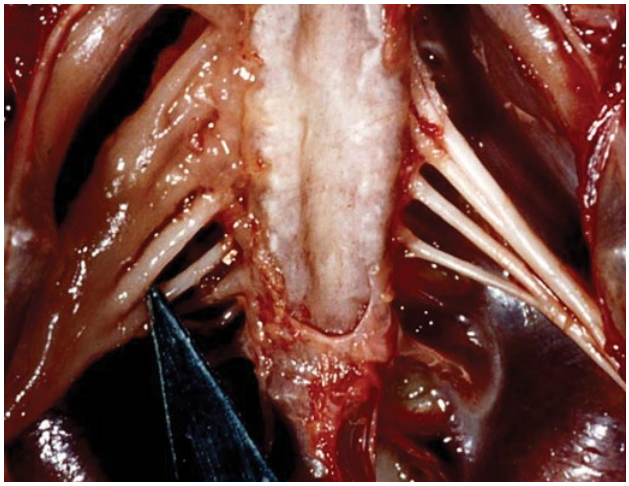


Figure 15.7 Enlarged sciatic plexus (left) and normal plexus (right). (Peckham).

Visceral Organs. Lymphomas may occur in one or more of a variety of organs and tissues. Lymphomatous lesions can be found in the gonad (especially the ovary), lung, heart, mesentery, kidney, liver, spleen, bursa, thymus, adrenal gland, pancreas, proventriculus, intestine, iris, skeletal muscle, and skin. Probably no tissue or organ is without occasional involvement. Both the genetic strain of chicken and the virus strain can influence the organ distribution of lesions. Visceral lymphomas are common in more virulent forms of the disease (597). Visceral tumors can occur in the absence of gross nerve lesions, especially in certain strains of chickens. Marek's disease lymphomas in most viscera appear as diffuse enlargements, sometimes to several times the normal size, and a diffuse white or grayish discoloration is often present (Figure 15.8B). Alternatively, lymphomas may occur as focal, nodular growths of varying size (Figures 15.8E and 15.8F). Nodules are white or gray in color and are firm, and the cut surface is smooth. Necrosis is rare but may occur in the center of rapidly growing lesions.

Diffuse infiltration of the liver causes loss of normal lobule architecture and often gives the surface a coarse granular appearance. Nodular tumors may also be seen in the liver. Lesions in the immature ovary are observed as small to large grayish translucent areas (Figure 15.8B). With large tumors, the normal foliated appearance of the ovary is obliterated. Mature ovaries may retain function, even though some follicles are tumorous. Marked

involvement is indicated by a cauliflower-like appearance. The proventriculus becomes thickened and firm as a result of focal leukotic areas within and between the glands, which may be seen through the serosal surface or, if involvement is diffuse, detected by palpation. Affected hearts are pale from diffuse infiltration or have single or multiple nodular tumors in the myocardium (Figure 15.8F). Pinpoint foci may be seen in the epicardium. Involvement of the lung (Figure 15.8E) may be indicated by increased firmness of the organ upon palpation. Muscle lesions may be present in both superficial and deep layers and are most common in the pectoral muscle (43). Gross changes vary from tiny whitish streaks to nodular tumors.

Integument. Skin lesions, probably the most important cause of condemnation in broiler chickens, usually are associated with feather follicles. The nodular lesions may involve few scattered follicles, or they may be numerous and coalesce. The distinct whitish nodules (Figure 15.8A), especially evident in dressed carcasses, may become scablike with brownish crust formation in extreme cases (43). Lapen and Kenzy (325) found the highest incidences of lesions in external and internal crural and dorsal cervical tracts. Erythematous involvement of the shank integument is seen, especially in virulent forms of the disease in broiler chickens (177) and is commonly known as "Alabama redleg." Swelling of the comb or wattles may indicate lymphoma growth in underlying tissues (177, 180). Interestingly, FFE is not essential for virus production or development of skin tumors, because scaleless chickens produced cell-free infectious virus in epithelial cells and developed skin tumors (250).

Eye. Gross ocular changes, including loss of pigmentation in the iris ("gray eye") and irregularity of the pupil, are caused by mononuclear infiltration of the iris (Figure 15.8C). Nearly all field isolates can induce ocular lesions in nonvaccinated or HVT-vaccinated chickens (597). Conjunctivitis, occasionally with multifocal hemorrhages and corneal edema have also been observed (198).

Other Syndromes. Gross lesions are associated with at least some of the other MDV-associated syndromes. The lymphodegenerative syndromes, related to intense cytolytic infections of lymphoid organs, usually are characterized by severe bursal and thymus atrophy. The cytolytic infection is first evident 3–6 days PI, but may persist becoming more obvious at 8–14 days PI (95, 571, 615). After inoculation with highly virulent field strains, some chickens may die at 20–50 days without gross lesions except severe bursal and thymic atrophy (597). Some chickens also develop a transient splenomegaly within 4–12 days PI (93). The splenomegaly is a non-neoplastic response to viral replication and is induced by all three serotypes. Vascular syndromes are manifested principally by occlusive atherosclerosis (192). Susceptible P-line chickens inoculated with the CU2 isolate of MDV developed grossly visible fatty atheromatous lesions in large coronary arteries, aortas, major aortic branches, and other arteries (Figure 15.8D). Lymphoid tumor transplants and local lesions are experimental syndromes characterized by nodular growths at the site of inoculation, although some transplantable tumors metastasize readily to the liver and spleen, causing diffuse enlargements (544).

Microscopic Pathology

Histopathologic changes associated with MD lymphoproliferative lesions have been described by numerous workers who are in general agreement about the types of histologic lesions and the cells involved (reviewed in 424, 425, 427, 499).

Nerves. In peripheral nerves, two main types of lymphoproliferative lesions are recognized, which are referred to as type A and B, respectively. Type A lesions consist mostly of $CD30^+CD4^+$ T cells (75) and some B cells and is considered neoplastic. In some cases, demyelination and Schwann cell proliferation are associated with A-type lesions. The second, B-type, lesions are essentially inflammatory and are characterized by diffuse, light-to-moderate infiltration by small lymphocytes and plasma cells, usually with edema, and sometimes, with demyelination and Schwann cell proliferation. A few macrophages may be found. The two types may be observed in different nerves of the same bird or even in different areas of the same nerve. Lawn and Payne (326) observed cellular infiltrations as early as five days PI, which gradually increased in intensity until three weeks when severe proliferative, type-A lesions were seen in the absence of paralysis or demyelination. Coincident with initial neurologic signs seen at four weeks PI, areas of widespread demyelination could be found within the proliferative lesions. Finally, characteristic inflammatory, type-B lesions (edema, sparse infiltrations) appeared, suggesting the occurrence of regression of A type lesions. A third, C-type, lesion consists of light infiltration of lymphocytes and plasma cells. The sequence of events has been reviewed in detail by Payne et al. (427). Characteristic changes in nerves are illustrated in Figure 15.9.

Brain. The principal CNS lesion in MD consists of mild, but persistent, perivascular cuffing usually accompanied by gliosis but without primary demyelination as the principal CNS lesion in MD (231, 427). Experimental infection with less virulent MDV strains only resulted in transient, moderately severe inflammatory lesions as early as 7–10 days PI. In contrast, lesions induced by

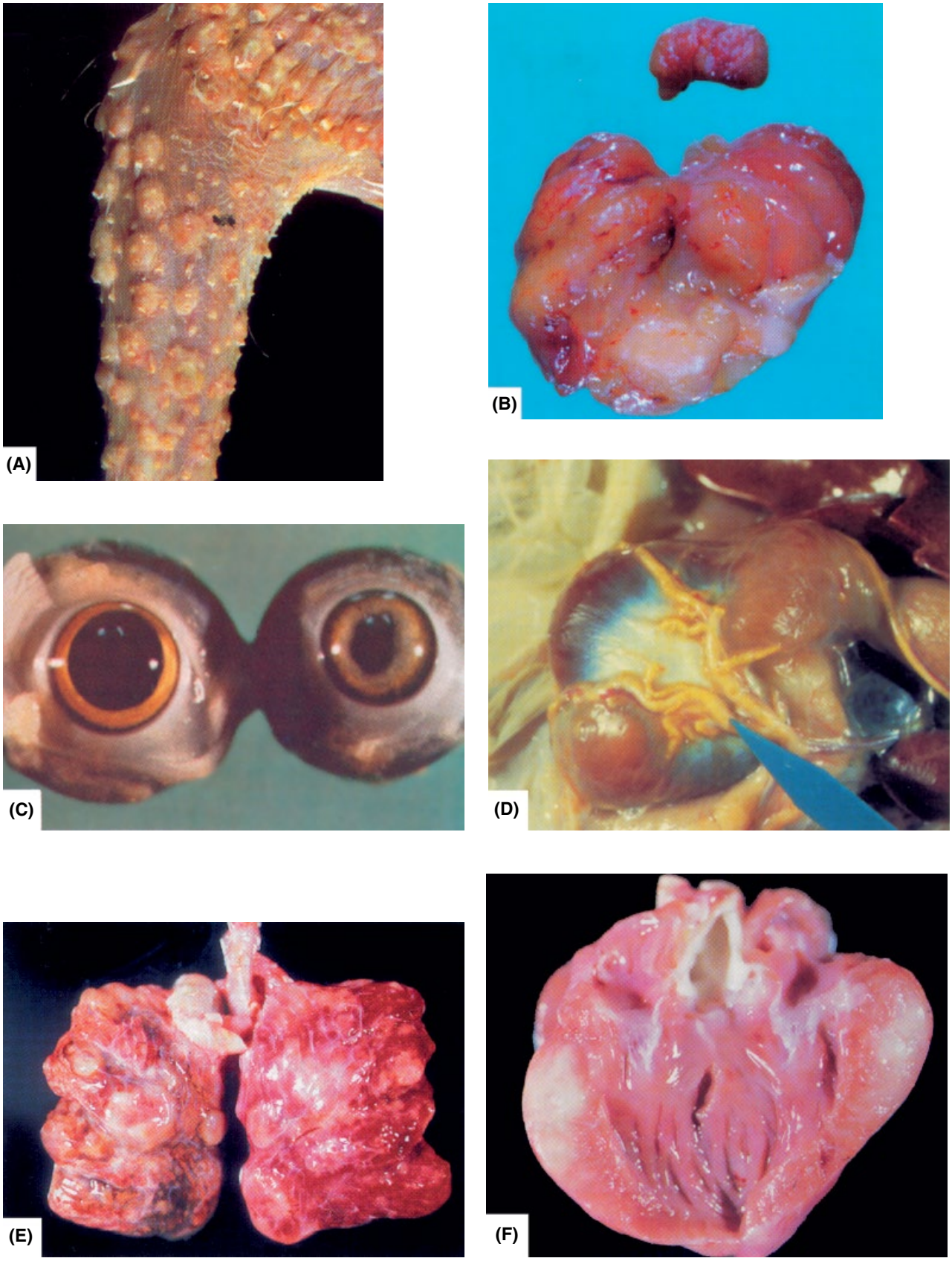


Figure 15.8 (A) Leukotic tumors involving feather follicles (skin leukosis). (Peckham) (B) Experimentally induced Marek's disease (MD) lymphoma in immature ovary (bottom) compared with normal ovary (top) (Witter). (C) Ocular lesions of MD. Note that the normal eye (left) has a sharply defined pupil and well-pigmented iris. Affected eye (right) has a discolored iris and very irregular pupil as a result of mononuclear cell infiltration. (Peckham). (D) Gizzard from a chicken infected with CU-2 isolate of MDV. Note the grossly obvious atherosclerotic change in the arteries. (C. Fabricant). Microscopic changes from similar arteries are shown in Figure 15.16B. (E) Multiple lymphomas in lungs. (F) Multiple lymphomas in heart. (Shivaprasad) (*For color detail, please see the color section.*)

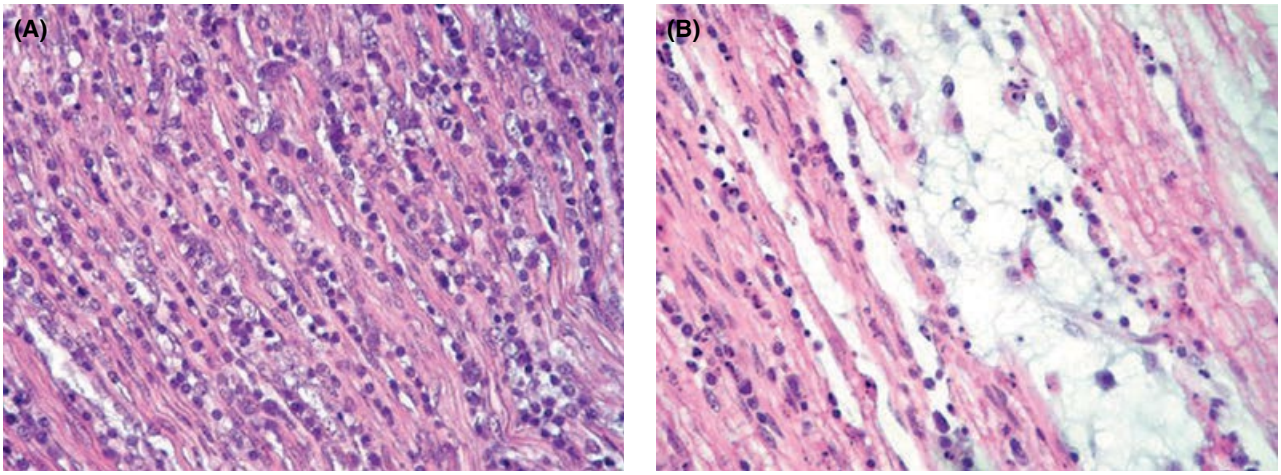


Figure 15.9 Microscopic lesions of Marek's disease in peripheral nerves. (A) Type A lesion characterized by marked cellular infiltration, numerous proliferating lymphoblastic cells, and no edema. H&E, ×550. (B) Type B lesion with edema, scattered infiltrating small and medium lymphocytes, and plasma cells. H&E, ×420. (Gimeno)

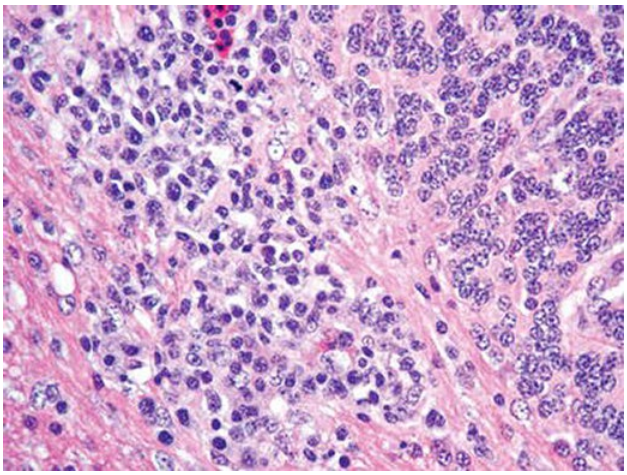


Figure 15.10 Extensive infiltration of lymphoblasts extending into the neuropil in the cerebellum of a Marek's disease virus-infected chicken three weeks postinfection (PI). H&E, ×400. (Gimeno)

vv+MDV strains appeared earlier, were more extensive, and were followed by proliferative lesions (227). The initial lesions involve vascular elements; endotheliosis occurs at 6 days PI and is followed at 8–10 days PI by a moderate to severe infiltration of lymphocytes and macrophages around blood vessels and scattered throughout the neuropil (227). The vasculitis and edema disappear and may be followed by lymphoproliferative infiltrations of large lymphocytes and glial cells. These lesions tend to persist and are associated with persistent neurological disease. Thus, as in nerves, brain lesions are both inflammatory and lymphoproliferative. Severe lymphoid infiltration in the cerebellum is shown in Figure 15.10.

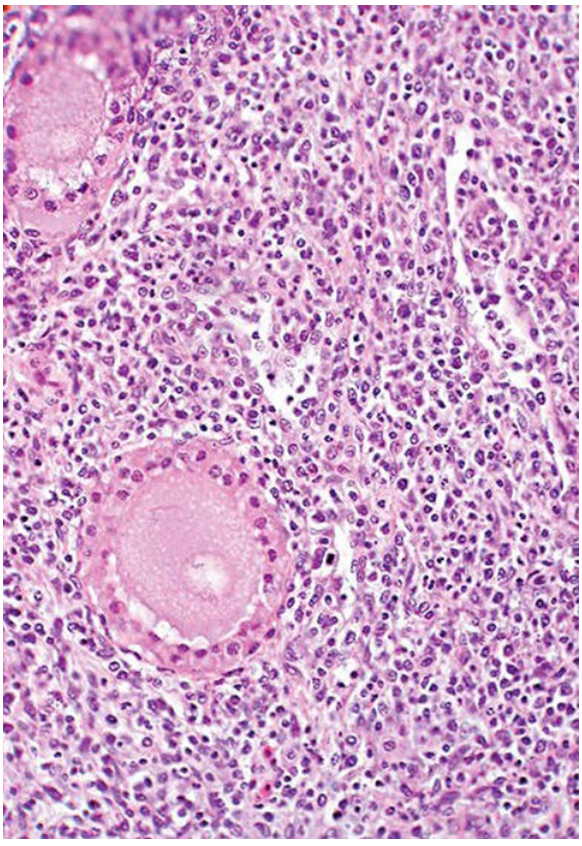


Figure 15.11 Lymphoid cell infiltration of ovary. Organ is composed largely of tumor cells, but a few ovarian follicles can be seen. H&E, ×116. (Witter)

Visceral Organs. Lymphomatous lesions in visceral organs (Figures 15.11 and 15.12) are more uniformly proliferative in nature than those in nerves and similar in appearance to A-type lesions consisting of diffusely

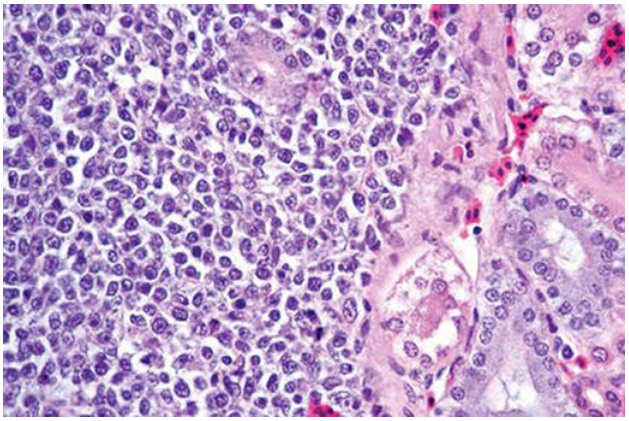


Figure 15.12 Higher magnification of a kidney lymphoma showing pleomorphic tumor cells. Kidney tubules (bottom) show degeneration caused by tumor cell pressure. H&E, ×450 (Gimeno)

proliferating small to medium T lymphocytes and lymphoblasts, NK cells, B cells and macrophages, while plasma cells are rarely present (reviewed in 73, 427). The cellular composition of tumors is similar from one organ to another, even though the gross pattern of involvement may vary. Tumor cells are characterized by large, pleomorphic nuclei with prominent nucleoli (169). The majority of transformed T cells express MHC class II and CD4 although $CD4^{-}CD8^{+}$, $CD4^{+}CD8^{+}$, and CD4–CD8– T cells can also be transformed by MDV (495). In addition the tumor cells express high levels of CD30 (77) and resemble Treg cells (511). Pradhan et al. (444) found immune complexes in the kidney, leading to glomerulopathy, in MDV-infected chickens. They suggested that these lesions might be one of the major causes of death in MD.

Integument. Lymphoproliferative nodules often surround feather follicles that contain MDV viral antigens and intranuclear inclusions in the FFE (92, 127). These lesions in the skin appear largely inflammatory but may also be lymphomatous. Massive proliferative lesions may cause disruption of the epidermis, resulting in an ulcer. Moriguchi et al. (377) described both inflammatory and lymphoproliferative lesions in the feather pulp; the latter were closely related to the incidence of MD and may be useful for antemortem diagnosis (128).

Eye. Smith et al. (534) and Pandiri et al. (414) examined the histology of eye lesions after experimental infection with the GA and Md5 strains, respectively. The former group found that apparently transformed cells could be detected as early as 11 days PI in the arachnoid layer of the optic nerve and subsequently in ciliary nerves and uvea. Pandiri et al. (414) distinguished early and late lesions. The early lesions, between 6–11 days PI, consisted of hypertrophy of endothelial cells, vasculitis, and infiltration of mostly CD8+ T cells, plasma cells, heterophils, and macrophages. The cellular infiltration probably represented an immune response to MDV viral antigens such as pp38, which is recognized by CTL (404). The late lesions appeared between 26–56 days PI and consisted of uveitis, keratitis, and retinal necrosis. Infiltrating cells included CD4+ and CD8+ T cells, macrophages, plasma cells, and granulocytes. Early and late lesions did not contain transformed lymphocytes based on the absence of meq expression.

The unusually severe ocular lesions described by Ficken et al. (198) include uveal changes with increased aqueous humor protein, vascular engorgement, mild hyperemia to severe swelling of the iris, severe inflammatory changes, and edema of the cornea, including intranuclear inclusion bodies.

Blood. Leukemia consisting of T lymphoblasts may occur occasionally (100, 430). Extravascular hemolytic anemia has been reported after infection with vvMDV strains in the absence of CIAV (209). The importance of this finding is not clear because hematocrit values are not routinely used as a MD parameter. Jakowski et al. (283) reported MDV caused anemia as a consequence of bone marrow aplasia, but their MDV was later found to be contaminated with CIAV (501). Blood lymphocyte numbers may vary during the infection cycle: T cells may be elevated while B cells are decreased (430). However, Morimura et al. (378) reported an initial increase of CD4+TCRαβ1 cells around 16 days PI followed by a decrease at 30 days PI. Infection with vv+ C12/130 caused significant increases in the absolute number of blood monocytes around 8 days PI. B cells, $CD4^{+}$ and $CD8^{+}$ T cells decreased during the early cytolytic infection followed by an increase between 8–10 days PI, but these changes were also seen for T cells after infection with HPRS-16 (37).

Lymphodegenerative Syndromes. Marek's disease virus replication in the bursa of Fabricius and thymus results in transient acute cytolytic changes accompanied by atrophy (citations in 89, 427). In experimental infections, bursal lesions consist of follicular degeneration, lymphoid necrosis with depletion, and cyst formation (Figure 15.13A). Thymic atrophy is often severe, and lymphocytes are depleted in both cortex and medulla (Figure 15.13B). Intranuclear inclusions can sometimes be found in cells associated with degenerative lesions. Viral antigens such as pp38 can be abundant during the acute cytolytic phase in infections with vv+MDV strains, especially in the medullary regions of the thymus and bursal follicles (Figures 15.14A and 15.14B). Infection in the absence of maternal antibodies may cause focal or generalized necrosis in a variety of organs, including the kidney (86, 199). Following the acute cytolytic phase,

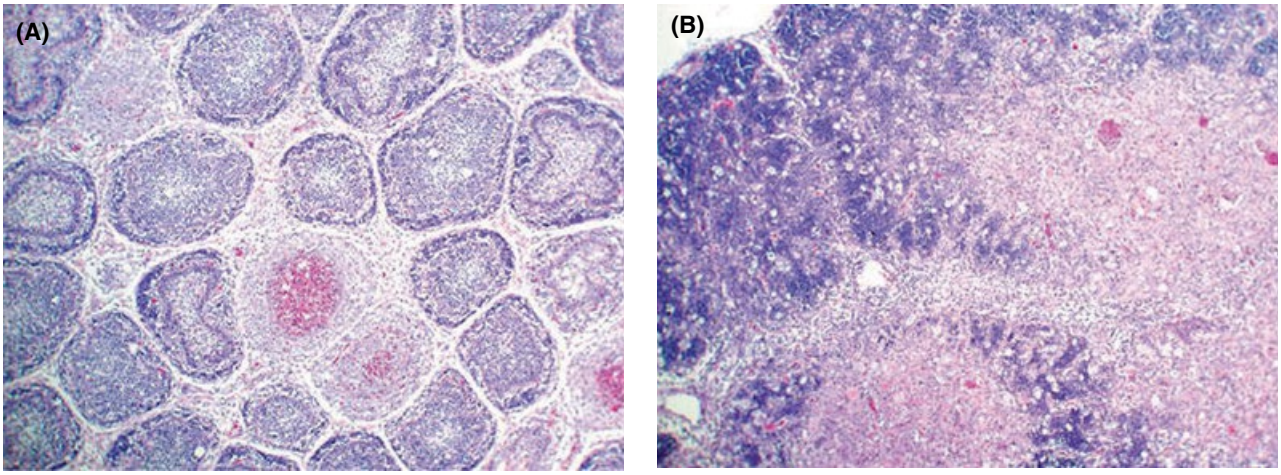


Figure 15.13 Degenerative lesions in bursa and thymus of chickens inoculated with the 648A (vv1) strain of Marek's disease virus. (A) Bursa at 10 days postinfection (PI) shows degeneration and atrophy of follicles. (B) Thymus at 6 days PI shows necrosis and lymphoid cell depletion. ×12 (Gimeno)

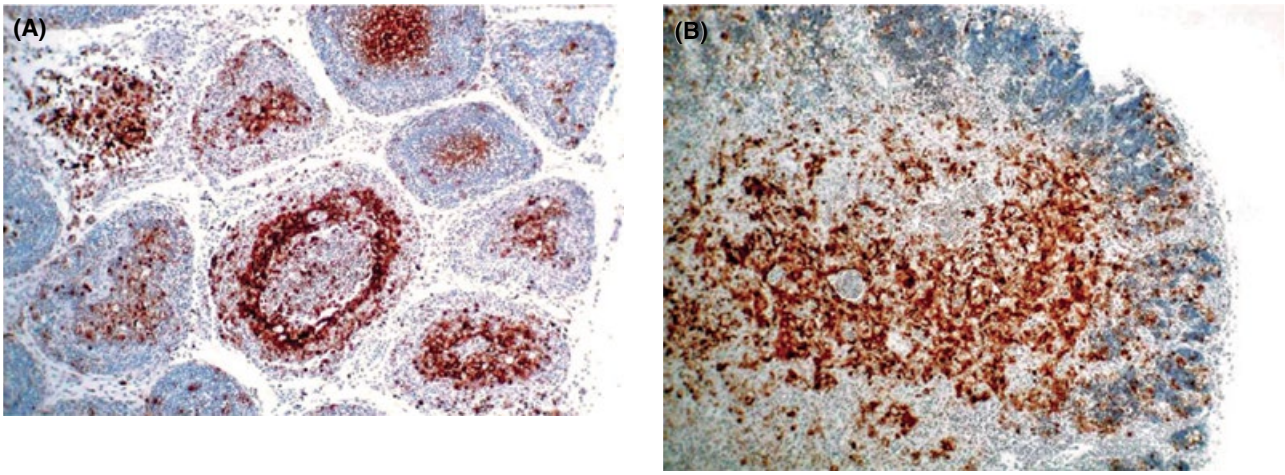


Figure 15.14 Acute cytolytic infection of lymphoid tissues 6 days postinfection (PI) with the 648A (vv+) strain of Marek's disease virus. The pp38 viral antigen is visualized by immunohistochemical staining (black). (A) Bursa of Fabricius. (B) Thymus. ×30 (649). (Witter)

antigen positive cells disappear, and at least partial repopulation with lymphocytes occurs. Bursal and thymic atrophy, however, may persist for several weeks or longer. In the bursa, some interfollicular lymphoid infiltration with T cells may occur. Early mortality syndrome is characterized by severe lymphoid degeneration and death, often with enlarged, necrotic spleens (615). Early mortality syndrome has been linked with CNS signs and transient paralysis (342, 607).

CNS Syndromes (Transient Paralysis). Lesions in classical and acute transient paralysis are very similar. The critical lesion in both types is vasculitis (Figure 15.15) leading to vasogenic brain edema (231, 550, 607). Leakage of albumin and IgG around affected vessels results in vacuolization. Edema and vasculitis develop coordinately with clinical flaccid paralysis and resolve in 2–3 days (549). Other, apparently unrelated, brain lesions (perivascular cuffing, lymphocytosis, and gliosis) may be observed after clinical recovery or in infected but clinically normal birds.

Vascular Syndromes. Arterial lesions associated with MDV-induced atherosclerosis include proliferative and fatty-proliferative changes in aortic, coronary, celiac, gastric, and mesenteric arteries (192, 372) (Figures 15.16A and 15.16B) (557). Internal and medial foam cells, extracellular lipid, cholesterol clefts, and calcium deposits characterized the fatty-proliferative lesions. Marek's disease virus antigens could be detected by IF adjacent to the arterial lesions.

Tumor Transplants and Local Lesions. Tumor transplants are composed of uniform, lymphoblastic cells with few, if any,

infiltrating host cells. In regressing tumor transplants small lymphocytes, heterophils, vascular invasion, and necrosis may be present (200). Local lesions induced in the wing web or pectoral muscle by inoculation of MDV-infected, allogeneic CKC are inflammatory in nature, consisting of lymphocytes and macrophages and sometimes accompanied by hemorrhages and necrosis (99).

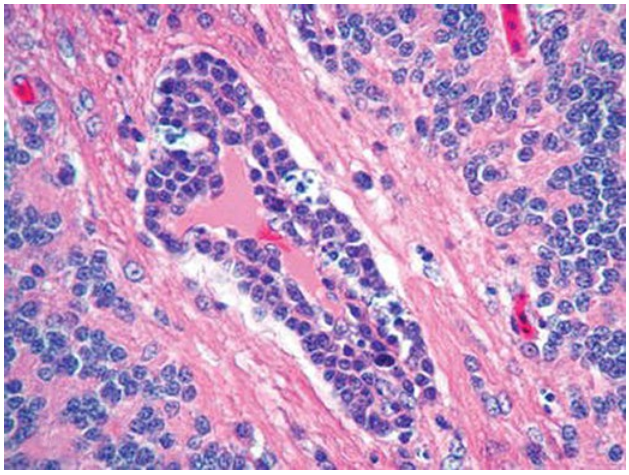


Figure 15.15 Transient paralysis lesions in the brain. Vasculitis in the cerebellum at 10 days postinfection (PI) showing endothelial cell necrosis, lymphoid cell accumulations, and vacuolization. Note intramural necrotic debris (arrow) as well as infiltration of heterophils in the vessel wall. H&E, ×250. (Gimeno)

Pathogenesis

Several reviews on the pathogenesis of MD have been published (27, 90, 502), which also provide references for older reviews. The use of BACs and overlapping cosmid technologies has allowed the deletion of specific genes. Using these technologies, the importance of several genes for the pathogenesis has been established.

Four phases of infection *in vivo* can be delineated. (1) Early productive–restrictive virus infection causing primarily degenerative changes. The infection is productive–restrictive because the virus remains cell-associated and is only transferred by cell to cell contact. (2) Latent infection. (3) A second productive–restrictive infection phase coincident with permanent immunosuppression. (4) The proliferative phase involving nonproductively infected lymphoid cells that may or may not progress to the point of lymphoma formation (Figure 15.17). This Division is somewhat arbitrary and phases 2–4 can coexist in different cells in the same bird. Infection with some of the vv+ strains may not follow this general pattern, and mortality can occur without even entering into the latent phase. The next section will describe the classical pathogenesis in lymphoid tissues, based mostly on studies in SPF chickens. The pathogenesis of infection in the FFE involves epithelial cells and will be discussed in the section on cytolytic infection in FFE.

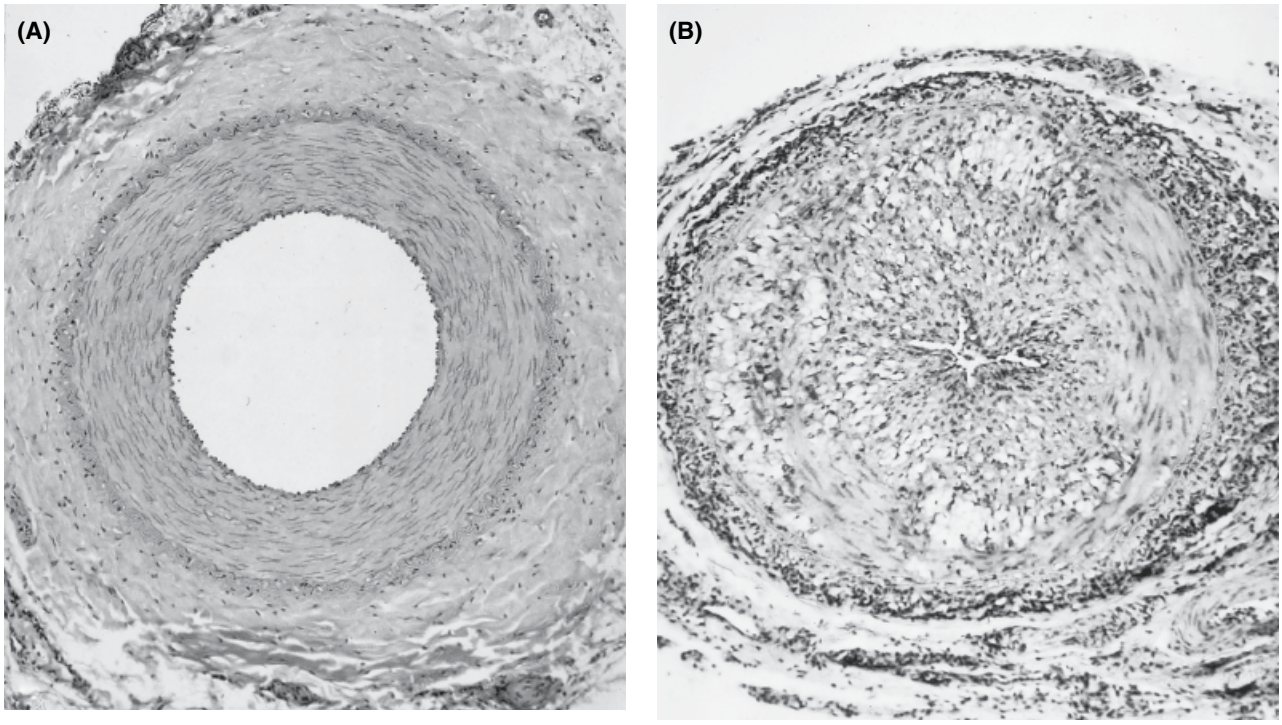


Figure 15.16 (A) Gastric artery of normal chicken. (B) Atherosclerotic artery in gizzard of chicken infected with CU2 isolate of Marek's disease virus. Lumen is occluded by thickened intima, and atheromatous changes have occurred deep in the intima and media. H&E, ×24. (C. Fabricant)

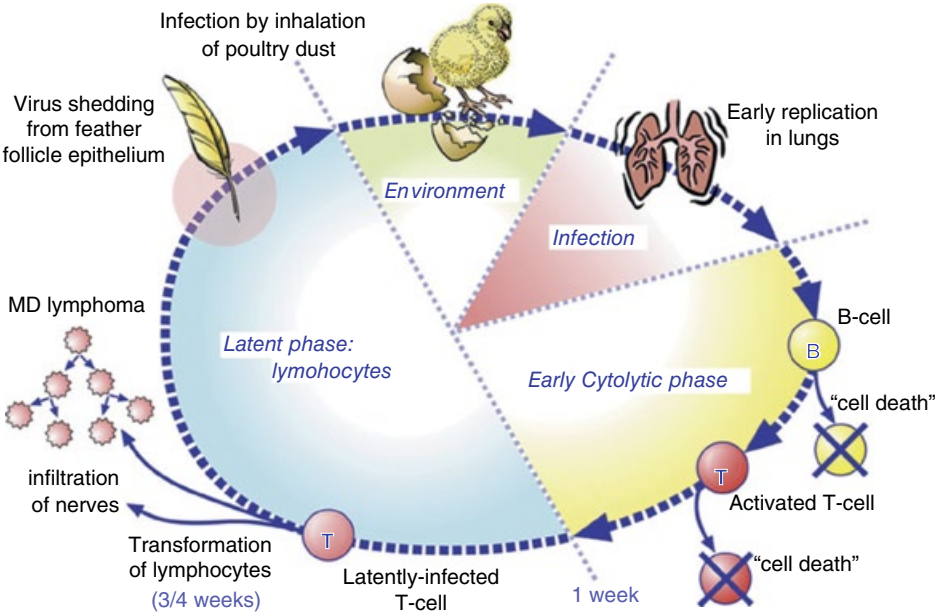


Figure 15.17 Schematic diagram showing the different stages of Marek's disease (MD) pathogenesis including the virus shedding from the feather follicle epithelium and the transformation of T lymphocytes in susceptible birds.

Early Productive-Restrictive Infection (Phase 1)

The virus enters the host via the respiratory tract and cell-free virus reaches the lymphoid organs within 24–36 hours after intratracheal inoculation (6, 482). Marek's disease virus is probably transferred to the lymphoid organs by phagocytic cells (90). Based on the presence of MDV proteins (36, 225) and transcripts (19) macrophages can become infected, but because virus particles could not be demonstrated, it is not sure if this is an abortive or productive–restrictive infection.

Shortly after infection either by inhalation of cell-free virus or by parenteral inoculation with cell-associated virus, cytolytic infection can be detected in the spleen, bursa of Fabricius, and thymus, peaking between 3–6 days. Splenectomy (483) and embryonal bursectomy (EBx) (491) delayed or reduced productive–restrictive infection and the development of lymphomas suggesting a central role for these two organs in the pathogenesis of MD. In contrast, neonatal thymectomy enhanced the pathogenicity of the low-oncogenic CU-1 and CU-2 strains (94). The explanation for these findings was provided by Shek et al. (521) discovering that the primary target cells in all three organs are B cells. Recently it has been shown that B cells are also infected in the lung two days after intratracheal inoculation (19). Infection of T cells requires activation based on the expression of MHC class II antigens and activated but not resting T cells can undergo cytolytic infection (102, 104, 105). Baigent and Davison (29) confirmed that the early cytolytic infection occurs mostly in B cells using dual staining techniques with mAb specific for B- and T-cell markers and MDV pp38. In addition, they demonstrated that a small percentage of $CD4^+$ and $CD8^+$ T cells expressing TCRαβ can become cytolytically infected during the early phase of the pathogenesis. The consequence is a transient atrophy of the lymphoid organs, especially the thymus and the bursa. Depending on the virulence of the challenge strain, birds may recover between 8–14 days PI, or the atrophy may become permanent (93, 95). The cytolysis is likely initiated by the activation of the host shut-off protein leading to cell-death by apoptosis (379). Although MDV-infected cells in the thymus are mostly B cells (521), thymocytes undergo massive apoptosis possibly as the consequence of viral infection (29) or virus-induced cytokine changes.

During phase one hyperplasia of lymphoid and reticulum cells occurs (427), causing splenomegaly between 4–7 days PI, which is also observed after infection with MDV-2 and HVT (93). The level of infection is in general similar in genetically resistant and susceptible strains during phase 1 (7, 295, 642). However, genetically resistant line 6 chickens have a significantly lower level of infected lymphocytes than susceptible line 7 chickens. Interestingly, line 6 has more B cells than line 7, thus the difference is not caused by a lack of target cells in line 6 (29). In spleens of line 7 birds, dramatic changes occur with irregular patches of $pp38^+$ B cells becoming surrounded by $TCR\alpha\beta1^+$ $CD4^+$ and $CD8^+$ cells, thus providing optimal conditions for virus transfer from B to T cells. These data suggest that MDV may replicate and spread more efficiently in line 7 than line 6 chickens (29, 104).

Recently several studies have been conducted to analyze the changes in transcriptome and proteome in various tissues after MDV infection (152, 242). Changes

have been found for many genes and pathways, but additional studies will be needed before general conclusions can be drawn for the understanding of the pathogenesis and immune responses.

Several factors can modify the early pathogenesis. Prior vaccination or the presence of maternal antibodies reduce the cytolytic infection (90). The reduction in cytolytic infection also will reduce the number of latently infected cells and reduce or delay tumor development. Exposure at one day of age prolongs the cytolytic infection compared to exposure at two or seven weeks of age (82). Likewise, the pathogenicity of the virus strain may affect the severity of early infection. The vv and vv+ strains can cause more severe lymphoid organ atrophy than the less oncogenic strains, resulting in an early mortality syndrome (95, 615).

The apoptosis of lymphocytes during the early cytolytic phase (379) may cause transient or permanent immunosuppression, depending on the virulence of the challenge strain. In addition, a transient suppression of mitogen stimulation has been reported, but this may actually represent a protective response (497, 502).

Latent Infection (Phase 2)

At about 6–7 days, the infection becomes latent when cytolytic infection can no longer be demonstrated, and tumors are not yet detectable. The development of latency coincides with the development of immune responses. The integration of the virus into the host telomeres is considered a key virus–host interaction for achievement of latency (367). Impairment of cell-mediated immunity (CMI) (82) or infection with the more virulent pathotypes delays the onset of latency (642). Several soluble factors have been implicated in the induction of latency including IFN-α, IFN-γ, latency maintaining factor, and NO (81, 573, 631). Based on infection of CEF with RB-1B in the presence of IFN-containing supernatants, Levy et al. (340) suggested that IFN may block virus replication before translation of late genes. In one study IL-10 expression leading to a T_{h2} response was the only cytokine upregulated in splenocytes during latent infection (253), but this could not be confirmed in another study (422).

Most latently infected cells are activated $CD4^+$ T cells, although $CD8^+$ T cells and B cells can also be involved (105, 337, 521). Infection in genetically resistant birds often remains latent and can last for the lifetime of the bird aside from a persistent low-grade productive infection in the FFE (90). Apoptosis of T cells during latent infection has been described (378, 380), although it cannot be excluded that MDV was reactivated in these cells. Susceptible birds or resistant birds infected with vv or vv+ strains may develop a second wave of cytolytic infections after the second or third week, coincident with permanent immunosuppression.

The extent to which nonlymphoid cells are latently infected is not known, although apparent latent infection has been observed in Schwann cells and satellite cells in spinal ganglia (434).

Second Phase of Cytolytic Infection (Phase 3)

The second cytolytic infection phase has not been studied in great detail and does not always occur depending on genetic resistance of the host and the virulence of MDV. If present, localized foci can be found in the lymphoid organs and in tissues of epithelial origin in various visceral organs (e.g., kidney, pancreas, adrenal gland, and proventriculus). Focal cell death and inflammatory reactions develop around affected areas (10, 90). If the second cytolytic infection occurs, many of the cytokines upregulated during the first cytolytic phase are again upregulated (422).

Cytolytic Infection in FFE

Starting around 14 days PI cytolytic infection occurs in the FFE (96), which is the only known site of complete virus replication. The replication occurs in genetically resistant as well as susceptible birds independently of the virulence of the MDV strain. Marek's disease virus most likely is transferred to the FFE by infected lymphocytes. Viral DNA of all three serotypes can be detected in feather tips as early as 5–7 days PI by qPCR (see Transmission, Carriers, Vectors). It is not known if this really represents infectious cell-free virus or viral DNA inside feather tips, because MDV-positive peripheral blood leukocytes (PBL) can be found in feather pulp as early as 4 days PI concomitant with increased IFN-γ transcription (4).

Lymphocyte aggregates consisting of small lymphocytes with nuclear inclusions can be detected in the perifollicular dermis as early as 7 days PI (126). The lymphoid aggregates can develop into either necrotic areas consisting of FFE cells and degenerating lymphocytes or into cutaneous tumors. The former is associated with strong expression of pp38, which is the first viral protein expressed in the FFE after reactivation followed by gB and gD (398). In contrast, the cutaneous tumors have only a few $pp38^+$ cells. It is likely that virus is reactivated from latency in the FFE, because mutant strains lacking vIL-8 (145) or pp38 (228) are able to produce virus in the FFE. Detailed dynamics of MDV interaction in the feather and its importance in spread has recently been reviewed (249, 286).

Development of Lymphomas (Phase 4)

Lymphoproliferative changes, constituting the ultimate response in the disease, may progress to tumor development. Death from lymphomas may occur at any time from about three weeks onward. Regression of lesions

has been reported after infection with vMDV strains and depends on the genetic resistance of the bird and the age at infection (75, 323, 519). However, it is not clear how important tumor regression is in commercial poultry.

The transformed T cells are mostly $CD4^+$ cells expressing TCRαβ1 or TCRαβ2 and MHC-II (495) with limited clonality (386). Other subsets (e.g., $CD8^+CD4^-$, $CD3^-CD4^-CD8^-$ and $CD3^+CD4^-CD8^-$) can be transformed under special conditions (99, 402, 495). Burgess and Davison (77) further characterized tumor cells using *ex vivo* lymphoma cells and tumor cell lines as MHC-I^{hi}, MHC-II^{hi}, $CD4^+$, TCRαβ1^+ or TCRαβ2^+, $CD25^+$, $CD28^-$, $CD30^{hi}$. This profile is compatible with Treg cells (420, 511). The expression of $CD30^{hi}$ suggests that MD could be a natural model for Hodgkin's disease (78). Marek's disease tumor cells may also express poorly characterized MATSA (623) and fetal antigens (442). In addition to tumor cells CD8+ T cells are present and are consisting of oligoclonal expansions with public and private CDR3 sequences (386).

The infection in transformed cells is nonproductive *in vivo* and *in vitro*. $CD4^+$ and $CD30^{hi}$ express Meq and SAR (see Viral Proteins), but are negative for pp38 and gB (77, 477). Meq is the key protein involved in the transformation of lymphocytes probably in conjunction with viral telomerase (see Genes Unique for MDV) (388, 565).

The possibility that MD tumors are of clonal origin has been proposed based on observations of random MDV DNA integration into the genomes of lymphoma cells (162). Analyzing the TCRβ repertoire of tumors, Mwangi et al. (386) concluded that most of the tumors are clonal in origin, but that birds could have tumors originating from different clones. In addition, PBL can be positive for these clonal tumor cells a few weeks before the birds succomb. Earlier studies by Schat et al. (495) showed that different lymphomas in the same bird could yield cell lines representing different T-cell phenotypes.

One of the major problems in understanding the molecular basis for transformation has been the lack of a reliable method for *in vitro* transformation of T cells, however such a model has been published which will allow analysis of interaction between virus and target cells in an easily accessible system (503).

Factors Influencing Pathogenesis

The pathogenesis of infection with oncogenic MDV, which has become attenuated by passage *in vitro*, has been studied by Bradley et al. (quoted in 427, 499) and Schat et al. (494). Attenuated viruses failed to cause cytolytic infection and cell-associated viremia levels were low. Moreover, attenuated virus was not infectious for lymphocytes *in vitro*, perhaps explaining the *in vivo* observations.

Virus strains differ in oncogenicity, but the molecular basis for differences in pathogenesis are not well defined. All cause similar early cytolytic infections, although the vv+ strains may cause a prolonged and more severe cytolytic infection (642). Some of the new strains are capable of infecting macrophages leading to increased death of macrophages (38). Murata et al. (385) suggested that point mutations in *meq* resulting in amino acid substitutions in the protein could change transactivation and hence change the pathogenicity.

The immune response itself may be responsible for some lesions characteristic of MD. Nerve lesions have some characteristics suggestive of an autoimmune disease (449, 505), although the presence of MDV was not positively shown. Additional evidence supporting an autoimmune component for MD comes from studies showing immune complexes in the kidneys of MDV-infected chickens and quail (306, 444).

The primary cytolytic infection is not an absolute prerequisite for tumor development. Schat et al. (491) found that MDV infection in EBx chickens resulted in the development of tumors in the absence of the primary cytolytic infection, which also is the case in vaccinated chickens. However, stress and immunosuppressive infections may induce secondary cytolytic infections, reducing the benefits provided by vaccination.

Pathogenesis of Non-Tumor Diseases

Marek's disease virus infection can cause several non-neoplastic disease syndromes (Table 15.3B). The pathogenesis of MDV-induced atherosclerosis has not been elucidated. Microscopic lesions consisting of fatty proliferative lesions with alterations in lipid metabolism in arterial smooth muscle cells could be detected as early as one month after infection (191, 237). In contrast to the original work by Fabricant et al. (192, 193), Njenga and Dangler (399) were unable to demonstrate arterial lipid accumulation without cholesterol supplementation. Cellular infiltrates were detected in the intima and serum cholesterol was increased significantly compared to non-infected control chickens.

The pathogenesis of the neurological lesion complex consisting of classical transient paralysis (TP), acute TP, persistent neurological syndrome (PND), and late paralysis (LP) (231) is not fully understood. The difference between classical and acute TP is somewhat arbitrary (607) and the early pathogenesis is probably similar. The development of both types of TP is influenced by the MHC and the virulence of the MDV strain with the more virulent strains causing acute rather than classical TP (504, 607). B cells are required for the induction of transient TP (418), probably because these cells are essential for the early cytolytic infection. The brain lesions start with vasculitis at 6–8 days followed by leakage of albumin from blood vessels into vacuoles (550). This vasogenic edema is transient and correlates with classical TP symptoms (548). Jarosinski et al. (288) noted that the development of neurological symptoms induced by

vv+RK-1 correlated with increased levels of iNOS mRNA in the cerebellum and NO in blood serum (see Nitric Oxide). Nitric oxide can cause vasodilation and could be the cause of the edema. Chickens inoculated with the vMDV JM-16 strain did not show neurological signs or iNOS mRNA in the cerebellum.

The degree of virus replication in the brain may be related to the severity of the disease. The absence or low levels of virus replication correlated with the absence of neurological symptoms (3, 229), while high levels of replication in MHC resistant and susceptible chickens resulted in neural lesions (288). Attenuation of the vv+648A strain resulted in reduced induction of TP coordinately with a reduction in viral replication in lymphoid organs and FFE (229).

The clinical signs of PND are associated with a strong infiltration of lymphoblasts in the neuropil, many of which express Meq protein. The later occurrence of PND after 3 weeks PI suggests that its pathogenesis may parallel that of lymphoma induction in other tissues. Moreover, PND was shown to be closely related with the onset of lymphoproliferative lesions in peripheral nerves and visceral organs (229).

Immunity

Infection with pathogenic MDV or vaccine strains not only results in the activation of innate, nonspecific and acquired, or specific immune responses but may also cause immunosuppressive effects especially after infection with pathogenic serotype 1 strains. The importance of the interactions between immune responses and immunosuppression for the pathogenesis of MD cannot be overemphasized. A distortion in the balance toward immunosuppression will lead to disease. Immune responses and immunosuppressive features of MD have been extensively reviewed (56, 157, 245, 497, 502).

Immune Responses

The immune responses developing during the early cytolytic phase of infection are crucial for the outcome of infection. Impairment of immune responses during this phase delays establishment of latency resulting in prolonging the lytic infection and the subsequent continued destruction of immune cells by virus-induced apoptosis. Impairments include infection at one day of age when the immune responses are not yet fully developed or treatment with cyclosporin or neonatal thymectomy combined with cyclophosphamide treatment (82). The importance of immune responses during latency is relevant for protection against the second cytolytic phase and is dependent on CMI. It has often been suggested that vaccine-induced immunity is an antitumor immune response because vaccination does not prevent superinfection with wild-type virus but does prevent tumor development. However, vaccination clearly reduces the early cytolytic infection (101, 252, 492), thus preventing extensive damage to the immune system and reducing the number of latently infected T cells. Lesion regression, however, has been described (75, 519) suggesting that immune responses against tumor cells may occur.

Initiation of Immune Responses. Professional antigen-processing cells (APC), such as dendritic cells, encountering pathogens are activated by interactions between the PAMP and the pattern recognition receptors (PRR), for example, TLR, on the APC. These interactions result in the activation of cytokines which direct both the innate and acquired immune responses. There is currently no information on PAMPs associated with any of the three MDV serotypes.

Although the distinction between innate and acquired immune responses has become less defined over the last few years, the innate and acquired responses will be discussed in separate sections.

Innate Immune Responses. Innate immune responses include changes in cytokine expression, natural killer (NK) cells, and macrophages.

Cytokine Responses. Infection with MDV results in the upregulation of a number of proinflammatory cytokines (see Early Productive-Restrictive Infection for details) driving a T_{H1} immune response.

IFN-γ is an important pleiotropic cytokine with many functions in antiviral immune responses, but few studies have been performed on the roles of IFN-γ in protective immunity to MD. *In vitro* studies indicate that IFN-γ is able to inhibit virus replication directly or indirectly through the induction of NO production and reactive oxygen intermediates (166, 631).

Detailed reviews on immunity to MD have been described elsewhere (56, 245, 246).

Nitric Oxide. Nitric oxide is synthesized by three isoforms of NOS with iNOS (NOS II) being inducible in macrophages, glial cells, astrocytes, and perhaps other cells as well. The induction of iNOS occurs as part of the nonspecific inflammatory immune response to microorganisms. Nitric oxide and other reactive nitrogen species are very versatile molecules with many functions. Nitric oxide has been linked to beneficial affects by killing pathogens but also to neurodegenerative processes in humans (65, 171).

Nitric oxide can inhibit MDV replication *in vitro* (166, 631). Increased transcription of iNOS has been reported between 6–12 days PI with MDV (630) resulting in increased levels of NO in the plasma of genetically resistant but not in genetically susceptible chickens (167, 290). Nitric oxide may be beneficial, because it inhibited MDV replication *in vivo* when genetically resistant chickens

were challenged with vMDV (631). However, pathology may be associated with very high levels of NO production especially in genetically resistant birds challenged with vv+MDV (288).

NK Cells. Natural killer cells are the first line of defense because these cells can lyse virus-infected and tumor cells without prior exposure to the pathogen. Natural killer cells are also potent inducers of IFN-γ. In order to lyse target cells, NK cells must recognize the target cells as foreign (e.g., the MHC-I has been altered or downregulated). Thus the downregulation of MHC-I during the lytic infection (268) supports a potential role for NK cells. Thus far functional NK cell assays have used total spleen cell populations and LSCC-RP9 as target cells in chromium release assays (CRA). The recent development of NK cell-specific mAb (284) will facilitate further examination of the importance of NK cells in MD immunity.

Macrophages. Activated macrophages can restrict MDV replication and perhaps as a consequence reduce tumor incidence. These effects are probably the result of NO production (497, 502) or by reactive oxygen intermediates (166). Increased numbers of macrophages have been noted shortly after infection in the lung in conjunction with increased transcription of iNOS. Macrophages harvested shortly after MDV infection can inhibit proliferation of MD cell lines *in vitro*, which was considered to be a transient immunosuppressive effect (334, 513). This inhibition may actually be a protective response, because it may limit the number of activated T cells during the critical switch of MDV from B to T cells (497, 502).

Acquired Immunity

Humoral Immunity. Chickens infected with MDV develop precipitating and VN antibodies within 1–2 weeks; a transient IgM response is replaced by IgY (256). Due to the cell-associated nature of MDV, antibodies are of limited importance in MD immunity. Virus-neutralizing antibodies are important only when cell-free virus infects chickens or when MDV proteins are expressed on the surface of cells. In the latter case, antibodies plus complement or antibody-dependent, cell-mediated cytotoxicity (ADCC) can lyse infected cells (317, 472). However, the target antigens and the effector cells involved in ADCC were not identified. *In vivo* VN has indeed been demonstrated using cell-free and cell-associated virus (79). Maternal antibodies reduce the cytolytic infection (86) and can reduce the efficacy of cell-associated vaccines with low titers or if cell-free HVT is used (111, 315).

The possibility that surface antigens found on MDV-transformed cells could be involved in immunity was suggested by protection against challenge after immunization anti-idiotype antibodies against MATSA (151). Similarly, antibodies against CD30 may provide protection against tumor cells (78).

Cell-Mediated Immunity. Cytotoxic T lymphocytes (CTL) recognize small peptide fragments of 8–12 amino acids presented in the context of MHC-I antigens. These peptides are generated from *de novo* synthesized proteins through a complex process involving the proteasome and transporters associated with antigen-processing (TAP) 1 and 2. *In vitro* demonstration of antigen-specific CTL requires effector and target cells expressing the same MHC-I antigens (515).

Recently, Mwangi et al. (386) found a limited number of CD8+ clonal cell populations, which were generated shortly after infection suggesting that MDV infection results in a limited number of antigen-specific clones. Earlier, Pratt et al. (448) stably transfected and expressed MDV genes in REV-transformed cell lines with known MHC antigens. These cell lines were used to show that CTL from infected or vaccinated chickens recognize peptides derived from pp38, Meq, ICP4, ICP27, gB, gC, gH, gI, and gE (361, 404, 502). The effector cells developed around 7 days PI and were characterized as typical CTL expressing CD3, CD8, and TCRαβ1 but not CD4 (405). Important differences were noted in the recognition of proteins by CTL from resistant and susceptible chicken lines. Cytotoxic T lymphocytes from resistant N2a (MHC: $B^{21}B^{21}$) but not from susceptible P2a (MHC: $B^{19}B^{19}$) chickens recognized ICP4 (404). Effective killing of infected cells as soon as ICP4 is expressed, for example, when latently infected cells are reactivated, and before virus replication is completed could be one of the contributing factors to MHC-based genetic resistance. Cytotoxic T lymphocyte-specific responses to gB, gI and pp38 also appear important to MD protection. (328, 394).

Cytotoxic T lymphocytes and NK cells can lyse target cells through the perforin/granzyme pathway. Increased transcription rates of Granzyme A occur between 4–15 days PI (251, 480, 481).

Vaccinal Immunity. Herpesvirus of turkey, attenuated MDV, and serotype 2 MDV protect against early replication of virulent viruses in the lymphoid organs of challenged birds, reduce the level of latent infection, but do not prevent infection (reviewed in 211, 497, 600). Based on current knowledge, the following sequence of events is proposed to explain vaccine-induced immunity with challenge occurring within three days after hatch as is typical in the field (Figure 15.14).

Vaccination with CVI988 results in upregulation of IFN-γ, IL-8, IL-18, and iNOS in lung and spleen as early as 3 days PI (213), which is probably also the case after vaccination with HVT and SB-1. IFN-γ can reduce virus replication and stimulate macrophages to initiate the

transcription of iNOS, producing NO between 3 and 7 days post vaccination, thus limiting the replication of challenge virus. Shortly afterwards, NK cells are activated (254, 514), probably producing more IFN-γ and killing virus-infected B cells.

Feather pulp can be used to examine temporal changes in immune responses in individual, vaccinated chickens. This will facilitate monitoring cytokine responses induced by different vaccines (5, 244). Vaccination with CVI988 showed a down regulation of RB-1B in the feather pulp (244). Challenge did not increase replication of CVI988 (34, 244) in contrast to the observation that HVT and SB-1 shedding increased after challenge with MDV (278).

Immunoevasion, caused by MDV, can interfere with vaccine-induced immunity. MHC-I downregulation is one strategy to reduce the host defenses, as reviewed elsewhere (245). The MDV gene MDV012 was recently demonstrated as capable of reducing surface expression of MHC-I on chicken cells (248). The host response can also be altered by differences between vaccines from different manufacturers, which probably relates to passage levels (331). Concurrent infections with immunosuppressive viruses, for example, CIAV (362) or stress may interfere with vaccine-induced CMI responses. Deletion of humoral immunity by bursectomy and X-irradiation does not seem to have a major effect on protection conferred by attenuated MDV (183), although similar treatment partially impairs HVT vaccine immunity (467).

Immunosuppression

Suppression of the immune response by MDV infection is a critical feature of the disease, contributing to the virulence of MDV isolates and altering susceptibility of the host to other pathogens (reviewed in 486, 500). Initial impairment of the immune response is the result of the lytic infection of lymphocytes during the first cytolytic infection (see Pathogenesis) (citations in 486, 500). The onset of immunosuppression later in life appears to be a unique feature of vv+MDV strains and is not eliminated by vaccination (196, 197). It is difficult to distinguish between cause and effect because tumor cells might have suppressor activity (69, 456). Because immunocompetence is required for the maintenance of latency (82) it might be that immunosuppression associated with the appearance of transformed lymphoblasts results in additional reactivation of the lytic infection. This, in turn, will cause the loss of additional B and T cells, thus compounding the situation and resulting in the bursal and thymic atrophy seen in birds destined to succumb to MD. However, immunosuppression may not be a prerequisite for the development of tumors, as observed with several experimental vaccine candidates (175, 611). Although virus-induced immunosuppression and oncogenicity are not invariably linked, they are often expressed concurrently and, in such cases, immunosuppression may serve to augment oncogenic potential.

Humoral and CMI can be suppressed by MDV infection leading to reduced antibody responses to a variety of antigens and alterations in T cell functions, such as skin graft rejection, mitogen stimulation of lymphocytes, delayed hypersensitivity, reduced NK cell activity, primary and secondary infections with coccidia, and impaired Rous sarcoma regression (citations in 427, 499).

Diagnosis

Techniques for diagnosis of infection with MDV are different from those needed for differential diagnosis of the disease. The infection is ubiquitous, but the disease is not. The principal methods to identify the presence of infection are isolation of the virus, demonstration of viral DNA or antigens in tissues, and detection of antibody. The applications of different diagnostic procedures have been recently reviewed (223, 401, 606).

Virus Isolation

Virus isolation is performed to confirm its presence for diagnostic purposes and to secure the infectious virus for further study. Techniques for isolation of all serotypes have been reviewed (223, 487).

Source of Virus

Marek's disease virus can be isolated as early as 1 or 2 days PI or 5 days after contact exposure and throughout the life of the chicken (reviewed in 499). Intact viable cells are the preferred inoculum because, in most cases, infectivity is avidly cell-associated, although cell-free preparations from skin, dander, or feather tips of infected chickens may contain the virus (92). Inocula may consist of blood lymphocytes, heparinized whole blood, splenocytes, or tumor cells. Marek's disease virus can often be recovered from infected cell suspensions following storage for 24 hours at 4°C, thus facilitating transport of samples (reviewed in 499).

Cell Culture Techniques

Probably, the most widely used method for primary isolation of MDV is inoculation of susceptible cell cultures with blood lymphocytes or single-cell suspensions from lymphoid tissues of infected chickens. Chicken kidney cell and DEF cultures are preferred substrates for primary isolation of serotype 1 MDV; whereas CEF normally are used for isolation of viruses of serotypes 2 and 3 as well as for attenuated serotype 1 vaccine strains. Although CEF are less permissive for growth of low passage serotype 1 virus (reviewed in 487), some contemporary isolates may grow well in CEF, even on primary isolation. Cultures are inoculated with $1–2 \times 10^6$ cells, although

some inhibition of viral plaque formation may be encountered with doses greater than 8×10^6 cells for some viruses (109).

Development of typical plaques (Figure 15.3) in inoculated cultures within 3–12 days and the absence of such changes in comparable uninoculated (or sham inoculated) control cultures are evidence for isolation of MDV. The plaques induced by serotype 1, 2, and 3 viruses can be distinguished, with practice, by morphologic criteria (484, 590), but IF staining with serotype-specific mAbs provides a more accurate differentiation. Optimal time for observation of plaques varies with the cell substrate and serotype of the virus. Marek's disease virus also has been isolated by direct culture of kidney cells from infected chickens or by inoculation of normal kidney cultures with trypsinized kidney cells from infected chickens (621).

Isolate Identification

Marek's disease virus serotype 1 isolates should be free of contaminating MD vaccine strains. It normally is useful for the isolate to be plaque purified or cloned at the earliest possible passage. Serotype identity and purity can be confirmed using staining techniques with serotype-specific mAbs (332). Freedom from extraneous viruses is critical, because contamination with passenger viruses may alter the apparent pathogenicity of the isolate (291, 409). Propagation of MDV isolates for up to six passages in CEFs or CKC cultures appears to exclude contaminants such as CIAV (641) and permits preparation of seed and working stocks, which can be more easily standardized and titrated. To preserve virulence, some workers have preferred to propagate serotype 1 viruses *in vivo*, preparing stocks of cryopreserved spleen or buffy coat cells from infected chickens. Pathotyping of serotype 1 MDVs, although not routine, may be accomplished by comparison of pathogenicity with that of prototype strains by inoculation of non-vaccinated chickens as well as chickens vaccinated with HVT or bivalent vaccines (604).

Virus Assay and Titration

Viruses of serotypes 1, 2, and 3 can be assayed by *in vitro* techniques similar to those described for virus isolation. Methods differ for different serotypes, but most rely on plaque induction in susceptible cell cultures. Enumeration should be done as soon as plaques become mature (time varies with isolate), because secondary plaques may occur when cultures are maintained with liquid medium. Procedures for titration of vaccine viruses have been reviewed (559) and are not fundamentally different from those for pathogenic isolates.

Viral Markers in Tissues

It is often desirable to detect the presence of viral infection in chickens without isolating the virus in culture. Such infection markers also have value for the identification of putative MDV isolates in cell cultures. However, only detection of oncogene meq and high load of MDV DNA in tumors can be used as diagnostic criteria for the disease (226).

Viral Antigen Detection

Monoclonal antibodies prepared against type-common and type-specific epitopes of all three MDV serotypes (332) are now used in preference to polyclonal antibodies for the detection of antigens in tissues. Monoclonal antibodies H19 and T65 can be used to differentiate CVI988 from other serotype 1 MDVs strains based on differences in the amino acid sequence of protein pp38 (147, 220). Viral antigens can be detected in feather tips and FFE, cytolytically infected lymphoid tissues, brain, or infected cell cultures with appropriate antibodies by fluorescent antibody tests, immunohistochemistry, dot-ELISA (322), agar gel precipitin (AGP) tests, and immunoassay (reviewed in 223, 499). In MD lymphomas, oncogene meq is the only antigen that is consistently expressed (226, 477) but pp38-positive cells occasionally are observed (226).

Viral Nucleic Acid Detection

Polymerase Chain Reaction (PCR) Assay. The availability of the nucleotide sequences of different genes from a large number of viruses including the complete genome sequence of the three serotypes of MDV allows the use of PCR-based methods of specific detection of MDV. Primers designed to amplify sequences specific for each serotype (277, 464) as well as to differentiate between serotype 1 oncogenic and attenuated strains based on the 132 bp (42, 525, 655), and between CVI988 and other serotype 1 MDV strains (30, 220, 468) have been described. However, PCR may not always be sensitive enough to detect latent infection due to the lower frequency of positive cells and the lower number of viral genomes per cell. Furthermore, detection of MDV DNA, even from an oncogenic MDV, does not have any diagnostic value as chickens get exposed to oncogenic MDVs, if properly immunized, they never develop MD.

Quantitative PCR (qPCR) assays using various primer sequences have been used to assay viral load in tissues from infected chickens (30, 31, 72, 76, 220, 277, 464) and are essential tools for diagnosis and epidemiological studies of MD. Unlike conventional PCRs, qPCR has been shown to be very valuable in the diagnosis of MD (218, 220, 226, 368, 582), for monitoring MD vaccination (136, 214, 216, 217, 220, 241, 276, 279, 469), and for studying different aspects of MD biology including replication kinetics of MDV in blood, feathers, lymphoid tissues, and dust (34, 136, 244, 276, 279, 280, 457–459).

DNA Probes. Methods using DNA–DNA dot-blot hybridization with DNA probes for the detection of

MDV DNA in feather tip extracts (154) have been used for detection of MDV (382–384). Furthermore, localization of virus-infected cells has been accomplished by *in situ* hybridization for both MDV (185, 434, 475) and HVT (265) and by fluorescence *in situ* hybridization (FISH) for MDV (302).

Loop-Mediated Isothermal Amplification. Loop-mediated isothermal amplification (LAMP) methods for the rapid detection of MDV-1 genome at high sensitivity were developed for detection of MDV in less-equipped laboratories as well as under field conditions (17, 175, 625, 627, 628).

Antibody Detection

Tests for identifying the presence of specific antibodies in chicken sera are useful in studies of viral pathogenesis and for monitoring SPF flocks. However, it does not have any value in the diagnosis of MD in commercial flocks. A number of procedures including AGP, fluorescent antibody ELISAs (373, 646), and VN tests are in common use (review in 223).

Diagnosis of the Disease

Despite long-established guidelines for the pathologic diagnosis of MD (524), diagnosis of the clinical disease remains difficult in practice because of the absence of truly pathognomonic gross lesions and the widespread nature, often with coinfection, of other pathogens such as ALV and REV (149, 153), complicating diagnostic efforts that depend on virological methods.

Diagnosis of MD must primarily be addressed by consideration of the characteristics of the proliferating cell populations that constitute the disease. Other disease-specific criteria, such as epidemiological factors, are also valuable. Detection of virus, viral DNA, or viral antigens (with the exception of oncogene meq) does not have any diagnostic value since infection is ubiquitous and vaccination does not prevent superinfection (226). However, quantification of MDV DNA load in tumors is a diagnostic criterion that can confirm the diagnosis of MD (218, 220, 226). The process commences with the acquisition of a flock history and a sufficient number (5 to 10) of representative sick and dead chickens showing the lesions of the disease and proceeds as a series of steps (606).

Step 1—Clinical Data and Gross Pathology

Although enlarged peripheral nerves and visceral lymphomas are common in MD and one or both are invariably present, neither lesion occurs consistently nor is pathognomonic. Thus, other criteria, such as age and lesion distribution, must be considered in the postmortem diagnosis of MD. Chickens may be diagnosed provisionally as MD if at least one of the following conditions is met: (1) leukotic enlargement of peripheral nerves; (2) lymphoid tumors in various tissues (liver, heart, gonad, skin, muscle, and proventriculus) in birds under 16 weeks of age; (3) visceral lymphoid tumors in birds 16 weeks or older that lack neoplastic involvement of the bursa of Fabricius; or (4) iris discoloration and pupil irregularity, as in Figure 15.8C. Proper examination of the bursa is particularly important and requires incision of the organ with close inspection of the epithelial surface. However, diagnoses based only on gross pathologic criteria are not definitive and additional steps are required.

Step 2—Histology, Cytology, and Histochemistry of Tumor Cells

Affected tissues, fixed in formalin or fresh-frozen, are used to prepare paraffin and cryostat sections, respectively. Impression smears of tumors may also be used (524). Histopathology can be very useful to confirm the diagnosis of lymphoma and to evaluate the morphology and distribution of tumor cells. Marek's disease tumors consist of a mixed population of small to large lymphocytes, lymphoblasts, plasma cells, and macrophages (222, 426). The presence of such infiltrates in the nerves (lesion type A) is the only pathognomonic lesion of MD (326, 426). However, MDV is also able to induce inflammatory lesions characterized by edema, demyelination, and plasma cell infiltration (lesion type B) that are not necessarily related with MD lymphoma and can be confused with peripheral neuropathy (25, 228, 326, 605). Likewise, minor infiltration of lymphocytes in peripheral nerves might be indicative of infection with MDV but not of MD lymphoma (review in 606).

Characterization of the cell phenotype by immunohistochemistry can aid in the differential diagnosis of MD lymphomas. Marek's disease tumor cells express MHC class II antigen and T-cell markers, especially CD4 (495). CD30 (477) and MATSA (623) are commonly found in MD tumor cells although they are also present on retrovirus-induced B cell lymphoma (226) and activated T cells (226, 365).

Step 3—Virologic Criteria

For tumors that satisfy MD criteria listed in steps 1 and 2 or for atypical tumors, the association of MDV with the tumor cell is a useful confirmation. Viral antigen Meq can be consistently detected in tumor cells by *in situ* hybridization, immunohistochemistry, or fluorescent antibody tests and can be used as diagnosis criterion to confirm MD tumors (226, 477). Detection of other viral antigens such as pp38 has been described in tumors (390) but expression is sporadic, it seems to be related to reactivation of virus in neoplastic cell, and it is not an adequate diagnostic criterion for MD tumors (226).

Polymerase chain reaction assays and virus isolation from buffy coat, spleen cells, or feather pulp samples as well as detection of viral proteins demonstrate the virus in the bird but do little to associate the virus with the tumor cells. However, there is a quantitative association between virus load and MD tumors. Low levels of virus or viral DNA may be detected in lymphocytes from nontumor-bearing chickens, but most tumor-bearing chickens have high viremia titers (622) and high load of MDV DNA measured by qPCR (33, 218, 220, 226, 459). Thus, the demonstration of high load of MDV DNA in tumor cells and the absence of other tumor viruses, along with the criteria in steps 1 and 2, is sufficient to establish MD diagnosis. Evaluation of MDV DNA load in blood of feather pulp of chickens as early as 21 days of age can also be used to predict the MD outcome on those flocks (218, 220, 459). Proper collection of samples for MD diagnosis using qPCR has been recently reviewed (223).

Pathotyping of MDV Strains

The concept of MDV pathotypes has arisen from the recognition of the existence of strains that are associated with increased virulence that show correlation with breaking of vaccinal immunity in the field (604). The ADOL (Avian Disease & Oncology Laboratory) method of pathotyping, based on induction of lymphoproliferative lesions in chickens vaccinated with different vaccination regimes is the most widely used. This method was used to characterize more than 45 isolates into distinct vMDV, vvMDV, or vv+MDV pathotype groups (597). Even though the ADOL method stipulates the use of line 15x7 chickens for pathotyping, experiments with other lines of birds have given similar results (83, 170, 604). The ADOL pathotyping assays are cumbersome and require infrastructure that is lacking in most laboratories. Therefore, different methods to differentiate between classical strains of MDV and the more virulent pathotypes have been examined including neuropathotyping (230), virus replication (172), lymphoid organ atrophy and immunosuppression (95, 196), and sequence of various genes (512, 555). However, classification obtained by the alternative methods do not always render the same results as ADOL pathotyping and most of these techniques are considered an adjunct to pathotyping more than substitutes of the "gold standard" ADOL pathotyping assay.

Differential Diagnosis

Details on how to do differential diagnosis of MD with other poultry tumor diseases has been reviewed (606). The major differential diagnosis for MD is lymphoid leukosis (LL). Lymphoid leukosis is a clonal, bursal lymphoma induced by ALV and, under some conditions, by REV in chickens older than 16 weeks of age. Chickens usually have gross tumors in the bursa of Fabricius, and tumor cells are uniform, blast-like, pyroninophilic, and express B-cell markers and IgM. Also, the tumor cells have clonal insertions of proviral DNA near the *c-myc* gene (see Leukosis/Sarcoma Group). Nerve enlargement, runting, and nonbursal T-cell lymphomas can be induced by REV but, thus far, have only been observed under experimental conditions or where chickens have been inoculated with contaminated vaccines. Lymphocytes obtained from REV-induced nerve lesions or tumors do not express Meq and have low MDV DNA load, if any (226). Cells from nonbursal RE lymphomas are negative for MHC class II and predominantly stain for CD8 antigen (606) (see Reticuloendotheliosis). Exclusion of ALVs or REVs, where possible, through negative PCR, histochemical assays on tumors, or antibody tests may provide strong support for a diagnosis of MD when other MD-related criteria are positive. However, it is possible to have mixed infections and in those cases further diagnostic assays (qPCR for MD and southern blot for retroviruses) are needed (190, 226).

Peripheral neuropathy is a neurological disease of uncertain etiology that causes paralysis and nerve enlargement in a low proportion of commercial chickens 6–12 weeks of age (25, 53) and is characterized by a Th1-to-Th2 shift (26). Affected chickens lack visceral lymphomas; the nerve lesions are uniformly B-type; and MDV is rarely, if ever, demonstrated. If MDV is present on those chickens, MDV DNA load is low (606). Other diseases that may present confusing gross lesions or paralytic signs are myelocytomatosis (myeloid leukosis), myeloblastosis, erythroblastosis, histiocytic sarcomas, carcinoma of the ovary, various other nonviral neoplasms, riboflavin deficiency, tuberculosis, histomoniasis, genetic gray eye, Newcastle disease, hepatitis E, and joint infections or injuries. Myeloid leukosis is a common tumor in broiler breeder flocks that superficially resembles MD but can be differentiated histologically. The tumor cells are myeloid in nature and lack T cell and MD viral markers.

Diagnosis of Other MD Syndromes

Transient paralysis occasionally is observed in the field, especially in chickens not vaccinated against MD once maternal antibodies have waned between 30–40 days of age (reviewed in 222). Development of TP in vaccinated commercial flocks would indicate failures on the vaccination process as proper vaccination against MD protects against the development of TP (reviewed in 222). Diagnosis of TP is done based on the history (sudden onset of flaccid paralysis that last 24–48 hours and can lead to death or total recovery), lack of gross lesions in nerves or viscera, and histopathology (brain edema and

vasculitis). There are three diseases that need to be included in the differential diagnosis with TP: botulism, neurological form of MD (fowl paralysis), and peripheral neuropathy. Both TP and botulism cause flaccid paralysis of the neck progressing to the limbs and are not associated with gross lesions in the nerves or viscera. Confirmation of TP can be done by histopathological examination of the brain as botulism does not produce brain edema and vasculitis characteristic of TP. Unlike TP, the neurological form of MD appears as permanent spastic paralysis with enlargement of peripheral nerves and pathognomonic type A lesions in the nerves. Peripheral neuropathy tend to last longer than TP and is also characterized by peripheral nerve enlargement with lesions that resemble MD B-type lesions. Davidson et al. (155) differentiated transient paralysis from peripheral neuropathy on the basis of PCR tests for MDV on brain tissue. However, brains from MDV-infected chickens without transient paralysis may also be detected as positive by PCR assays (607). In contrast, detection of viral antigens in the brain appeared to correlate with the onset of paralytic signs (227).

Skin leukosis (the skin form of MD) can be differentiated from dermal squamous cell carcinoma, which is commonly observed in defeathered broiler chickens at processing (236, 391). Marek's disease lesions are nodular and contain lymphoid cells, whereas squamous cell carcinomas have a craterlike gross appearance and are composed of squamous epithelial cells.

Due to its complexity, the most difficult syndrome to diagnose is MDV-induced immunosuppression (MDV-IS). Early MDV-IS is associated with replication of MDV in the lymphoid organs of unvaccinated chickens lacking maternal antibodies and it is characterized by atrophy of the bursa and thymus. However, under field conditions lymphoid organ atrophy could be due to several infectious and noninfectious diseases. Experimentally it is possible to detect MDV antigens in the lymphoid organs before the atrophy occurs, however such expression occur for a short period (1–2) days and is not associated with clinical signs or gross lesions. Late MDV-IS is even more complicated to diagnose than early MDV-IS as it is not associated with lymphoid organ atrophy and/or tumors (195–197). Because late-MDV-IS cannot be protected by currently vaccination protocols (197), it is very likely causing problems in commercial flocks and should be considered in the differential diagnosis when immunosuppression is suspected.

Intervention Strategies

The development of successful vaccines for control of MD (132, 403, 470, 488) was a significant achievement. Vaccination represents, for now and the foreseeable future, the central strategy for the prevention and control of MD. Genetic resistance and biosecurity, however, are critical adjuncts to properly executed vaccination procedures. No effective practical treatment exists for the disease in individual chickens or infected flocks. An integrated strategy to prevent early infection, to slow the acquisition of virulence of field strains, and to provide superior immune responses seems most likely to succeed. Various reviews on MD vaccines and control procedures are available (67, 211, 462, 489).

Vaccination

Types of Vaccines

Several different types of MD vaccines are in common use, both individually and in various combinations. The most widely-used products are low pathogenic serotype 1 MDV attenuated in cell culture (470) and naturally nononcogenic serotype 3 (HVT) (403), and serotype 2 viruses (490, 618). The latter usually are combined with HVT to take advantage of the synergistic activity documented between serotypes 2 and 3 (492, 589). All vaccine types are protective but to varying degrees. Herpesvirus of turkey virus, mainly strain FC126 (613) is extensively used because it is effective and economical to produce and combines well with other products. Although both cell-free and cell-associated forms of HVT are available, the latter has been most widely used because it is more effective than cell-free virus in the presence of maternal antibodies (603). Bivalent vaccines consisting of HVT and SB-1 (490) or 301B/1 (618) strains of serotype 2 MDV were introduced in the mid-1980s. The CVI988 strain (470) used in the Netherlands (356) and other countries since the early 1970s, was introduced to the United States in the early 1990s. Serotype 1 and 2 vaccines are available only as cell-associated products.

Besides the traditional vaccines, in the recent years a number of recombinant vaccines have been developed, licensed, and are currently commercialized (reviewed in 462). Efforts to develop recombinant MD vaccines started in the early 1990s using either fowlpox virus (394) or HVT (374, 474) as vectors. However, it has been in the last few years that several products using HVT as a vector for various diseases (infectious laryngotracheitis, infectious bursal disease, Newcastle disease, and avian influenza) have been licensed and widespread used; either alone or in combination with MD vaccines of other serotypes (188, 189, 205, 208, 232, 292, 339, 411, 435, 568). In addition to HVT vector vaccines, recombinant vaccines based on serotype 1 MDV strains have been developed either by using attenuated serotype 1 MDV strains as vector (275, 349, 566, 647), by modifying or deleting genes (62, 144, 333), by insertional mutation of the long terminal repeat (LTR) region of the REV in various MDV serotype 1 strains (149, 313, 353, 364, 611), or by combining

various techniques (i.e., REV LTR insertion and deletion of meq, meq-deleted vaccines as vectors) (215, 545). To date, the only recombinant vaccine based on serotype 1 MDV strains that is licensed and commercialized in some countries is a recombinant CVI988 vaccine with an insertion of the REV LTR (66, 353). This vaccine has been shown to replicate and protect better than the parental CVI988 and does not induce lymphoid organ atrophy in susceptible chickens lacking maternal antibodies (66). However, experimental vaccine based on vvMDV strain Md5 with deletion of both copies of the oncogene meq (352, 526) has been shown to be the most protective vaccine against early challenge with vv+MDV (115, 331). This vaccine protects not only against the development of tumors but also against the development of late MDV-IS in commercial meat type chickens (197). Furthermore, it can be successfully used as a vector vaccine for other poultry diseases (215, 651). Unfortunately, the deletion mutant is causing severe thymus and bursa atrophy in maternal antibody-negative, one-day-old chicks (175) preventing licensing in the United States under current regulations. The negative effect on the lymphoid organ in chickens lacking maternal antibodies can be reversed by attenuation in cell culture or by adding a UL5 helicase-primase subunit point mutation; albeit at a cost of reducing protection (257, 330).

Other strategies to improve vaccines through recombinant DNA approaches that might have potential use in the future includes DNA vaccines (563), incorporation of cytokines (168, 243, 554), and Toll-like receptor-based adjuvants (419, 421).

Vaccine Administration

Marek's disease vaccines are administered to chicks at hatch by subcutaneous or intramuscular inoculation or *in ovo* at ED18 (67, 516) *In ovo* vaccination is now performed by automated technology and is used in more than 90% of commercial broiler chickens in the United States (586). *In ovo* vaccination not only reduces labor costs and has greater precision of vaccine administration but also confers better protection against early challenge with MDV than one-day-old vaccination (649). Furthermore, it has been recently shown that *in ovo* vaccination with HVT hastens the maturation of the immune system of the chicken embryo rendering chicks more immunocompetent at hatch (221). Deposition of the vaccine by the amniotic or intraembryonic route is essential for optimal protection (580, 585). Proper handling of vaccine during thawing and reconstitution is crucial to ensure that adequate doses are administered (207, 239).

Factors Affecting Efficacy

Vaccines typically are given at doses of 2,000–6,000 plaque forming units (PFU) per chick, but these are often significantly reduced in broilers. The older literature suggests that increased doses of HVT above a threshold level of around 400 PFU did offer little improvement (178). However, recent studies indicate that a substantially higher threshold is needed to protect against challenge with vv and vv+ strains (214, 216, 217).

Revaccination has been recently shown to increase protection against early challenge with MDV (219, 224, 629). The benefits of double vaccination has been reproduced under laboratory conditions (224) when the second vaccine is more protective than the first vaccine administered and both vaccinations occur before challenge with oncogenic MDV (224). It has been suggested that the best revaccination program will include a low protective vaccine *in ovo* (i.e., HVT) followed by a high protective vaccine at day of age (i.e., HVT+SB-1 or/and CVI988) (219).

Several other factors may influence vaccine efficacy such as the passage level of the vaccine viruses. Increased passage level may cause a decrease in vaccine replication resulting in decreased immunity (330, 614). Unfortunately, passage levels of commercial vaccines are in general not available. Maternal antibodies against the three serotypes are typically present in commercial one-day-old chicks. These antibodies may reduce the effectiveness of cell-associated vaccines but do not abrogate the protective effect (315), if sufficient PFU are administered.

In addition to improper vaccination techniques (381), early exposure is undoubtedly one of the most important causes of excessive MD in vaccinated flocks because field exposure usually occurs very soon after placement of chickens (612) and because at least seven days is required to establish solid immunity after vaccination (281) (reviewed in 502). The vaccine strain of virus also has a major influence on vaccine efficacy. Immunity induced by weaker vaccines such as HVT may be excellent against low virulence challenge but can be completely overwhelmed by early challenge with highly virulent strains (597). Protection conferred by CVI988, however, does not seem to be as affected by the pathotype of the challenge strain (459) as vaccines of other serotypes. Although high virulence strains commonly are invoked to explain field outbreaks of disease, many alternate causes should be considered.

Stress appears to interfere with the maintenance of vaccinal immunity. In chickens properly vaccinated at hatch and well-protected following challenge, Powell and Davison (441) induced MD lesions and mortality by immunosuppressive treatment at 10 weeks of age. The onset of egg production deserves consideration as a stress factor precipitating vaccine breaks (600). Infection with other pathogens such as IBDV, REV, CIAV, and reoviruses have been reported to interfere with the induction of vaccinal immunity (reviewed in 500), although very specific conditions are sometimes required. T2-toxin has been

recently shown to have detrimental effect on the MD outcome of nonvaccinated chickens but it did not affect protection conferred by HVT (321).

The strain of chicken is also an important determinant of vaccine efficacy. Schat et al. (492) found that HVT vaccine in genetically resistant chickens resulted in a stronger immunity than did the bivalent (HVT+SB-1) vaccine in susceptible chickens. Chang et al. recently demonstrated that in genetically resistant lines of chickens, HVT can induce as much protection as some of the commercial CVI988 strains (117). In commercial chickens, protective immunity conferred by vaccines is influenced by the B-haplotype (22) as well as by non-MHC genetic variation (116).

Vaccination Strategies

Marek's disease vaccines as a class are unusually effective, often achieving greater than 90% protection under commercial conditions (600). However, attention is often focused on flocks in which MD losses are perceived to be excessive (381). Causes for such vaccine failures are difficult to ascertain by retrospective analysis (381, 600). However, there are several checkpoints that could be evaluated in the event of an immunization failure (212). Auditing at the hatchery to evaluate improper vaccination techniques, titration of the batch of vaccines used, replication of the vaccine in the chickens, early exposure to oncogenic MDV, coinfection with other immunosuppression agents (especially with CIAV infection), and the emergence of new MDV strains with increased virulence need to be considered (212).

Efficacy data comparing certain groups of vaccines are available (citations in 485, 600). Bivalent serotype 2+3 vaccines are clearly more effective than HVT, but the original CVI988 vaccine seems to be the most effective (593, 608), a result consistent with earlier reports from Europe (572). However, differences in the level of protection conferred by CVI988 from different sources have been documented (214, 608). Recent studies demonstrate that a recombinant vaccines with the deletion of both copies of the oncogene meq provides even better protection than the most protective CVI988 strain (115).

The propensity of MDVs to evolve to greater virulence is critical to the strategic use of vaccines for MD control (210). Vaccination itself no doubt contributes to this virulence increase, which, in turn, tends to make earlier vaccines obsolete. Kreager (320) noted that the useful life of a MD vaccine has been about 10 years under current management conditions. Although this is perhaps an overstatement, the implications are serious. Since CVI988 was introduced in the United States, some evidence already suggests that contemporary strains have increased their virulence in CVI988-vaccinated chickens (599). In a recent study, Dunn et al. demonstrated that in those farms where vv+MDV were isolated in late 1990s current MDV isolates are still vv+MDV strains suggesting that once vv+MDV infection gets established in a farm it will stay (173).

The emergence of increasingly virulent viral strains, coupled with an apparent reduction of vaccine efficacy during the past 20 years, has prompted justifiable concern. This suggests that vaccination by itself does not provide a complete control program and is not the ultimate solution for MD. In fact, recent evidence also points towards the potential contribution of the early generations of vaccines themselves driving virulence and selection of more virulent MDV pathotypes (461). Strict biosecurity procedures to reduce early exposure and the presence of genetic resistance are essential adjuncts to a successful vaccination program. Furthermore other than increased protection against tumors vaccine research should focus on protecting against other aspects of MDV infection such as infection, transmission, and MDV-IS.

Genetic Resistance

The well-known variation in susceptibility of different lines of chickens to MD challenge is determined by genetic factors (20, 71, 120) and provides a unique opportunity to include genetic approaches to control MD. Indeed, poultry breeders have included resistance to MD in selection programs for many years (366). However, genetic resistance can be overcome by challenge with highly virulent MDVs and is best applied in concert with vaccination and biosecurity to achieve optimal control. Genetics influences virtually every aspect of host response to MD. However, only those issues germane to disease control programs are considered here.

For successful incorporation of selection for resistance in breeding programs several conditions need to be met (reviewed in 20). The heritability of resistance is relatively large, thus selection has a considerable impact. Selection for resistance is at least neutral for production traits or is correlated positively with production traits (13). Sufficient heterogeneity exists in single-sire families to warrant selection for resistance in commercial chickens, which is still the case in recent commercial genetic stocks (184).

Selection Methods

Selection programs for resistance historically have been based on progeny testing or family selection (133) or reproduction from survivors of exposed nonvaccinated breeding flocks through mass selection (355). Bacon and Witter (21) found that resistance may better be determined in vaccinated stocks. Under certain conditions acquisition of resistance can be obtained within four to six generations (133, 355). Family selection may be more appropriate than mass selection for commercial breeders in order to avoid high loss of genetic material on initial challenge exposure (20). Resistance has been considered

dominant, although this varies to some extent (255). In most cases, resistance of crosses has been intermediate to that of the parent strains (55).

In addition to selection based on challenge with MDV, blood typing for MHC can be used based on the close relationship between MD resistance and certain alleles of the B-F region of the MHC, especially B^{21} (61, 71). However, the value of selection for MHC-associated markers may vary considerably among commercial lines and crosses (55, 247).

Several non-MHC genes may also be involved in resistance. For example, line 6 and line 7 chickens differ markedly in MD susceptibility, but both lines are both homozygous for the MHC B^2 allele (143). Because non-MHC effects were considered more important than MHC effects in several commercial lines (234) the need to identify genetic markers associated with MD resistance became priority in all genomic studies. Various strategies have been used to identify genetic markers of MD resistance including identification and mapping of map quantitative trait loci (QTL), RNA expression profiling, expression quantitative trait loci, and allele-specific expression (ASE) screens (reviewed in 120). Genome-wide QTL scans with microsatellite markers have identified 14 or more putative QTL associated with MD resistance (70, 569, 632). To complement the QTL scans, gene expression profiling using microarray technology has been integrated. Gene profiling has been conducted to identify differentially expressed genes between MD-resistant and MD-susceptible lines after MDV challenge (346); among MHC-congenic lines of chickens following inoculation with different MD vaccines(375); and in CEF transformed with Meq (341). Marek's disease virus-chicken protein–protein interactions have been very useful to confirm genes associated with MD resistance(397) such as growth hormone (GH1) (346), stem cell antigen 2 (SCA2) (347), and MHC class II β chain (B-LB)(396). However, purely genetic-driven approaches to identify high-confidence candidate genes have not been as successful as first thought. A combination of genetic and functional genomic approaches such as ASE, epigenetics, and RNA expression profiling seems to be more useful in identifying candidate genes (121, 308, 350, 358, 436, 634, 640).

Applications to Control

The knowledge that genetically resistant chickens are protected by vaccination to a greater extent than more susceptible strains (542) has fueled interest by commercial breeders to emphasize MD resistance in selection programs. However, synergy between host genetics and vaccines is complex. Some resistant B-haplotypes were demonstrated only by challenge of previously vaccinated chickens (21). However, the relative efficacy of MD vaccines is also influenced by B-haplotype, leading to the concept to select the most appropriate vaccine based on the predominant B-haplotypes in a particular strain (23). In practice, this issue has been either ignored or addressed through the use of vaccines containing multiple serotypes. Recently, vaccine efficacy has been shown to be influenced by non-MCH genetic variation (116).

In light of the selection tools available, the absence of negative correlations, and the major benefits to be derived, it is not surprising that breeders place a high priority on this approach. Although selection for B-haplotype has been practiced with variable success and has proven to be complex, especially in meat strains (366), breeders acknowledge the value of improved genetic resistance to offset virulence increase by viral strains and the limitations of current vaccines (319, 624).

Management Procedures

Strict biosecurity practices to limit the extent of early MDV exposure, although impractical as a primary control procedure, are a crucial and cost-effective adjunct to vaccination. Marek's disease control is compromised because modern poultry management decisions are often linked to cost analyses. As a consequence replacement flocks of different ages are placed in close proximity to each other, vaccine doses are reduced and/or litter from a previous broiler flocks is used (489). The failure to prevent early exposure is perhaps the most important single cause of vaccine failures. Improved hygiene has often appeared to play a key and cost-effective role in the elimination of excessive MD losses in vaccinated flocks. Relevant sanitation principles have been reviewed (423).

For SPF flocks, higher standards of biosecurity are required and become cost effective. Most SPF operations use filtered-air, positive-pressure houses, which, along with strict biosecurity measures, successfully can maintain large flocks free of MDV infection. In this case, biosecurity becomes a substitute for vaccination and provides a practical demonstration that MDV infection can be prevented, at least under specialized conditions.

Nononcogenic Avian Herpesviruses

As mentioned earlier, two additional groups of nononcogenic herpesviruses, MDV-2 (*Gallid herpesvirus 3*) and HVT (*Meleagrid herpesvirus 1*), isolated from chickens (50, 125) and turkeys (307, 613), respectively, are considered part of the *Mardivirus* genus. The classification of these two viruses as two distinct serotypes, originally based on the recognition of common and distinct antigenic epitopes (576, 577), has been justified from the data on the complete sequence for serotype 2 strains HPRS-24 and SB-1 (282, 540), and serotype 3 FC126 virus strains (11, 316).

Interest in MDV-2 and HVT derives mainly from their use as live vaccines against MD. However, both viruses occur in nature independent of vaccination, and it seems appropriate to also consider some aspects of their epizootiology and pathogenesis in their natural or alternate avian hosts. Reviews providing additional details on pathogenesis of these infections may be consulted (88, 90).

Turkey Herpesvirus

Herpesvirus of turkey is endemic and ubiquitous in domestic turkeys (619) and has also been isolated from wild turkeys (135). In chickens, the virus has also become ubiquitous because of its widespread usage as vaccines (67). Increasingly, HVT is used successfully as recombinant live virus vectored vaccines against a number of diseases (review in 462).

The 160 kb-long HVT genome encodes nearly 100 functional genes that include sets of homologous and unique genes (11, 316), as well as novel microRNAs (579, 637). The function of HVT genes in relation to their distinct properties of high immunogenicity, lack of oncogenicity, and ability to produce cell-free virus are not fully understood. The availability of infectious BAC clones of HVT (32) will be helpful in gaining further insights into HVT gene functions.

In turkeys, HVT spreads rapidly through exposed flocks by contact exposure as no evidence of vertical transmission has been demonstrated (620). Virtually all individual turkeys become viremic and develop antibodies within a few weeks (619). The virus appears to mature in the FFE, because cell-free skin extracts are infectious (620) although viral antigen was found only infrequently and at low levels in the FFE (194). Herpesvirus of turkey may be transmitted from turkeys to chickens under experimental conditions (620), although this is considered extremely rare. Only limited contact spread occurs among chickens, but transmission could not be demonstrated by the airborne route (122). Replication of virus in the FFE of infected chickens appears limited and transient (122, 446), although increased levels of HVT DNA were observed in FFE of HVT-vaccinated chickens after MDV challenge (278).

Fabricant et al. (194) studied the early pathogenesis of HVT infection in chickens and turkeys. Chickens had no cytolytic infections in any lymphoid organ. Turkeys infected with HVT had some viral antigen-positive cells at 4–14 days PI in the spleen, but no cytolytic infections in bursa or thymus were seen. In chickens, there was no depression of bursa or thymus size, although a transient splenomegaly was variably present (93, 194). Expression of gB was detected in spleen, with limited levels in thymus and bursa of Fabricius (264). B cells are rarely infected, but latent infection is probably established in MHC class II-positive T cells (88). Natural killer cell activity was stimulated through at least 8 week PI (517). Herpesvirus of turkey can be recovered from infected chickens for long periods, and antibodies persist for life (451, 617). The virus is apparently nononcogenic in turkeys (588), but the possibility of fertility problems in HVT-infected toms has been raised (560). The virus generally causes no clinical disease in intact or immunosuppressed chickens (518, 613). However transient B-lymphocyte dysfunction (203), atrophy of the bursa and thymus with high doses (238), and minor cellular infiltrations in nerves (194, 617) have been reported. In contrast, up to 19% of S-line chickens infected with HVT *in ovo* (ED 8) were reported to develop clinical paralysis and gross nerve enlargement due to inflammatory type lesions (101). Chickens exposed to HVT at ED 14 or earlier showed higher incidence of immunological tolerance resulting in a persistent HVT viremia (650). A possible role for HVT as a predisposing factor in autoimmune disease is suggested by its implied involvement in autoimmune vitiligo in certain strains of chickens (187).

Herpesvirus of turkeys seems to have an adjuvant effect when administered with other vaccines such as serotype 2 MDV strains (103, 609) and fowlpox virus (395). Furthermore, recently it has been shown that administration of HVT to chicken embryos at 18 ED hastens the development of the immune system (221).

Serotype 2 Marek's Disease Virus

Apathogenic strains isolated from clinically normal chickens (50, 125) subsequently were determined to be a separate serotype based on antigenic properties (577) and genome sequences. The 166 kb-long MDV-2 genome encodes several homologous and unique genes (282, 540) as well as novel microRNAs (638). Availability of infectious BAC clones of SB-1 virus (437, 529) could facilitate functional analysis of MDV-2 genes.

Serotype 2 viruses are widespread, although not universal in commercial chicken flocks (50). The epizootiology has been complicated by distribution of the virus through a seeder chick program (644) and widespread use of MDV-2 vaccines (103, 616). Other unique features of this virus group were further elucidated following the isolation of the SB-1 strain (490).

Serotype 2 viruses replicate in the FFE (124) and spread readily by contact (490, 618). Following inoculation of day-old chickens, the virus can be first isolated 5–6 days PI, reaching peak titers at 2–4 weeks, persisting thereafter for long periods (93, 618). A transient splenomegaly was induced between 4–12 days PI with SB-1, but no bursal atrophy and only occasional thymic atrophy was seen and cytolytic infection of lymphoid organs was not observed (93). In contrast, Lin et al. (345) found viral antigens in spleen and bursal tissues 5–14 days PI, primarily in B cells, but no gross or microscopic changes were observed.

Calnek (88) considered B cells and macrophages relatively refractory to infection, and the cells supporting latent infection lacked MHC class II antigens, thus differing from those in HVT infections. T cells also may not be very susceptible because of the poor re-isolation rates of SB-1 from CD4+ and CD8+ cells of infected chickens (337), although MDV-transformed MSB-1 cells can be dually infected with MDV-1 and MDV-2 viruses (638). SB-1 is not normally considered immunosuppressive (181) although a diminished response to a B lymphocyte-specific mitogen and decreased antibody responses to bovine serum albumen have been reported in chickens vaccinated with SB-1 strain in combination with HVT. However, as it has not been associated with neoplastic lesions in chickens or embryos, SB-1 is designated nononcogenic rather than apathogenic (490). The absence of lymphoma was confirmed by other workers (50, 123, 618), although there was one report of visceral lymphomas in 2 of 48 chickens inoculated with the HPRS-24 strain (440).

Vaccination with serotype 2 viruses causes a pronounced enhancement of B-cell lymphomas in certain genetic strains of chickens exposed at an early age to subgroup A ALV (24) or REV (12) (see Leukosis/Sarcoma Group). The ability of serotype 2 virus to enhance LL was attenuated without abrogation of its protective properties against MD challenge (594). Avian leukosis virus-A has now been eradicated from most of the chicken lines susceptible to serotype 2 enhancement and field problems due to serotype 2 enhancement of LL are rare (see Leukosis/Sarcoma Group). Recently, it has been shown that serotype 2 MDV can enhance the development of spontaneous ALV-like bursal lymphomas in ALVA6 transgenic chickens (resistant to infection with subgroups A and E of ALV) (112). Such enhancement is independent of age of vaccination (*in ovo* vs hatch) and might explain some of the spontaneous lymphomas observed in commercial flocks that are not related to ALV or REV (112).

Leukosis/Sarcoma Group

Venugopal Nair

Summary

Agent and Disease. Avian leukosis sarcoma group of pathogens are retroviruses associated with a number of neoplastic diseases in poultry. These viruses are grouped into different envelope subgroups and induce diseases such as lymphoid leukosis (LL) and myeloid leukosis (ML) that are widespread in many countries causing major economic losses and animal welfare issues.

Diagnosis. Major challenge in the clinical diagnosis of the disease is because of the difficulty in differentiating from other avian neoplastic diseases. Flock-level monitoring for the presence of viral antigens, combined with serological and molecular diagnostic tests are important disease diagnosis and eradication.

Intervention. Eradication of the pathogen by application of flock monitoring tests to eliminate infected birds at the pedigree breeding flock level is the best intervention strategy. Selection of birds for genetic resistance to infection will also be a useful adjunct in the control strategy.

Introduction

Definition and Synonyms

The leukosis/sarcoma (L/S) group of diseases designates a variety of transmissible benign and malignant neoplasms of chickens caused by members that belong to the family Retroviridae (201). Because of the expansion of the literature on this disease, it is no longer feasible to cite all relevant publications that provide the scientific basis for our current knowledge of the disease. Hence the literature is cited selectively, with reviews often used instead of original papers. For more detailed references to the literature, readers are advised to refer to the chapter in the previous edition (266).

Lymphoid leukosis (LL) has been the most common form of L/S group of diseases seen in field flocks, although myeloid leukosis (ML) has also become prevalent. The neoplasms and their synonyms are listed in Table 15.4. Members of this group of avian viruses are characterized, as are all members of the Retroviridae, by possession of an enzyme reverse transcriptase, which directs the synthesis of the proviral DNA form of the RNA virus that forms part of the retroviral life cycle and from which the family name is derived.

Sections reflecting the host response (Pathology and Pathogenesis of the Infectious Process) are discussed under the pathologic entities without regard for the properties of the inducing virus(es) other than their inclusion in the L/S group.

Economic Significance

Infection of chickens with avian leukosis virus (ALV) is the most common L/S virus infection encountered in field flocks and is known to be of significant economic

Table 15.4 Neoplasms caused by viruses of the leukosis/sarcoma group.

Neoplasm	Synonyms
Leukoses	
Lymphoid leukosis	Big liver disease, lymphatic leukosis, visceral lymphoma, lymphocytoma, lymphomatosis, visceral lymphomatosis, lymphoid leukosis
Erythroblastosis	Leukemia, intravascular lymphoid leukosis, erythroleukosis, erythromyelosis, erythroblastosis, erythroid leukosis
Myeloblastosis	Leukemic myeloid leukosis, leukomyelosis, myelomatosis, myeloblastosis, granuloblastosis, myeloid leukosis
Myelocytoma(tosis)	Myelocytoma, aleukemic myeloid leukosis, leukochloroma, myelomatosis
Connective tissue tumors	
Fibroma and fibrosarcoma	
Myxoma and myxosarcoma	
Histiocytic sarcoma	
Chondroma	
Osteoma and osteogenic sarcoma	
Epithelial tumors	
Nephroblastoma	Embryonal nephroma, renal adenocarcinoma, adenosarcoma, nephroblastoma, cystadenoma
Nephroma	Papillary cystadenoma, carcinoma of the kidney
Hepatocarcinoma	
Adenocarcinoma of the pancreas	
Thecoma	
Granulosa cell carcinoma	
Seminoma	Adenocarcinoma of the testis
Squamous cell carcinoma	
Endothelial tumors	
Hemangioma	Hemangiomatosis, endothelioma, hemangioblastomas, hemangioendotheliomas
Angiosarcoma	
Endothelioma	
Mesothelioma	
Related tumors	
Osteopetrosis	Marble bone, thick leg disease, sporadic diffuse osteoporostitis, osteopetrosis gallinarum
Meningioma	
Glioma	

importance. Economic losses from ALV-induced diseases are attributed to two sources. First, tumor mortality commonly amounts to around 1–2% of birds, with occasional losses of up to 20% or more. Second, subclinical infection by ALV, to which most flocks are subject, produces a depressive effect on a number of important performance traits, including egg production and quality (152). Economic losses due to ALV tumor mortality and reduced productivity are estimated to be in millions of US dollars each year (266). Currently the virus is causing huge economic losses to the poultry industry in China (229).

Public Health Significance

Recent studies have addressed the relationship between avian tumor viruses, particularly ALVs, and human health. Evidence for the presence of antibodies to ALVs in humans usually has been lacking or at best is presumptive (192). In a serological survey that included 549 human subjects, including groups exposed and not exposed to chickens, significant differences between men and women were found for the prevalence of antibodies to ALV but were not related to exposure to chickens (70).

Detection of reverse transcriptase activity as a sensitive assay for the presence of ALV in human vaccines derived from chicken cells was known to be of public health significance (332). While the test is of great value in detecting contaminating viruses in vaccines (35), no evidence of antibodies or proviral sequences of ALV was found in the vaccines or in the sera of vaccine recipients (187, 352).

History

The earliest reports of leukotic diseases in fowl are those of Roloff (334), who described a case of "lymphosarcomatosis" in 1868, and of Caparini (58), who in 1896 described cases of "fowl leukemia." Observations of osteopetrosis lesions in the bones of chicken recovered from ancient Roman burial sites were probably associated with avian leukosis (42).

Viral oncology was initiated as a discipline from the work with the demonstration of transmission of erythroleukemia and myelogenous leukemia by inoculating cell-free filtrates (116). Peyton Rous received the Nobel Prize in 1966 for his seminal work on transplantable tumor that could be transmitted by cell-free filtrates (336). Detailed reviews of the history of avian retrovirus research are available elsewhere (107, 266, 298, 306, 338, 389–391, 409, 415, 427).

Etiology

Classification

As per the latest classification of viruses (1), avian L/S group are placed in the *Alpharetrovirus* genus of the family Retroviridae. Members of this family are RNA viruses characterized by the possession of the enzyme reverse transcriptase, which is necessary for the formation of a DNA provirus that is integrated in the host genome during virus replication. Avian leukosis virus is the type species of the genus (Figures 15.18 and 15.19). Rous sarcoma virus (RSV) and a number of replication-defective, acutely transforming viruses such as MC29 and MH2 that carry oncogenes are also in the genera.

Morphology

Ultrastructure

In thin-section electron microscopy, avian leukosis/sarcoma viruses (ALSV) have an inner, centrally located, electron-dense core about 35–45 nm in diameter, an intermediate membrane, and an outer membrane. This appearance typifies the C-type retroviral morphology. The overall diameter of the virus particle is 80–120 nm, with an average of 90 nm. Immature virions budding from the cell membrane can be visualized (Figure 15.20). Characteristic knobbed spikes about 8 nm in diameter

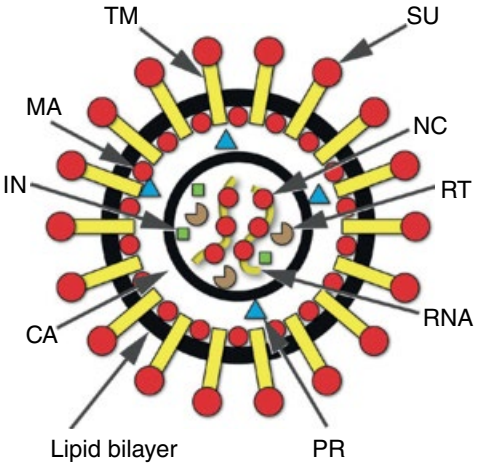


Figure 15.18 Schematic diagram of avian leukosis virus particle. The viral envelope is a lipid bilayer in which the gp37 transmembrane (TM) and the gp85 surface (SU) proteins, encoded by the env gene, are inserted. Internal components encoded by the gag/pro gene are p19 matrix (MA) protein, p27 capsid (CA) protein, p12 nucleocapsid (NC) protein, and p15 protease (PR). The pol gene encodes the reverse transcriptase (RT) and p32 integrase (IN). The core of the particle contains two viral RNA strands.

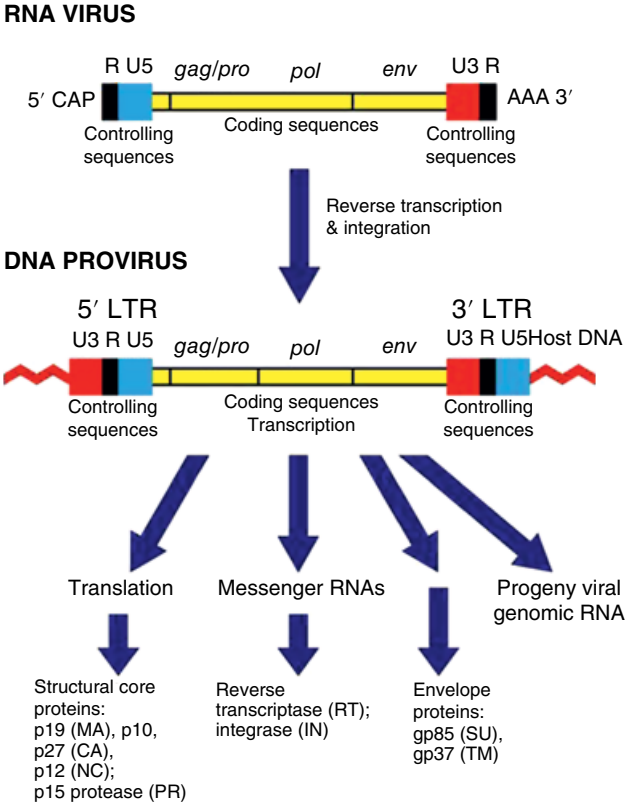


Figure 15.19 Key features of the viral RNA and proviral DNA forms of the genome of avian leukosis virus. CAP, 5′ end structure; AAA, polyadenylation of 3′ end; R, repeat sequence; U5, unique 5′ end sequence; U3, unique 3′ end sequence; LTR, long terminal repeat. For other abbreviations, see Figure 15.18 and the text.

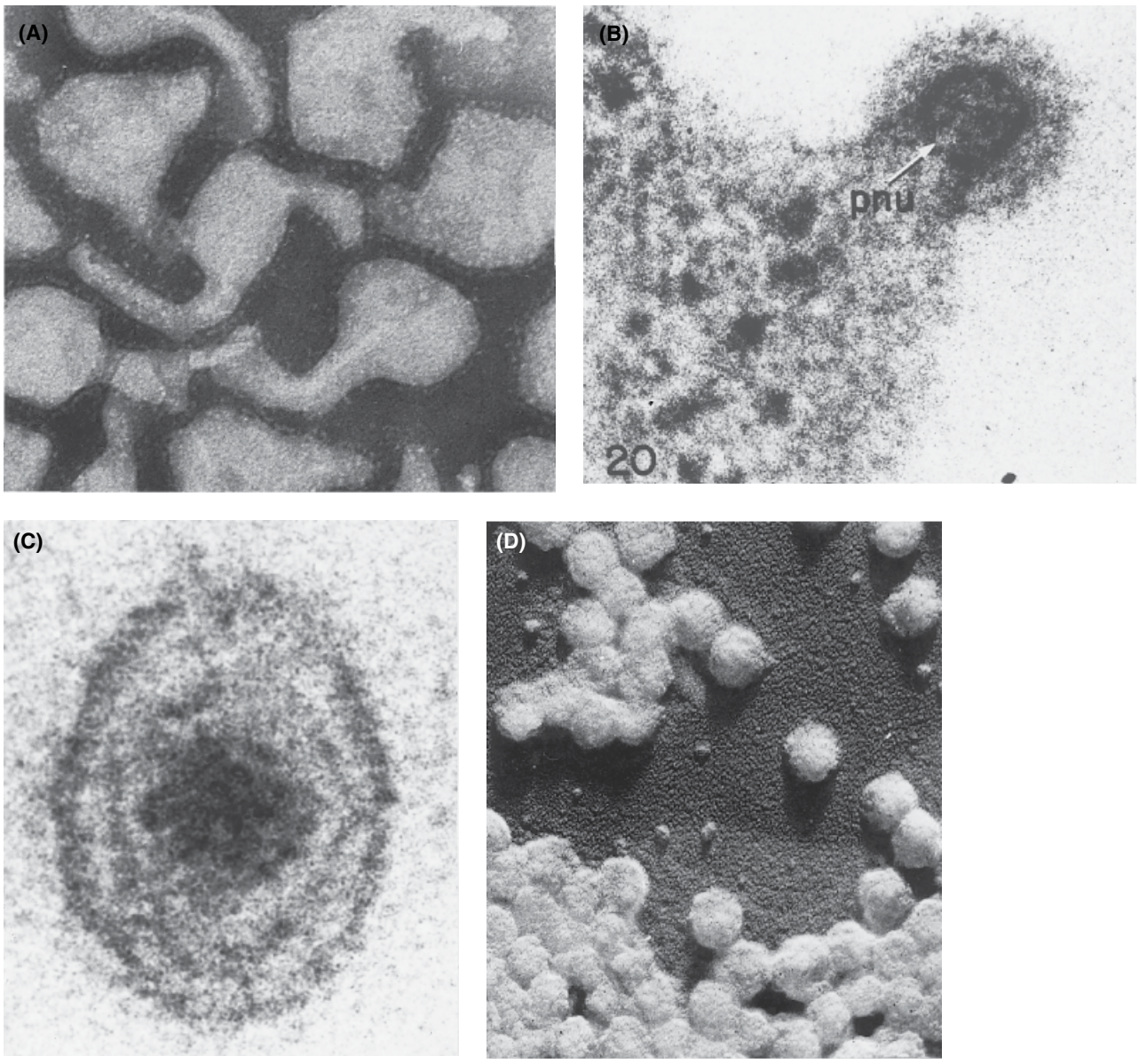


Figure 15.20 Ultrastructure of leukosis/sarcoma viruses. (A) BAI-A of avian myeloblastosis virus (AMV), unfixed, and negatively stained with neutralized phosphotungstic acid. Peripheral fringe about particles is resolved in some places into discrete "knobs." ×150,000. (B) Ultrastructure of leukosis/sarcoma virus release. Virus budding at cell membrane of a leukemic myeloblast. Surface of buds and particles peripheral to outer membrane is irregular and indistinct (pnu, dense prenucleoid). ×215,000. (C) Thin section of BAI-A of AMV sedimented from plasma, fixed in osmium tetroxide, and stained with lead subacetate. Inner and outer membranes and granular character of nucleoid can be seen. Impression of granules might be derived from sectioning of filaments. Some granules appear to be hollow. ×510,000. (D) Purified BAI-A of AMV fixed and shadowed with chromium. ×50,000. (Bonar and de Thé).

are present on the surface of the particles and comprise the viral envelope glycoproteins. These projections can also be seen in thin sections. Significant advances have recently been made in ultrastructural studies of retroviral capsids (311, 356).

Size and Density

By filtration through membranes of graded pore size, ultracentrifugation, and electron microscopy, viruses have a diameter of 80–145 nm. The value of 1.15–1.17 g/mL for the buoyant density in sucrose is characteristic for C-type retroviruses (266).

Chemical Composition

The overall composition of avian myeloblastosis virus (AMV), which has been studied extensively, is 30–35% lipid and 60–65% protein, of which 5–7% is glycoprotein, 2.2% is RNA, and small amounts of DNA are present, apparently of cellular origin (266). Quantitative

proteomics analysis of virus-infected cells using improved mass spectrometric methods has identified a number of host proteins associated with these viruses (225).

Viral Nucleic Acids

The major class sizes of RNA sediment at 60–70 S, which is the viral genome, and at 4–5 S, most of which is host tRNA, are thought to be accidentally included in the virion. A tRNA is also associated with the 70 S RNA and serves as a primer for the DNA polymerase during transcription of viral RNA to DNA. Small amounts of 18 and 28 S RNA, viral and cellular mRNA, and DNA are also present. The 60–70 S genomic RNA is a dimer and can be split into two subunits of about 34–38 S, which are believed to represent the diploid genome. These subunits of genomic RNA are mRNAs, and their genes have been mapped for a number of avian retroviruses.

The sequence of the structural genes of ALV, from the 5' end to the 3' end of the RNA molecule, is *gag/pro-pol-env*; these genes encode, respectively, the proteins of the virion group-specific (gs) antigens and protease, RNA-dependent DNA polymerase (reverse transcriptase, or RT), and envelope glycoproteins (Figure 15.19). The structural genes are flanked by terminal genomic sequences with gene promoter and enhancer activities, and that, in the DNA provirus, form the long terminal repeat (LTR) regions. The viral genome is about 7.3 kb in size.

Acutely transforming viruses possess additional transduced oncogene sequences that initiate neoplastic transformation. Acquisition of a viral oncogene usually is accompanied by genetic defects elsewhere in the viral genome (see Pathogenicity). Non-defective RSV has the genetic composition *gag/pro-pol-env-src*. The additional gene, *src*, responsible for sarcomatous transformation, evidently was acquired originally from a normal cellular oncogene, cellular *src*. The gene cellular *src* is an example of a number of host cell genes, termed proto-oncogenes or *onc* genes, concerned with acute transformation (211, 424). Viral and cellular versions of *onc* genes, and of the specific varieties such as *src*, are distinguished by the prefixes v- and c-. Specific v-*onc* genes, with c-*onc* counterparts in normal cells (Table 15.5).

Viral Proteins

The nature, location, and synthesis of proteins that constitute avian retroviruses have been extensively studied (394) (Figures 15.18 and 15.19). The virion core contains five non-glycosylated proteins encoded by the *gag/pro* gene: MA (matrix, p19); p10; CA (capsid, p27), which is the major gs antigen (Gag) in the core shell; NC (nucleocapsid, p12), involved in RNA processing and packaging; and PR (protease, p15), involved in cleavage of protein precursors. Other minor polypeptides have been reported.

The *pol* gene encodes the enzyme reverse transcriptase (RT) present in the core. It is a complex consisting of the b subunit (95 kDa) and the a subunit (68 kDa) derived from it and has RNA- and DNA-dependent polymerase and DNA–RNA hybrid-specific ribonuclease H activities. The b subunit also contains the IN domain (integrase, p32), the enzyme necessary for integration of viral DNA into the host genome. Recent structural studies on the catalytic core domain of the ALSV integrase suggested that it can dimerize in more than one state allowing the flexibility for multifunctionality during different steps of retroviral life cycle (19).

The virion envelope contains two glycoproteins encoded by the *env* gene: SU (surface, gp85), the viral surface knob-like structures that determine viral envelope subgroup specificity of the ALSV; and TM (transmembrane, gp37), representing the transmembrane structure that attaches the knobs to the envelope. These two envelope (Env) proteins are linked to form a dimer, termed virion glycoprotein (VGP).

Enzymes and other proteins are found in virions and are considered to be cellular components incorporated during virus maturation (393). Of practical value is the presence in AMV obtained from blood of infected chickens, or from myeloblast cultures, of adenosine triphosphatase derived from the cell membrane and incorporated into the virus particle during maturation.

Virus Replication

As with other retroviruses, replication of ALSV is characterized by the formation, under the direction of reverse transcriptase, of a DNA provirus that becomes linearly integrated into the host cell genome (Figure 15.19). Subsequently, the proviral genes are transcribed into viral RNAs, which are translated to produce precursor and mature proteins that constitute the virion.

Penetration of the Host Cell

Detailed reviews describing the recent understanding of the early ALV interactions with the host cells are available (266, 393). Although adsorption of the virion to the cell membrane is nonspecific, occurring even in cells resistant to infection, penetration of cells is dependent on the presence, in the cell membrane, of host gene-encoded receptors specific for particular virus envelope subgroups and on fusion of viral and cell membranes. The receptor for subgroup A ALV, designated TVA, is related to the human low-density lipoprotein receptor (167, 244). Decreased susceptibility subgroup A ASLV *in vitro* and *in vivo* from intronic deletions in close-bred line of domestic chickens resulting in inefficient splicing of the tva mRNA has been reported (63, 328). The receptors for ALV subgroups B, D, and E, designated TVB^{s3} and TVB^{s1}, resemble a receptor for cytokines of the tumor necrosis

Table 15.5 Acutely transforming avian sarcoma and leukemia viruses classified according to viral oncogene.

Virus strain	Oncogene(s) carried	Oncogene product	Predominant neoplasm(s)	Cells transformed *in vitro*
RSV, B77, S1, S2	*src*	Nr ptk	Sarcoma	Fibroblast
FuSV, UR1, PCR II, PCR IV	*fps*	Nr ptk	Sarcoma	Fibroblast
Y73, ESV	*yes*	Nr ptk	Sarcoma	Fibroblast
UR2	*ros*	R ptk	Sarcoma	Fibroblast
RPL30	*eyk*	R ptk	Sarcoma	Fibroblast
ASV-17	*jun*	Tf	Sarcoma	Fibroblast
ASV-31	*qin*	Tf	Sarcoma	Fibroblast
AS42	*maf*	Tf	Sarcoma	Fibroblast
ASV-1	*crk*	Ap	Sarcoma	Fibroblast
AEV-ES4,	*erb*A, *erb*B	Tf, R ptk	Erythroblastosis, sarcoma	Erythroblast, fibroblast
AEV-R	*erb*A, *erb*B	Tf, R ptk	Erythroblastosis	Erythroblast
AEV-H	*erb*B	R ptk	Erythroblastosis, sarcoma	Erythroblast, fibroblast
S13	*sea*	R ptk	Erythroblastosis, sarcoma	Erythroblast, fibroblast
E26	*myb, ets*	Tf	Myeloblastosis, erythroblastosis	Myeloblast, erythroblast
AMV	*myb*	Tf	Myeloblastosis	Myeloblast
MC29	*myc*	Tf	Myelocytoma, endothelioma	Immature macrophage, fibroblast
CMII	*myc*	Tf	Myelocytoma	Immature macrophage, fibroblast
966 ALV-J	*myc*	Tf	Myelocytoma	Immature macrophage
OK10	*myc*	Tf	Endothelioma	Immature macrophage, fibroblast
MH2	*myc, mil*	Tf, S/tk	Endothelioma	Immature macrophage, fibroblast

Note: Ap 5 Adaptor protein; Nr ptk 5 Nonreceptor protein tyrosine kinase; R ptk 5 Receptor protein tyrosine kinase; S/tk 5 Serine/threonine kinase; and Tf 5 Transcription factor.

factor family (2–4, 205, 329). Polymorphisms within the TVA and TVB receptors have been reported among chicken populations (228, 450). Editing of TVB receptor sequences in DF1 cells can induce resistance to ALV B infections (218). The receptor for the subgroup C avian sarcoma and leukosis viruses, Tvc, is related to mammalian butyrophilins, members of the immunoglobulin superfamily (115, 263). The host cell receptor used by the ALV subgroup J, which has a distinct envelope with limited homology to those of other subgroups, has been identified as the chicken Na(+)/H(+) exchanger type 1 (chNHE1) protein (59, 61, 138, 279), with the nonconserved tryptophan 38 residue critical in discriminating resistant and susceptible avian species (206, 327). While Japanese quail was shown to be resistant to ALV-J, some of the new world quail species with tryptophan 38 residue are susceptible and could potentially serve as reservoirs of infection (315). Recent study also showed that genetic resistance to ALV-J could be engineered in DF-1 cells by genome editing of the receptor sequence (217).

Synthesis and Integration of Viral DNA

Detailed reviews on the synthesis and integration of viral DNA have been provided elsewhere (71, 117, 166, 222, 383). Major stages in formation of retroviral DNA are: (1) synthesis of the first (minus) strand of viral DNA by reverse transcription of viral RNA by reverse transcriptase, forming an RNA : DNA hybrid; (2) removal of RNA from the hybrid by RNase-H and formation on the template of minus-strand DNA of second (plus) strands of viral DNA, giving rise to linear DNA duplexes; and (3) migration of linear DNA to the cell nucleus. Linear viral DNA becomes linearly integrated into the host DNA under the influence of the enzyme integrase. This integration can occur at many sites, and infected cells can contain up to 20 copies of viral DNA. Recent studies have demonstrated the importance of host factors such as the SSRP1 and Spt16 of the FACT protein complex as a principal cellular binding partner of ALV integrase (435). The proviral genes occur in the same order as their RNA copies occur in the virion, and they are flanked on

either side by identical sequences of nucleotides—the long terminal repeats (LTRs) (Figure 15.19).

Transcription

Formation of new virions in the infected cell is the result of transcription and translation of proviral DNA involving multiple steps (reviewed in 266, 417). Viral RNA molecules give rise to mRNA in association with polyribosomes, and they also serve as genomic RNA in newly formed virions. The mRNA species are translated to form the *gag*, *pol*, and *env* gene-coded proteins that compose the virion. The *env* gene product is a precursor protein gPr92 (92 kDa) from which the viral envelope proteins SU (gp85) and TM (gp37) are derived. Translation of *env* is from a spliced subgenomic RNA. The viral proteins localize at the plasma membrane of the cell, where crescent-shaped structures develop and virions that bud off from the cell may be visualized.

Defectiveness and Helper Viruses

A number of avian retroviruses (Table 15.6) have been shown to have defective genomes and arise either spontaneously or as a result of experimental mutagenesis (177, 238). They will transform cells but require the presence of a helper leukosis virus to enable them to replicate (e.g., BH-RSV and AMV lack the *env* gene, and AEV and MC29 lack the *pol* and *env* genes). Other acutely transforming viruses, such as certain strains of RSV, have lost their v-*onc* gene and ability to transform rapidly. They are called transformation defective (*td*) mutants and have an oncogenic potential similar to that of nondefective ALVs. Stocks of *rd* mutant RSV must by their existence contain helper viruses; these originally were referred to as Rous-associated viruses (RAVs). Infectious RSVs formed in these circumstances are called pseudotypes, and their designation includes the helper virus when this is identified (e.g., BH-RSV(RAV-1) when RAV-1 strain ALV helper virus is used). Use of defective strains of RSV enables tailor-made RSV to be produced with envelope properties of the helper ALV. Determinations of host range, interference pattern, and neutralization can be performed more easily with the appropriate RSV pseudotype than with the ALV, because the former can be readily quantified in cell culture.

Table 15.6 Phenotypic expression of representative endogenous avian leukosis viral (*ev*) genes in normal chicken cells.

Phenotype	Symbol	*ev* locus
No detectable viral product	gs^2chf^2	1, 4, 5
Expression of subgroup E envelope antigen	gs^2chf^1	9
Coordinate expression of group-specific and envelope antigens	gs^1chf^1	3
Spontaneous production of subgroup E virus	$V\text{-}E^1$	2

Source: Adapted from Smith (464).

Endogenous Leukosis Viruses

Avian leukosis viruses that are transmitted as infectious virus particles are termed exogenous viruses. The normal chicken genome also contains several classes or families of avian retrovirus-like elements that are transmitted genetically and are termed endogenous viruses. Extensive literature on earlier studies on these elements can be accessed in earlier editions of this book (266). Certain retroelements are believed to represent stages in the evolution of retroviruses from cellular movable genetic elements (transposons); whereas others are thought to be degenerate proviral forms of exogenous retroviruses that have lost the ability to produce infectious virus due to mutations.

The genetic sequences of the *ev* loci are related to subgroup E ALVs and are present as either complete or defective genomes in almost all normal chickens (Figure 15.21). The chromosomal locations of a number of *ev* loci have been determined using new sequence analysis pipelines on the chicken genome (32, 342–344). The phenotypic expressions of these loci vary, depending on the viral genes present and on poorly understood control mechanisms (Tables 15.6 and 15.7). More recent studies have demonstrated the role of endogenous retroviruses in generating genomic variations (219) and that the PIWI-interacting RNAs (piRNAs) can protect germ line from targeting endogenous retroviruses (385). The expression of endogenous *ev* genes is thought to be

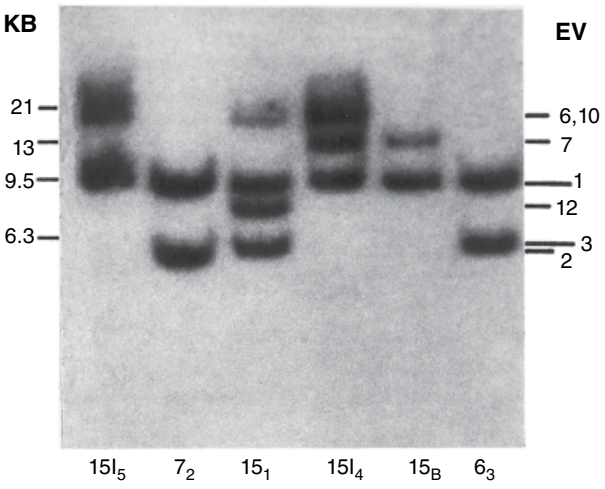


Figure 15.21 Endogenous viral (ev) loci detected in six inbred lines of White Leghorns by restriction fragment polymorphisms generated after Sac-1 endonuclease digestion of red blood cell DNA and hybridized to 32P-labeled RAV-2 genomic sequences (469).

responsible for a dominant form of genetic resistance of chicken cells to infection by subgroup E ALV (ALV E) from a block of virus receptors by envelope protein (239).

Endogenous viruses have either beneficial or detrimental effects, perhaps by their induction of immunity or tolerance to tumor virus antigens, depending on when they are expressed. Long-term selection studies showed that only a few of the integrations contributed to the high ALV E expression which seemed to correlate with lower body weights for the females indicating potential linkage to the loci regulating growth (197). Endogenous viruses of the *ev* family are not essential, because it has been possible to produce chickens free of *ev* genes (8). A line of such chickens has been produced, designated line 0 (81), that is of value in research studies and certain diagnostic tests in which birds or cells free from *ev* loci are needed.

Of particular importance is the *ev*21 locus, which is linked tightly in White Leghorn stock to the dominant sex-linked gene, *K*, on the Z chromosome (13), which regulates slow feathering (79). The *ev*21 gene is expressed as an infectious endogenous ALV, EV21, in the dam, which is transmitted congenitally to the progeny, inducing immunological tolerance and, consequently, increased susceptibility to infection by exogenous ALV (13, 175, 363, 365). The biologic functions, if any, of the other endogenous elements such as endogenous avian retrovirus (EAV), ART-CH, and CR1 are not fully understood. EAV family are not expressed as infectious virus, but RT activity can be expressed and has been found in live virus vaccines (404, 428). A member of the EAV family, EAV-HP (also termed ev/J), is believed to be the origin of the *env* gene of subgroup J ALV (17). Strongest evidence of the role of EAV-HP in the emergence of ALV-J by recombination was obtained from the identification of an intact chicken EAV-HP locus showing a uniquely close relationship to the ALV-J prototype clone HPRS-103 *env* region (345, 346).

Table 15.7 Phenotypes of endogenous avian leukosis (*ev*) genes in inbred and commercial lines of White Leghorn chickens.

ev	Phenotype	Line or Source[a]
1	gs^2chf^2	Most lines
2	$V\text{-}E^1$	RPRL72
3	gs^1chf^1	RPRL63
4	gs^2chf^2	SPAFAS
5	gs^2chf^2	SPAFAS
6	gs^2chf^1	RPRL151
7	$V\text{-}E^1$	RPRL15B
8	gs^2chf^2	K18
9	gs^2chf^1	K18
10	$V\text{-}E^1$	RPRL 15I4
11	$V\text{-}E^1$	RPRL 15I4
12	$V\text{-}E^1$	RPRL 151
14	$V\text{-}E^1$	H & N
15 (C)	None	K28 3K16
16 (D)	None	K28 3K16
17	gs^2chf^2	RC-P
18	$V\text{-}E^1$	RI
19	$V\text{-}E^1(?)^b$	RW
20	$V\text{-}E^1(?)^b$	RW
21	$V\text{-}E^1$	Hyline FP

*Note: Ev*13 is associated with the gs^2chf^2 phenotype, but restriction fragments have not been characterized.

[a] Not exclusive to line or source. K, Kimber; R, Reaseheath; H & N, Heisdorf and Nelson; for references see Smith (464).

[b] The presence of 5 *ev* loci in Reaseheath line w birds precludes definitive assignment with the $V\text{-}E^1$ phenotype. Definitive association requires further segregation of *ev* genes. Hyline FP birds also carry *ev*1, *ev*3, and *ev*6.

Susceptibility to Chemical and Physical Agents

Details of the susceptibility to chemical agents, conditions of thermal inactivation, pH stability and susceptibility to ultraviolet radiation can be found in the same chapter of the previous editions of this book (266).

Strain Classification

Antigenicity

Avian leukosis/sarcoma viruses that occur in chickens have been divided into six envelope subgroups, A, B, C, D, E, and J, on the basis of differences in their viral envelope glycoproteins, which determine antigenicity, viral interference patterns with members of the same and different subgroups, and host range in chicken embryo fibroblasts (CEF) of different phenotypes (266, 306). The other subgroups, F, G, H, and I, represent endogenous ALVs occurring in pheasants, partridge, and quail (266). Occurrence of new ALV subgroup K (ALV-K) has been reported in China based on sequence analysis (107, 224), although additional criteria based on the results of subgroup-specific neutralization, interference, and host-range analysis will be required for absolute confirmation of its status as a new subgroup.

Viral interference patterns (Table 15.8) and host range patterns (Tables 15.9 and 15.10) are the most reliable methods for subgroup classification. Antigenicity, as determined by the production of neutralizing antibodies or neutralization by known subgroup-specific antibodies, can also be used for strain classification, but is less dependable. Viruses within a subgroup usually cross-neutralize to varying extents, but with the exception of partial cross-neutralization between subgroup B and D viruses, viruses of different subgroups do not.

Table 15.8 Interference patterns between avian leukosis virus (ALV) and Rous sarcoma virus (RSV) of subgroups A–E and J.

Subgroup of interfering ALV	Subgroup of challenge RSV					
	A	B	C	D	E	J
A	1	2	2	2	2	2
B	2	1	2	1	1	2
C	2	2	1	2	2	2
D	2	2	2	1	2	2
E	2	2	2	2	1	2
J	2	2	2	2	2	1

Note: Susceptible avian embryo fibroblast cultures are infected with ALV of each subgroup and challenged several days later with RSV of each subgroup. Reduction in RSV foci in infected cultures compared with uninfected controls is indicative of viral interference. 1, interference; 2, no interference.

Table 15.9 Examples of host range of subgroup A–E and J avian leukosis/sarcoma viruses in chicken embryo cells of different phenotypes.

Phenotype of cells	Examples (chicken or cell lines)	Subgroup of virus					
		A	B	C	D	E	J
C/0	15B1	S	S	S	S	S	S
C/AE	C, alv6	R	S	S	S	R	S
C/A,B,D,E	7_2	R	R	S	R	R	S
C/E	0, 15l, BrL	S	S	S	S	R	S
C/EJ	DF-1/J[a]	S	S	S	S	R	R

Note: S, susceptible; R, resistant. The cell phenotype designation denotes chicken (C) cells resistant to (/) the specified subgroup (0, no subgroup; AE, subgroups A and E; etc.).
[a] Cell line, Hunt et al. (265).

Table 15.10 Host range of different subgroups of Rous sarcoma virus (RSV) in embryo fibroblasts of various avian species.

Avian species	Subgroup of RSV					
	A	B	C	D	E	J
Red jungle fowl	S	S	R	R	R	S
Common pheasant	S	R	R	R	S	R
Japanese quail	S	R	R	R	S	R
Guinea fowl	S	S	S	S	S	R
Turkey	S	S	S	S	S	S
Peking duck	R	R	S	R	R	R
Goose	R	R	S	R	R	R

Note: Embryo fibroblast cultures from avian species are challenged with RSV and susceptibility to RSV focus formation determined. S, susceptible; R, resistant. Data from Payne et al. (389).

Molecular Characteristics

Sequence analysis of the gp85 encoding sequences of the *env* genes of ALVs of subgroups A–E have identified two hypervariable regions, hr1 and hr2, and three less variable regions, vr1, vr2, and vr3, in which differences between the subgroups are present (37, 38, 109). Studies of recombinants indicated that hr1 and hr2, and to a lesser extent vr3, play the major role in determining receptor tropism (108). However, the exact locations and nature of the differences that determine host range and antigenicity have not yet been identified. The gp85 sequence of the *env* gene of subgroup J ALVs differ more extensively from those of the other five subgroups, notably at hr1, hr2, vr2, and vr3, and to a lesser extent also between these regions (17, 18). Different subgroup J isolates also vary at particular hypervariable regions of gp85 (227, 245).

Pathogenicity

Numerous strains of ALSVs exist, many of which were isolated from naturally occurring or experimentally-induced neoplasms over many years. Many induce a predominant type of neoplasm and can be named accordingly; for example, lymphoid leukosis virus (LLV), although ALV is more commonly used than LLV; avian erythroblastosis virus (AEV); avian myeloblastosis virus (AMV); and avian sarcoma virus (ASV) (Table 15.11). RPL12 strain of ALV induces lymphoid leukosis, erythroblastosis, osteopetrosis, hemangiomas, and sarcomas; the BAI A strain of AMV induces myeloblastosis, lymphoid leukosis, osteopetrosis, nephroblastomas, sarcomas, hemangiomas, thecomas, granulosa cell tumors, and epitheliomas (266).

Strains of ALSV can also be placed into two major classes in respect of rapidity of induction of tumors:

1) Acutely transforming viruses. These viruses can induce neoplastic transformation, *in vivo* or *in vitro*, within a few days or weeks. They cause various types of acute leukemia (leukosis) or solid tumors (usually sarcomas) (211, 266). The acutely transforming viruses are those that carry viral oncogenes in their genome (Table 15.5). Details of viral oncogenes and the biochemical functions of their products have been reviewed elsewhere (211, 242).
2) Slowly transforming viruses. These ALVs do not carry viral oncogenes. They induce tumors by a "promoter insertion" or a related mechanism that activates a cellular oncogene to bring about neoplastic transformation, with the development of tumors taking several weeks or months (211, 242).

Nomenclature

A variety of conventions, which reflect the classification methods outlined previously, are used in designating

ALSVs; many of these are illustrated in Table 15.11. They are given a full and an abbreviated designation based on the predominant neoplasm they induce, with an affix to indicate their origin with an individual (e.g., Rous sarcoma virus [RSV]) or a location (e.g., Regional Poultry Research Laboratory isolate 12 [RPL12 of ALV). Substrains of RSV are designated according to individuals who worked with them (e.g., Bryan's high-titer strain [BH-RSV]) or to location (e.g., Prague [PR-RSV]). Subgroups (e.g., subgroup A) may be designated also: PR-RSV-A. The general terms *avian leukosis* (or *leukemia*) *virus* (ALV) and *avian sarcoma virus* (ASV) are used widely to designate members of the group.

Helper viruses isolated from stocks of defective viruses are named, for example, as Rous-associated virus (RAV) or myeloblastosis-associated virus (MAV), and isolates

Table 15.11 Laboratory strains of avian leukosis/sarcoma viruses of the chicken classified according to predominant neoplasm induced and virus envelope subgroup.

	Virus class according to envelope subgroup						
Virus class according to neoplasm	**A**	**B**	**C**	**D**	**E**	**J**	**No subgroup (defective virus)[a]**
Lymphoid leukosis virus (LLV)	RAV-1	RAV-2	RAV-7	RAV-50	RAV-60		
	RIF-1	RAV-6	RAV-49	CZAV			
	MAV-1	MAV-2					
	RPL12						
	HPRS-F42						
Avian erythroblastosis virus (AEV)							AEV-ES4
							AEV-R
							AEV-H
Avian myeloblastosis virus (AMV)							AMV-BAI-A
Avian sarcoma virus (ASV)	SR-RSV-A	SR-RSV-B	B77	SR-RSV-D	SR-RSV-E		BH-RSV
	PR-RSV-A	PR-RSV-B	PR-RSV-C	CZ-RSV	PR-RSV-E		BS-RSV
	EH-RSV	HA-RSV					FuSV
	RSV29						PRCII
							PRCIV
							ESV
							Y73
							UR1
							UR2
							S1
							S2
Myelocytoma and endothelioma						HPRS-103	MC29
viruses						ADOL-Hc1	966
							MH2
							CMII
							OK10
							RAV-0
Endogenous virus (EV) (no neoplasm)					EV21 ILV-E		

[a] Defective viruses have the envelope subgroup of their helper virus.

are numbered (RAV-1, MAV-1, etc.). Where a helper virus is used for replication of a defective virus, this is indicated. Thus, BH-RSV grown with RAV-1 as a helper is designated BH-RSV(RAV-1). Endogenous ALV is abbreviated EV (e.g., EV21). Strains of ALV that act as resistance-inducing factors (see Diagnosis) were designated RIFs, but this term is now rarely used. Further details of the origins of the abbreviations (297) are given in Table 15.11.

Laboratory Host Systems

Chick Inoculation

Rous sarcoma and other sarcoma viruses produce tumors when injected by the subcutaneous (SC), intramuscular (IM), or intra-abdominal (IA) routes and at times by contact with inoculated chickens. Subcutaneous injection into the wing web or IM injection can be used for sarcoma virus isolation and propagation (Reviewed in 266). In susceptible chickens, these may grow rapidly, ulcerate, and metastasize; in resistant chickens, the sarcomas may regress. Virus isolates that caused LL also caused erythroblastosis. Osteopetrosis, hemangiomas, and fibrosarcomas were also observed in chickens of certain strains and passages (see 266).

Avian myeloblastosis virus can be titrated in susceptible chicks by IV inoculation at 1–3 days of age. Avian myeloblastosis virus in chicken plasma can be assayed by its adenosine triphosphatase activity, a method useful for routine and large-scale studies. Osteopetrosis-inducing activity of virus strains can be examined by IV or IM inoculation of day-old chicks. Guinea fowl are particularly susceptible to osteopetrosis induced by MAV-2(O) virus (see 266).

Embryo Inoculation

When RSV and other sarcoma viruses are inoculated onto the chorioallantoic membrane (CAM) of 11-day-old susceptible embryos, tumor pocks develop (Figure 15.22), which can be counted eight days later and are related linearly to virus dose (112). This technique is also useful for detecting genetic resistance to infection.

Avian leukosis viruses have been quantitated by IV inoculation into 11-day-old susceptible chicken embryos. Depending on the virus, within two weeks of hatching, a high incidence of neoplasms (mainly erythroblastosis) can occur, although hemorrhages and solid tumors can develop including fibrosarcomas, endotheliomas, nephroblastomas, and chondromas. When chicks are held for a postinoculation period of 46 days, responses are higher by 1–2 $\log_{10}$ dilutions than those following chicken inoculation. Most chickens that survive the acute neoplasms develop LL after 100 days PI (312).

Avian myeloblastosis virus produced a myeloblastosis response within a few weeks when injected by IV into susceptible embryos (20–22). When the HPRS-103 strain of subgroup J ALV was inoculated by IV into 11-day-old embryos, first death from tumor (myelocytoma) was not until 9 weeks of age, and median tumor mortality was at 20 weeks (301).

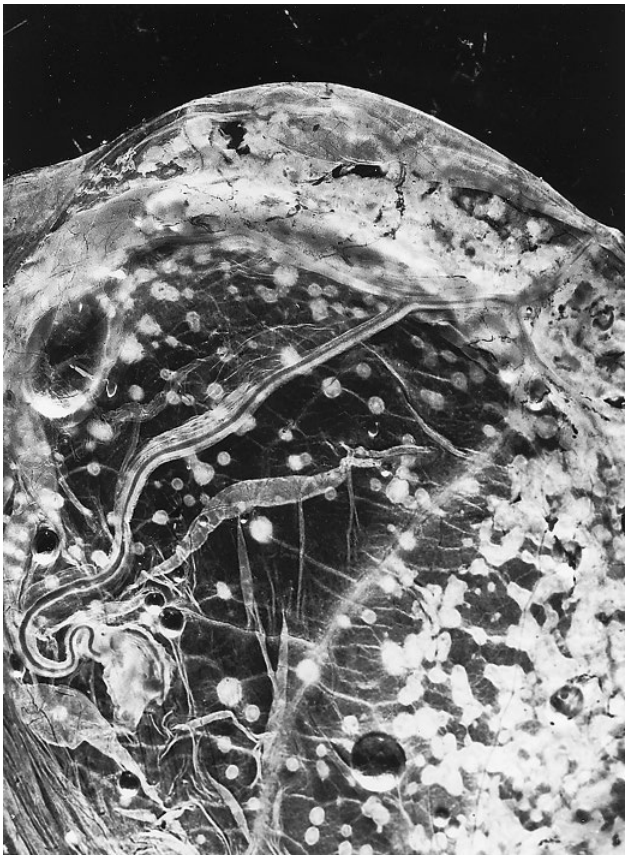


Figure 15.22 Pocks induced by BH-RSV on the chorioallantoic membrane (CAM) of a chicken embryo. (Piraino).

Cell Culture

Rous sarcoma virus and other sarcoma viruses induce rapid neoplastic transformation of cells when inoculated onto monolayer cultures of chicken embryo fibroblasts (323). The transformed cells proliferate to produce within a few days discrete colonies or foci of transformed cells (Figure 15.23), which under agar can be used for quantitative assay of virus (400).

Most leukosis viruses replicate in fibroblast cultures without producing any obvious cytopathic effect. Their presence can be detected by a variety of tests (see Diagnosis). Avian leukosis viruses of subgroups B and D may induce cytopathic plaques that may be used for virus assay (163). The cytopathic effect of these two subgroups is explained by their use of the death receptor of the tumor necrosis factor receptor family (41, 69, 100, 101).

Acutely transforming ALVs will transform hematopoietic cells *in vitro* (257). Yolk sac and bone marrow cells in

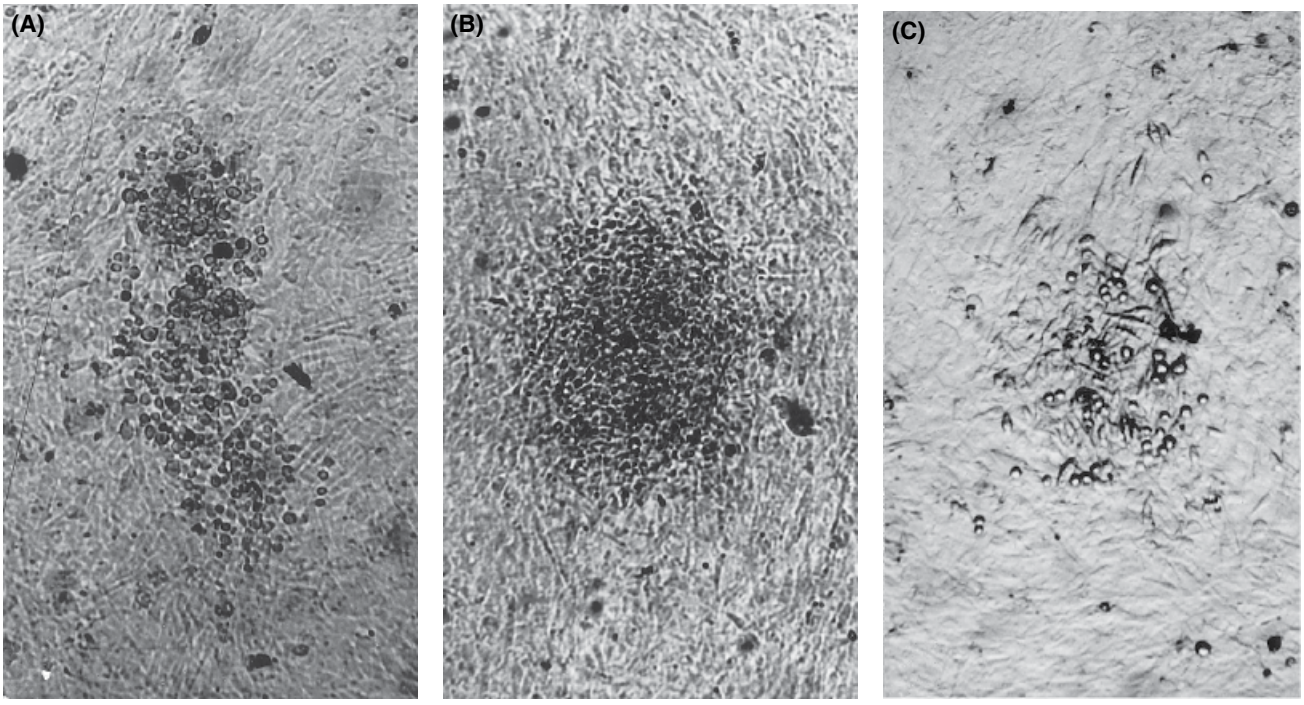


Figure 15.23 Foci induced by Rous sarcoma virus (RSV) in cell culture. (A) Unstained focus of transformed spherical, refractile chicken embryo cells infected six days previously with Bryan's standard strain of RSV. ×100. (B) Unstained focus of transformed, polygonal, opaque Rous sarcoma cells infected six days previously with Bryan's high-titer strain. ×100. (C) Unstained focus of transformed round and fusiform cells infected six days previously with Popken's preparation of RSV. ×100.

culture are transformed to neoplastic myeloblasts on infection with AMV (258), and bone marrow cells transform to erythroblasts with AEV (164). Transformation of hematopoietic cells by MH2, MV29, and OK10 viruses was observed by Graf and Beug (165). Acutely transforming variants of subgroup J ALV can also transform bone marrow cells and blood monocytes *in vitro* (66, 302). *In vitro* transformation of B-lymphocytes by nondefective ALV has not been reported, and bone marrow cultures were not transformed by nondefective subgroup J ALV (302).

The properties of ALSVs in cell culture are described in more detail under Diagnosis.

Pathogenicity

As discussed previously (see Strain Classification), strains of ALVs may produce more than one type of neoplasm, and the oncogenic spectrum of each strain tends to be characteristic but often overlaps with responses to other strains. Viral factors including the origin and dose, and host factors such as route of inoculation, age, genotype, and sex influence the oncogenic patterns of different virus strains.

Origin of Virus

Differences in tumor spectrum may be seen in virus strains newly isolated from the field, as exemplified by tumor spectrums of RPL26, RPL27, and RPL28 isolates of ALV (143). Please refer to the chapter in the previous edition for earlier studies in this area (266).

Virus Subgroup

Usually no relationship has been observed between virus subgroup and oncogenicity except for endogenous E subgroup ALVs, such as RAV-0, which have little or no oncogenicity (261). However, the low oncogenicity of RAV-0 is believed to be related to the weak promoter activity of the subgroup E LTR and not to the *env* gene. Subgroup J ALV-induced myelocytomatosis (301), and the *env* as well as other elements are thought to be associated with the unique oncogenicity (66–68, 234, 235).

Virus Dose

High doses of RPL12 ALV mainly induced erythroblastosis; whereas doses close to the endpoint predominantly induced LL (50). Sarcomas, endotheliomas, and hemorrhages were also more common with high virus doses. Occurrence of osteopetrosis showed no dependency on dose (144).

Route of Inoculation

Responses obtained after virus administration by less efficient portals of entry into the host apparently reflect the decreased effective dose. Thus, exposure of susceptible birds by contact with birds inoculated with a high

dose of strain RPL12 of ALV resulted in a LL response similar to that expected with 1/1000 of the inoculated dose (see 266). Intramuscular inoculation of strain RPL26 of ALV favored sarcoma induction; whereas IV inoculation mainly produced erythroblastosis and hemorrhages (see 266). These differences may reflect variations in amounts of virus that reach the target cells by different routes.

Age of Host

In general, resistance of birds to the development of neoplasms of all types increases with age, the rate varying with route of inoculation. Resistance increases rapidly between 1 and 21 days of age with oral or nasal administration but relatively slowly when virus is inoculated intravenously (48).

Genotype and Sex of Host

The genetic constitution of the host has a strong influence on response to ALSVs (see Pathobiology and Epizootiology). Females are more susceptible to LL than males. Castration increases the incidence of disease, and treatment with testosterone increases resistance of males and capons (51). These effects are probably a consequence of hormonal effects influencing regression and, hence, target cell numbers in the bursa of Fabricius.

Pathobiology and Epidemiology

Incidence and Distribution

Notwithstanding the eradication programs instituted by many primary breeding companies, ALV infections still occur in many flocks. The incidence of subgroup A ALV-induced LL, the most common neoplasm observed in infected flocks, is usually low, in the order of 1 or 2%, although losses of up to 20% can occur. Diseases associated with subgroup J continue to be a problem in many parts of China (306).

Incidence of Disease

Earlier reports on the incidence of ALV-associated diseases can be seen in this chapter in the previous edition of this book (266). Although sporadic cases of ALV-induced neoplasia occur in most flocks, it is only occasionally that even the most common neoplasm, LL, produces heavy losses. Lymphoid leukosis mortality in the Netherlands during 1973–1979 was 2.18% of 11,220 white layers and 0.57% of 7,920 brown layers (96, 97), although the incidence has been reduced significantly since these studies. Serotype 2 MDV was found to enhance the development of LL in certain lines of chickens following exposure to ALV after hatch (16, 124, 128, 133). Molecular and *in situ* hybridization analysis of the bursa from chickens coinfected with ALV and serotype 2 MDV proved that MDV was closely associated with transformed, but not with nontransformed, bursa cells (148, 240).

Until recently and before the recognition of subgroup J ALV (299, 308), myelocytomatosis was mainly a sporadic disease seen among young and adult birds (321). An overall incidence of 27% myelocytomatosis was reported in meat-type chickens inoculated with strain HPRS-103 of ALV-J (301). High incidence of ALV-J tumors have been reported in many countries with mortalities up to 1.5% in excess of normal levels per week in some commercial broiler breeder flocks (306). Avian leukosis virus-J-induced disease in China is characterized by the occurrence of the disease in meat-type as well as layer population including the native breeds (229, 246) and is characterized by a high incidence of hemangiomas (215, 451).

Connective tissue tumors, which are often not the primary cause of death, make up about 20% of nonlymphoid tumors in broilers (57). The incidence of connective tissue tumors in chickens is probably less than 1 in 1,000 (321), but epizootics have occurred. An outbreak of histiocytic sarcomas was reported in a flock of 600 1-year-old hens, during which tumors were found in 90% of 400 birds examined during a 4-month period (310). Low incidence of histiocytic sarcomatosis associated with ALV-J has also been reported (5).

Osteopetrosis occurs much less frequently than LL, and epizootics occur sporadically in broilers. In all types of chicken, males are more frequently affected than females. Examination of a 1986 outbreak of osteopetrosis revealed ALV sequences (23).

Incidence of Virus Infection

Subgroup A ALV is the most common subgroup of L/S viruses isolated from field outbreaks of LL; it is encountered more frequently than subgroup B. In general, fewer studies of the prevalence of ALV in meat lines have been made compared with those in egg lines. Antibodies to the novel subgroup J ALV were found in three of five meat-type chicken lines, but not in seven layer lines examined in the United Kingdom (305). Using virological and serological assays, the incidence of ALV-J infection in affected broiler breeder flocks was reported to be as high as 87% (132). Similarly high levels of ALV-J infection have been reported in other countries also (139, 226, 229, 246). The incidence of infection with subgroup ALV-J was also influenced by other factors such as age at exposure (433, 434).

Subgroups A, B, C, and D of ALV have been isolated from commercial flocks in Finland; 5 of 10 flocks surveyed had antibody to all 4 subgroups (348).

Antibodies to subgroups A and B are common among wildfowl and domestic chickens in Kenya and Malaysia, and some evidence exists of antibody to subgroup

D viruses in Kenya. Subgroup F viruses have been found in ring-necked and green pheasants, and subgroup G viruses in Ghinghi, silver, and golden pheasants. Subgroup H virus has been isolated from Hungarian partridges and subgroup I virus from Gambel's quail (see Virus Subgroups). Viruses that do not fit within known subgroups have been isolated from Mongolian and Swinhoe pheasants, Chinese quail, and chickens. However, none were found in Japanese quail, pigeons, geese, and Pekin and Muscovy ducks (64, 315).

In most vertebrate species, endogenous retroviral genomes are inherited in a Mendelian fashion and occur at distinct chromosomal loci. DNA sequences related to RAV-0, the endogenous avian retrovirus, occur in the germ lines of most domestic chickens and several species of galliform birds. For example, partridges, true pheasants, grouse, and jungle fowl contain sequences complementary to RAV-0; whereas guinea fowl, quail, peafowl, ruffed pheasants, gallo-pheasants, and turkeys do not (145). The structure, function, and regulation of endogenous retroviruses in the genome of the chicken have been reviewed (79, 219, 344, 385).

Natural and Experimental Hosts

Chickens are the natural hosts for all viruses of L/S group (296); these viruses have not been isolated from other avian species except pheasants, partridges, and quail (see Virus Subgroups). Experimentally, however, some members of the L/S group of avian retroviruses have a wide host range and can be adapted to grow in unusual hosts by passage in very young animals or through the induction of immunologic tolerance prior to inoculation of the virus. Rous sarcoma virus has the widest host range; it will cause tumors in chickens, pheasants, guinea fowl, ducks, pigeons, Japanese quail, turkeys, and rock partridges. Ducks were shown to be an ideal experimental system for studying persistence of ALV as the virus appeared to persist even up to three years with no viremia or neutralizing antibodies after embryonic infection (272). However ducks infected as embryos with subgroup C showed wasting disease soon after hatching (382, 392, 403). There has been one report of lymphoid leukosis in ostriches (150). Some strains of RSV induce tumors in mammals (414), including monkeys (207–210). Osteopetrosis can be produced in turkeys by inoculation of fresh whole blood from affected chickens (181). Also, turkeys were susceptible to ALV-J infection and tumors induced by the acute form of strain HPRS-103 of ALV-J (412).

Transmission

Exogenous ALVs are transmitted in two ways: vertically from hen to progeny through the egg and horizontally from bird to bird by direct or indirect contact (Figure 15.24) (reviewed by 266, 306). Although usually only a small percentage of chicks are infected vertically, this route of transmission is important epizootiologically because it affords a means of maintaining the infection from one generation to the next. Most chickens become infected by close contact with congenitally infected birds. Although vertical transmission is important in the maintenance of the infection, horizontal infection may also be necessary to maintain a rate of vertical transmission sufficient to prevent the infection from dying out (300). The infection does not spread readily from infected birds to birds in indirect contact (in separate pens or cages), probably because of the relatively short life of the virus outside the birds (see Thermal Inactivation). However, contact exposure at hatch was shown to be an effective method of spread of ALV-J among broiler breeder chickens (132, 436, 437) and was prevented by small group rearing (440).

Four classes of ALV infection are recognized in mature chickens: (1) no viremia, no antibody (V–A–-); (2) no viremia, with antibody (V–A+); (3) with viremia, with antibody (V+A+); and (4) with viremia, no antibody (V+A–) (reviewed by 266, 306). Birds in an infection-free flock and genetically resistant birds in a susceptible flock fall into the category V–A–-. Genetically susceptible birds in an infected flock fall into one of the other three categories. Most are V–A+, and a minority, usually less than 10%, are V+A–. Most V+A– hens transmit ALV to a varying but relatively high proportion of their progeny (266, 306). A small proportion of V–A+ hens transmit the virus congenitally and do so more intermittently; the tendency for congenital transmission of ALV in this category was found to be more frequent in hens with low antibody titer (406). Congenitally infected embryos develop immunologic tolerance to the virus and after hatching make up the V+A– class, with high levels of virus in the blood and tissues and an absence of antibodies. By 22 weeks of age, up to 25% of meat-type chickens exposed to ALV-J at hatch were found to be V+A–, although this could be affected by a number of factors including the virus strain (288, 291).

The role of males in the transmission of ALV is at best equivocal. Infection of the cock apparently does not influence the rate of congenital infection of progeny (339, 376). The genetics of the host and the strain of ALV influence shedding and congenital transmission after horizontal infection (90). With electron microscopy, virus budding has been seen on all structures of reproductive organs of cocks except germinal cells (99), indicating that the virus does not multiply in germ cells. The cock, therefore, acts only as a virus carrier and source of contact of venereal infection to other birds or through semen (226, 366). Congenital infection of embryos is strongly associated with shedding by the hen of ALV into egg albumen and with presence of virus in the vagina of hens (304, 375). These traits are also highly correlated with viremia.

Shedding of ALV into egg albumen and transmission to the embryo is a consequence of virus production by albumen-secreting glands of the oviduct. In most hens congenitally transmitting ALV, the highest titers of virus were found in the ampulla of the oviducts, suggesting that embryo infection is closely related with ALV produced at the oviduct but not with ALV transferred from other parts of the body (405). Electron microscopy studies have revealed a high degree of virus replication in the magnum of the oviduct (102). Virus budding also occurs in various cell types in the ovary but not in the follicular cells or ovum, and transovarial infection does not seem to be important (304). Not all eggs that have ALV in the albumen give rise to infected embryos or chicks; in the studies of Spencer et al. (375), Payne et al. (304), and Tsukamoto et al. (406), only about one-half to one-eighth of embryos were infected from eggs with virus in the albumen. This intermittent congenital transmission may be a consequence of neutralization of virus by antibody in the yolk and of loss because of thermal inactivation.

In flocks infected with subgroup A ALV, only a minority of ALV-infected birds develop LL; the others remain as carriers and shedders. Viremic-tolerant (V+A–) birds are reported to be several times more likely to die of LL than those with antibody (V–A+) (339). Incidence of leukosis decreases rapidly if infection by natural routes occurs after the first few weeks of age (48); there are well-established genetic differences in susceptibility to LL development in chickens that are equally susceptible to virus infection (88).

Endogenous ALVs (see Etiology) usually are transmitted genetically in germ cells of both sexes (Figure 15.24). Many are genetically defective and incapable of giving rise to infectious virions, but some are not and may be expressed in an infectious form in either embryos or hatched birds. In this form, they then are transmitted similarly to exogenous viruses, although most chickens are genetically resistant to such exogenous infection. Endogenous viruses have little or no oncogenicity but may influence response of the bird to infection by exogenous ALV. Immunodepression induced by

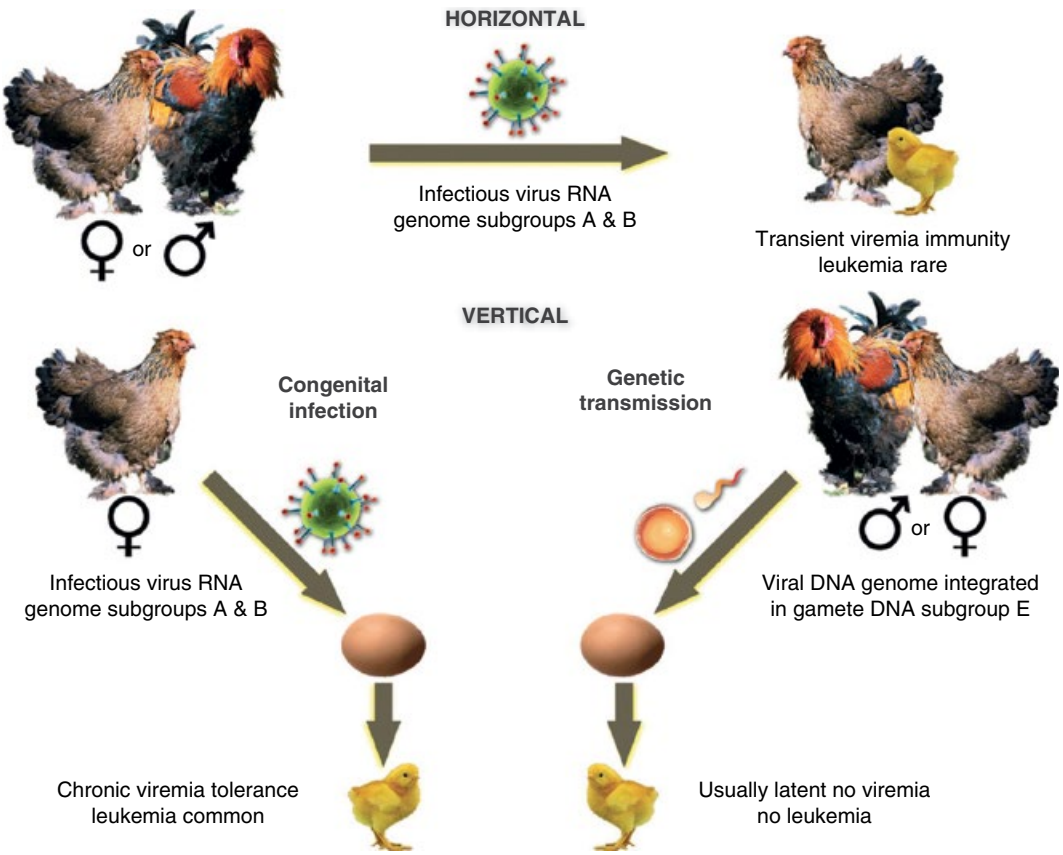


Figure 15.24 Horizontal and vertical transmission of exogenous avian leukosis virus (ALV) (e.g., subgroups A and B) and genetic transmission of endogenous (subgroup E) virus with potential outcomes (115).

infectious bursal disease virus increased the rate of shedding of ALV (135); also strain of virus can influence the incidence of tolerantly infected chickens (127).

Incubation Period

Members of the L/S group of viruses are multipotent viruses capable of inducing a variety of neoplastic diseases. The incubation period for these diseases is dependent on strain and dose of virus, route of infection, age at exposure, and genetic constitution of the host. Susceptible chicks inoculated as embryos or at 1–14-days-of-age with a standard strain of ALV-RPL12 (50), B15, F42 (34), or RAV-1, developed LL between weeks 14 and 30 weeks of age. It is very seldom that LL cases occur in chickens under 14 weeks. Certain laboratory recombinant viruses have been shown to cause LL within 5–7 weeks (198), although such short incubation periods are not found in field outbreaks.

Another determining factor is whether the virus strain lacks or possesses a viral oncogene. For example, diseases such as erythroblastosis, induced by slowly transforming viruses lacking a viral oncogene, usually develop after a long latent period (67, 146, 211, 422), as in such cases transformation is induced by promoter insertion activation of the cellular oncogene c-*erbB*. After IA inoculation of the slowly transforming RPL12 strain virus into susceptible day-old chicks, the incubation period varies from 21–110 days (50). On IV inoculation of 11-day-old embryos, chicks occasionally have been found to have erythroblastosis on hatching. Field strains and viruses passaged in cell culture induce erythroblastosis after a longer incubation period (49). Passage from donors with erythroblastosis greatly shortens the incubation period (143).

Other strains of virus including F42 (34), ES4, and strain 13 (28) also produce erythroblastosis. Field cases usually occur in birds older than three months of age. Viruses such as RPL12 and F42 are non-defective and slowly transforming; whereas ES4 and R are defective and acutely transforming (165).

Strain BAI-A of ALV predominantly induces myeloblastosis. Virus stocks are defective and contain helper viruses of both A and B subgroups (191). After inoculation of susceptible day-old chicks with large doses of virus, changes in the blood can be observed in 10 days, and birds die a few days thereafter. Mortality continues for about one month, and only a few deaths occur after this (53, 114). The virus E26 (165) also predominantly induces myeloblastosis.

Virus-induced myelocytomatosis generally has a longer incubation period than erythroblastosis and myeloblastosis induced by the acutely transforming virus strains, but shorter than LL. On IV injection of MC29 into young chicks, myelocytomas were obtained in 3–11 weeks (253). The incubation period in field cases is unknown, but most cases are observed in immature birds. The virus CMII also induces myelocytomas (165). Myelocytomatosis induced by the HPRS-103 strain of ALV, which lacks a viral oncogene, had a long latent period (median time to death was 20 weeks) (301). However, median time to death with the acutely transforming 879-strain variant of HPRS-103, believed to carry a viral oncogene, was nine weeks (302). Field cases of subgroup J-induced myelocytomatosis were reported in broiler breeder chickens as young as four weeks of age (132).

Most strains of ALV have been found to cause hemangiomas (45, 144). These tumors can be found in birds of various ages. In naturally occurring outbreaks, most mortality from hemangiosarcomas occurred at 6–9 months (54, 55). Induction of lung angiosarcomas by subgroup F ALVs is reported (362). After experimental inoculation of young chicks with field strains of virus (143), hemangiomas appeared in three weeks to four months.

In field cases, ALV-induced renal tumors are rarely seen in chickens younger than five weeks; most cases are seen in birds between two and six months of age. Nephroblastomas induced by strain BAI-A may reach an incidence of 60–85% in birds not dying of myeloblastosis (53). Renal carcinomatous lesions induced by strain MC29 are found as soon as 18 days or as late as 7 weeks after virus inoculation.

Osteopetrosis may develop any time after one month following experimental inoculation of day-old chicks with strain RPL12-L29 of ALV (349) or other viruses (179, 181, 320); it is most commonly seen in birds 8–12 weeks of age. The disease probably has a similar incubation period in the field. MAV-2(O) virus will induce palpable osteopetrosis 7–10 days after hatching in chicks inoculated at 1 day of age or as 11–12-day-old embryos (142).

Sarcomas may occur any time after inoculation with ALVs but are most frequently observed in the first 2–3 months (52). In field flocks, connective tissue tumors may occur in birds at any age (448). Sarcomas also develop readily and are palpable within three days after inoculation of chicks with high doses of acutely transforming RSV.

Clinical Signs

Outward signs of the leukotic diseases are mostly nonspecific. They include inappetence, weakness, diarrhea, dehydration, and emaciation. In LL especially, there may be abdominal enlargement. The comb may be pale, shriveled, or occasionally cyanotic. In erythroblastosis and myeloblastosis, hemorrhage from feather follicles

also may occur. After clinical signs develop, the course is usually rapid, and birds die within a few weeks. Other affected birds may die without showing obvious signs.

In myelocytomatosis, skeletal myelocytomas may cause protuberances on the head, thorax, and shanks. Myelocytomas may occur in the orbit of the eye, causing hemorrhage and blindness. Hemangiomas may occur in the skin, appearing as "blood blisters," and these may rupture causing hemorrhage (136, 196). Renal tumors may cause paralysis due to pressure on the sciatic nerve. Sarcomas and other connective tissue tumors may be seen in the skin and musculature. When advanced, these various other tumors may be accompanied by the nonspecific signs given previously. Benign tumors may follow a long course, malignant tumors a rapid one.

In osteopetrosis, the long bones of the limbs are commonly affected. Uniform or irregular thickening of the diaphyseal or metaphyseal regions can be detected by inspection or palpation. The affected areas are often unusually warm. Birds with advanced lesions have characteristic "bootlike" shanks. Affected birds usually are stunted and pale and walk with a stilted gait or limp.

Avian leukosis virus also has been shown to be associated with the "so called fowl glioma" (281), associated with cerebellar hypoplasia and myocarditis (269, 270).

Pathology

Introduction

One or more specific neoplasms induced by ALSVs may occur in a given flock of chickens, and more than one type of neoplasm may occur in an individual bird. This is particularly true in flocks infected with subgroup J ALV. The presence of a tumor similar to that produced experimentally is only provisional evidence that a bird was infected with a virus of this group. Firmer evidence is provided by ALSV detection or isolation, and if appropriate by experimental reproduction of tumors by a virus isolate.

In this section, the pathology of the different neoplasms is discussed without regard for virological properties of the inducing agent(s). Only entities that have been reproduced with ALSVs are described.

Nonneoplastic Conditions

The clinical consequences of infection of chickens with exogenous ALV vary. Some chickens, principally those with a tolerant viremic infection (arising from congenital or early neonatal infection, see Immunity), may show a variety of clinical signs, as detailed later in this chapter, including depression of body weight and of other production traits. Birds with tolerant viremic infections are also those most likely to develop neoplasia. Ultrastructural and virological studies of congenitally or neonatally infected birds have shown the virus to be widespread in most tissues and organs of the body (6, 7, 104, 111, 223, 333, 381, 402). DiStefano and Dougherty (103, 111) observed virus budding in cells of every type of tissue examined, except germ cells and neurons.

Avian leukosis virus infection in the absence of overt disease can adversely affect the productivity of egg-laying chickens. Compared with nonshedders, hens that shed virus produced 20–35 fewer eggs per hen housed to 497 days of age; matured later sexually (i.e., age at first egg); and produced smaller eggs (374), at a lower rate, and with thinner shells. Mortality from causes other than neoplasms was 5–15% higher, fertility was 2.4% lower, and hatchability was 12.4% lower in shedders than nonshedders (154). In this study, shedding refers to the transfer of ALV to egg albumen and nonshedders, as well as shedders, would likely be ALV-infected; shedders are mostly viremic birds; whereas nonshedders are immune with ALV antibody. Avian leukosis virus infection has similar effects on broiler breeders and causes a consistent, although often small, reduction in broiler growth rate (87, 153). However, these effects are more marked in meat-type chickens infected with subgroup J ALV (377, 378, 380, 381). In broiler breeder flocks affected by myeloid leukosis caused by subgroup J ALV, smaller eggs were associated with the presence of gs antigen in allantoic fluid and virus in the embryo (374). The most critical time for the immunosuppressive effects of ALV-J infection in experimental studies was the 3–4 week period after infection (420). Other studies on reduced productivity in chickens with ALV infections and the genetic consequences have been reviewed (152, 189, 373). The presence of ALV in semen was not associated with reduced semen production, but some evidence suggested an effect on semen quality and fertility (357). The physiological bases for these effects have not been studied.

A number of other nonneoplastic effects of ALV infection have been observed, mostly in experimental infections.

Chickens, turkeys, and jungle fowl exposed when young to certain ALVs (RAV-1, RAV-60, MAV-2(O)), and ALVs of subgroup B and D develop anemia, hepatitis, immunodepression, and wasting; some may die (82, 371). A myocarditis and chronic circulatory syndrome was reported in chickens inoculated with RAV-1 ALV (158). Intracytoplasmic viral matrix inclusion bodies have been observed in the myocardium of adult ALV infected chickens (156, 267). Chickens inoculated with RAV-7 develop neurologic signs including ataxia, lethargy, and imbalance resulting from a nonsuppurative meningoencephalomyelitis (429). A persistent infection of the central nervous system (CNS), with inflammatory lesions and clinical signs, followed *in ovo* infection with RAV-1 (120). Fowl glioma-associated virus also shows involvement of brain with cerebellar hypoplasia (270, 402).

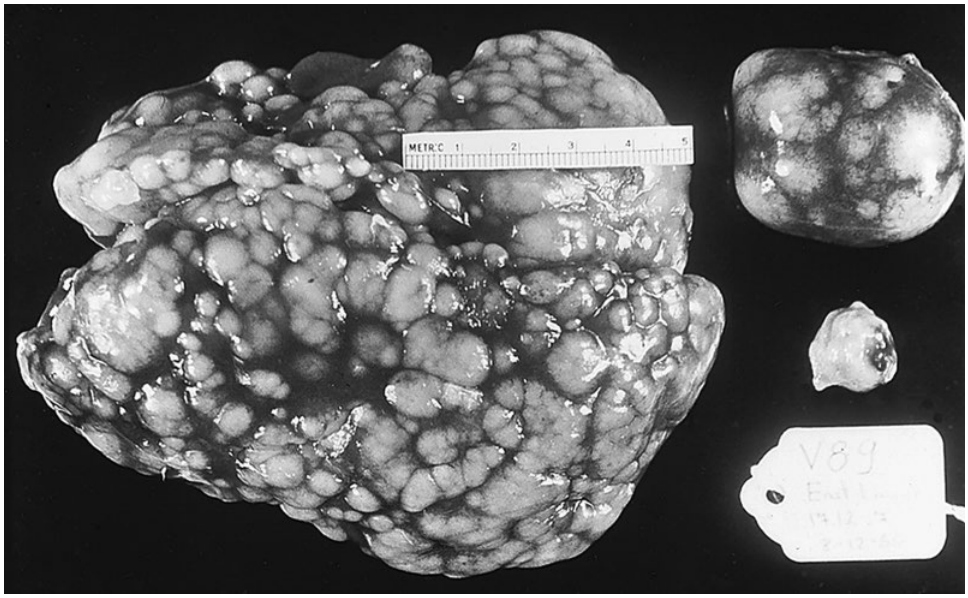


Figure 15.25 Nodular lesions in liver and spleen of bird with lymphoma leukosis (LL) inoculated at one day of age with RPL12 virus. Bursa also has a small tumor.

In MAV-2(O) infection, anemia occurs due to an aplastic crisis in the bone marrow in which erythrocytes fail to incorporate iron into hemoglobin and exhibit a decreased survival time (93). Administration of antiviral antibody will prevent anemia (320). The immunodepression may involve atrophy or aplasia of lymphoid organs, hypergammaglobulinemia, decreased mitogen-induced blastogenesis, and decreased antibody response (371). The changes in the immune system are likely a result of cessation of B-cell maturation and a block in the development of T-suppressor cells, possibly due to interference with the synthesis of functional interleukin-2 (179, 214).

In addition to stunting and atrophy of the lymphoid organs, RAV-7 caused obesity, high triglyceride and cholesterol levels, reduced thyroxine levels (hypothyroidism), and increased insulin levels. The frequent occurrence of stunting may relate to the virus's suppression of thyroid function. Stunting of chicks with congenital subgroup J ALV infection was also associated with hypothyroidism, possibly mediated via effects on the pituitary (44) or other effects (378).

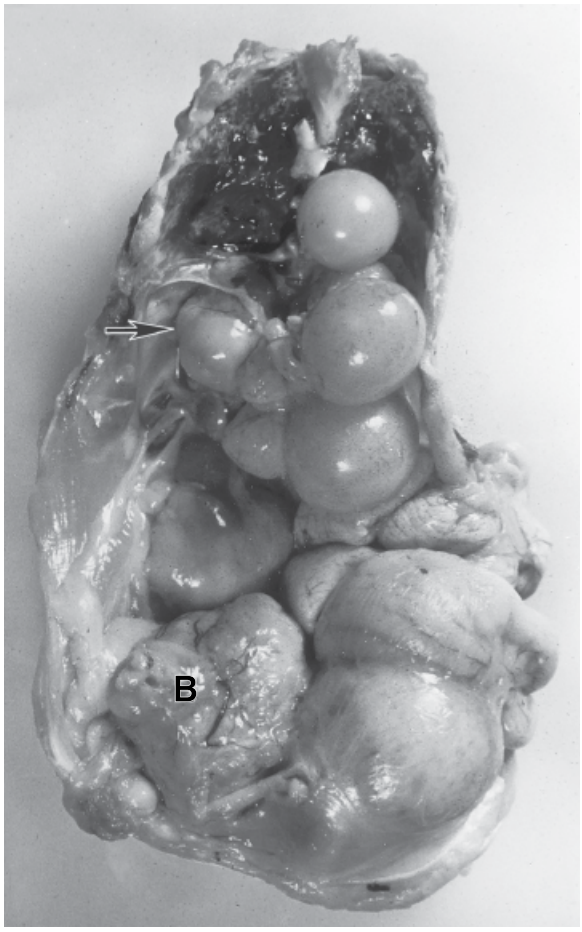


Figure 15.26 Large tumor of bursa of Fabricius (B) and kidneys (arrow) in a naturally occurring case of lymphoid leukosis (LL) in the adult hen.

Lymphoid Leukosis

Gross. Fully developed LL occurs in chickens of about four months of age and older. Grossly visible tumors almost invariably involve the liver (Figures 15.25 and 15.30A), spleen, and bursa of Fabricius (Figures 15.26 and 15.30G). Other organs often grossly involved include kidney, lung, gonad, heart, bone marrow, and mesentery.

Tumors are soft, smooth, and glistening; a cut surface appears grayish to creamy white and seldom has areas of necrosis. Tumor growth may be nodular (Figure 15.25),

miliary, diffuse (Figure 15.30A), or a combination of these forms. In the nodular form, the lymphoid tumors vary from 0.5–5 cm in diameter and may occur singly or in large numbers. They are usually spherical but may be flattened when they are close to the surface of an organ. The miliary form, which is most obvious in the liver, consists of numerous small nodules less than 2 mm in diameter uniformly distributed throughout the parenchyma. In the diffuse form, the organ is uniformly enlarged, slightly grayish in color, and usually very friable. Occasionally, the liver is firm, fibrous, and almost gritty.

Microscopic. All tumors are focal and multicentric in origin. Even in organs appearing diffusely involved when examined grossly, the microscopic pattern is one of coalescing foci. As tumor cells proliferate, they displace and compress cells of the organ rather than infiltrate between them (Figure 15.27). Nodules in the liver usually are surrounded by a band of fibroblast-like cells that have been shown to be remnants of sinusoidal endothelial cells (168). In the bursa, a follicular pattern of tumor growth usually can be seen.

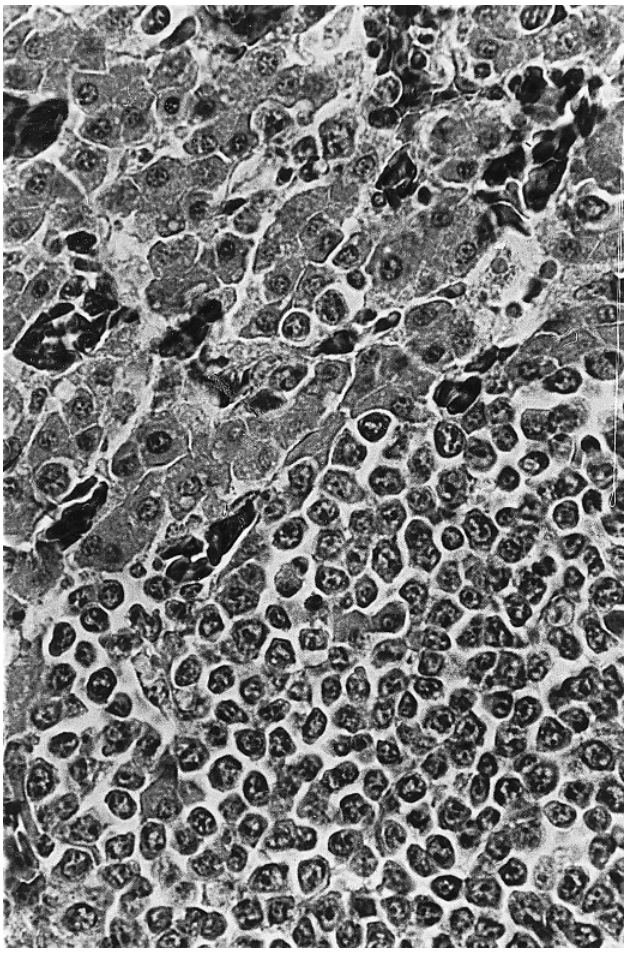


Figure 15.27 Liver tumor from a 20-week-old chicken inoculated at one day of age with RAV-1 ALV. Note displacement and compression of hepatic parenchyma. ×700 (179).

Tumors consist of aggregates of large lymphoid cells (lymphoblasts) that may vary slightly in size but are all at the same early developmental stage. They have a poorly defined cytoplasmic membrane, much basophilic cytoplasm, and a vesicular nucleus in which there are margination and clumping of the chromatin and one or more conspicuous acidophilic nucleoli (297).

The cytoplasm of most tumor cells contains a large amount of RNA, which stains red with methyl green pyronin, indicating that the cells are immature and rapidly dividing see (266). Characteristic features of the cell can best be seen in wet-fixed impression smears that have been stained with May–Grunwald–Giemsa, methyl green pyronin, or other cytological stains. The tumor cells have B-cell antigen markers and produce and carry IgM on their surface (77, 307).

Ultrastructural. Vacuoles are found infrequently in lymphoid cells of birds with LL, but some virus particles have been observed budding from the plasma membranes of lymphoblasts (for details of references to this work, see 266). Inclusion bodies in enteric smooth muscle and virions resembling avian retroviruses in the adjacent intercellular spaces of a bird positive for ALV-J PCR (171).

Pathogenesis. Avian leukosis viruses multiply in most tissues and organs of the body (111). Transitory lymphoid foci may occur in various tissues and are considered to be inflammatory in nature (Figure 15.28). The infection persists longer in bursal lymphocytes than in other hematopoietic tissues (9, 10), and cells of the bursa of Fabricius are the target cells that neoplastically transform. The target cells must be resident in the bursa, because surgical bursectomy up to five months of age and other treatments that destroy the bursa of Fabricius will eliminate the disease. (For details of references, please refer to 266). Medullary macrophages appear to be the principal bursal cells for virus replication and may be important in transmitting infection to the lymphoid cells (157). At a variable time after infection, which can be as short as four weeks in experimental studies, a proliferation of lymphoblasts occurs in one or more lymphoid follicles in the bursa. These altered bursal follicles are termed transformed follicles (9, 275, 321), and the change is regarded as a focal preneoplastic hyperplasia (182, 183) (Figure 15.29). The transformed follicle is a consequence of activation of the c-*myc* gene by nearby insertion of ALV. This places the c-*myc* gene under the control of the enhancers of the viral LTR, resulting in over-expression of *myc*, causing a maturation arrest and proliferation of bursal stem cells, associated with changes in global gene expression profiles and genomic instability (276). Arrest of maturation

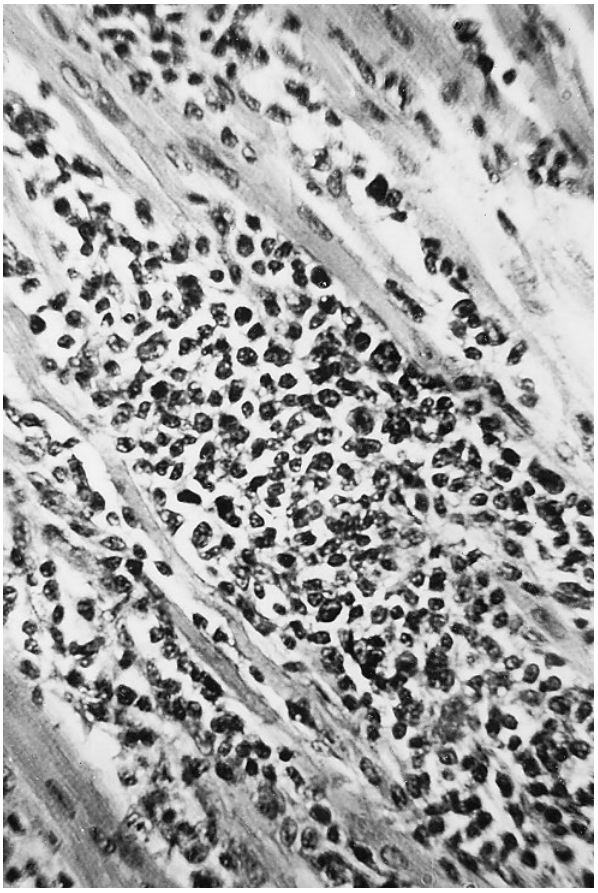


Figure 15.28 Lesions in young chickens induced by leukosis virus. Heart from a 4-week-old RIF3-infected chick showing diffuse accumulations of lymphoid cells among myocardial fibers ×430. (Calnek).

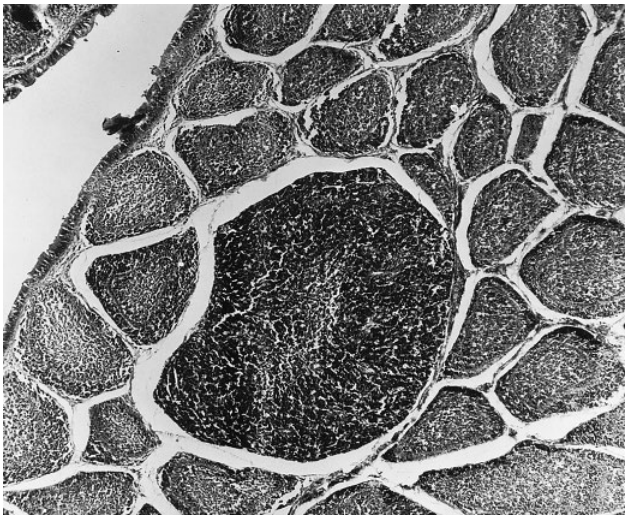


Figure 15.29 Lymphoblastic transformation in single bursal follicle in chicken with lymphoma leukosis (LL). All surrounding follicles are histologically normal in this and other sections from a 16-day-old chicken infected with RPL12 virus at hatching. Methyl green pyronin, ×40. (Dent).

of the transformed B cells results in interference of the normal intraclonal switch of immunoglobulin production from IgM to IgG, hence the surface IgM that characterizes LL cells. The cells grow within the confines of the bursal follicle and are not neoplastic. Sometimes, many follicles are transformed, but the majority of these appear to regress, and only a few continue to grow to give rise to nodular tumors in the bursa, which are visible grossly from about 14 weeks of age (76, 275). Progression of the transformed follicle to the fully neoplastic state requires additional genetic changes, and other putative oncogenes, *Blym*-1 (162, 273) Mtd/Bok (43) and c-*bic* (72, 395), have been implicated. Studies have suggested that the oncogenicity associated with the non-coding c-*bic* transcript is due to a novel microRNA designated miR-155 (396). Oncogenicity assays demonstrated that *bic* can cooperate with c-*myc* in lymphomagenesis and erythroleukemogenesis, providing direct evidence for the involvement of untranslated RNAs in oncogenesis (397). Genome-wide analysis of palindrome formation has shown that *myc*-induced genomic instability from palindrome formation in many sites including the c-*bic*/miR-155 locus is a major factor triggering the bursal lymphoma (274). Analysis of the proviral integration sites in B-cell lymphomas showed that the telomerase reverse transcriptase (TERT) promoter/enhancer region was a common integration site, suggesting that upregulation of cellular TERT by insertional activation is a factor initiating/enhancing B-cell lymphomas (445). Recent studies have also identified other integration sites that contribute to the development of tumors (195, 196, 309). Evidence also suggests that apoptosis of neoplastic bursal cells is inhibited by an antagonist of apoptotic cell death, NR-13, related to the *Bcl*-2 proto-oncogene (221, 280). Induction of angiogenic factors from the transformed cells also contributes to the generation of *myc*-induced lymphomas (40). From about 12 weeks of age, cells in the clonal bursal tumors metastasize to other organs and tissues and result in the terminal disease. Metastatic tumors in the viscera usually have the same DNA fragments as bursal tumors from the same birds, supporting their clonal origin (83), but multiple bursal tumors can give rise to polyclonal metastatic disease (368).

Experimentally, B-cell lymphomas also have been induced by c-*myb* activation, following embryonic infection with ALV (198, 313). The tumors were unusual in that metastatic disease occurred within seven weeks of infection, and preneoplastic and primary bursal neoplasms were not detected. Spontaneous bursal lymphomas of unknown etiology have also been observed in lines of chickens free of exogenous ALV and including line 0 free of *ev* loci (91, 92). Reticuloendotheliosis virus (see Reticuloendotheliosis) can also induce LL associated with c-*myc* activation.

Transplantable LL tumors can be developed from ALV-induced tumors. The RPL12 transplantable tumor,

from which the RPL12 strain of ALV was isolated, is a well-known example. Several other new LL transplantable tumors have been described (284). Transplantable LL tumors grow to a palpable size within 5–10 days and become widely disseminated, inducing rapid mortality.

Erythroblastosis

Gross. Natural cases of erythroblastosis (erythroid leukosis) usually occur in birds between three and six months of age. The liver and kidney are moderately swollen, and the spleen often is greatly enlarged. The enlarged organs are usually cherry red to dark mahogany (Figure 15.30B) and are soft and friable. The marrow is hyperplastic, semi-liquid, and red in color. Petechial hemorrhages occur in various organs such as muscles, subcutis, and viscera. Thrombosis, infarction, and rupture of the liver or spleen may be observed. Edema of the lungs, hydropericardium, and a fibrinous clot on the liver may occur.

With severe anemia, atrophy usually is seen in visceral and lymphoid organs, particularly the spleen.

Changes in the blood reflect those in other organs, such as liver, spleen, and bone marrow, and depend largely on the extent of anemia or leukemia. When severe anemia exists, the blood is watery and light red and clots slowly. In contrast, acute cases may show no grossly apparent changes, although usually the blood appears dark red with a smoky overcast.

Microscopic. Examination of the marrow in early cases reveals blood sinusoids filled with rapidly proliferating erythroblasts that fail to mature. In advanced cases, marrow consists of sheets of homogeneous erythroblasts with small islands of myelopoietic activity and little or no adipose tissue. With concurrent anemia, the number of erythropoietic cells may be reduced.

Alterations in visceral organs are primarily due to hemostasis, resulting in an accumulation of erythroblasts in the blood sinusoids and capillaries (Figure 15.31). The liver sinusoids, splenic red pulp, bone marrow, and sinusoids of other organs are filled with proliferating erythroblasts.

The sinusoids become greatly distended, resulting in pressure atrophy of the parenchyma. Although accumulations of erythroblasts may be extensive, they always remain intravascular, unlike those in LL and myeloblastosis.

Varying degrees of anemia may occur. Sometimes erythroblastosis occurs, and there may be only severe anemia. Extramedullary erythropoiesis is common.

The primary cell involved is the erythroblast. The cell has a large round nucleus with very fine chromatin, one or two nucleoli, and a large amount of cytoplasm that is basophilic. A perinuclear halo, vacuoles, and occasionally fine granules are present. The cell is irregular in shape and often has pseudopodia. Erythroblasts have cell markers that identify them as members of the erythrocytic series.

Stained blood smears reveal a variable number of erythroblasts (Figure 15.30D). These vary in maturity from the early erythroblast, which is the dominant cell, to the various stages of polychrome erythrocytes. The more mature cells often appear early in the course of the disease or during remission, if it occurs. The thrombocytic series of cells may be somewhat increased in number and immaturity. Similarly, in most naturally occurring cases, immature cells of the myelocytic series appear in the peripheral circulation. Occasionally, they are as prominent as the erythroblasts. Cases of mixed erythroblastosis and myelocytomatosis may occur.

Ultrastructural. Numerous studies have been made of the primitive cells in erythroblastosis induced by different strains of ALV and RPL12 (for details of these references please refer to 266). Neoplastic erythroblasts are for the most part indistinguishable from corresponding cells in the normal bird, except that virus particles may be present in extracellular spaces and within vacuoles inside cells. In erythroblasts in the circulating blood, as in cell culture, there is a great increase in membrane activity, with vacuolization of the cytoplasm and budding of virus particles from the cell membrane.

Pathogenesis. Inoculation of slowly transforming strains (i.e., those lacking a viral oncogene) of ALV such as RPL12 into 11-day-old chick embryos induces erythroblastosis from the first week of age (312). When day-old chicks are inoculated, the incubation period varies from 21 to more than 100 days (50). Induction of erythroblastosis by slowly transforming ALV involves activation of the cellular oncogene c-*erb*B by LTR insertion (67, 147, 213), and new acutely transforming AEV strains with transduced c-*erb*B genes may arise (for references to these, please refer to (266). Whether such acutely transforming viruses spread naturally and induce more erythroblastosis is not clear.

Experimentally, acutely transforming AEV strains, such as ES4 and R, cause mortality from erythroblastosis 7–14 days after inoculation (165). ES4 carries the gene v-*erb*A, which blocks erythroid precursor cell differentiation, in addition to the v-*erb*B oncogene (for details of these references, please refer to 266). Two subgroup J ALV isolates, 1B and 4B, have been shown to be acutely transforming and to induce erythroblastosis as well as myelocytomatosis and other tumors (411). Their viral oncogenes have not yet been identified. More recently ALV-J-associated outbreak of erythroblastosis has also been reported (421).

When birds are exposed to an acutely transforming AEV, the first alterations are found in three days as foci of

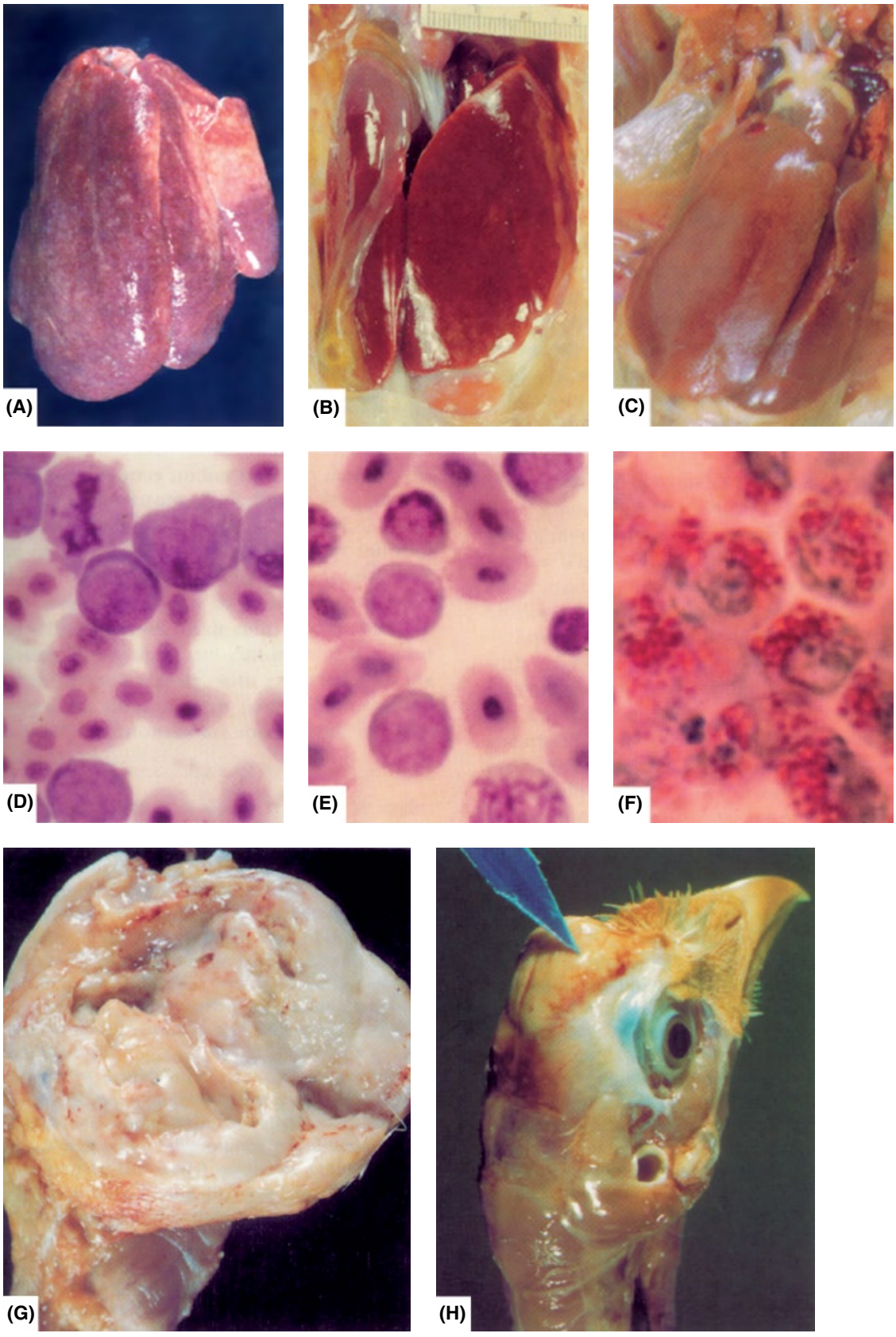


Figure 15.30 Comparison of leukosis. (A) Lymphoid leukosis (LL): Diffuse form affecting the liver. Lesion is grossly indistinguishable from those in Marek's disease (MD); (B) Erythroblastosis: enlarged cherry red liver and spleen, note the fibrinous exudates; (C) Myeloblastosis, with enlarged gray-red liver; (D) Erythroblastosis, note the basophilic cytoplasm and perinuclear halo, blood smear, Giemsa stain, ×975; (E) Myeloblastosis: myeloblasts are slightly smaller than erythroblasts; cytoplasm is not as basophilic, nucleus is less vesicular, and nucleoli are as not frequent or conspicuous, blood smear, Giemsa stain, ×975; (F) Myelocytomatosis, note myelocytes are packed with acidophilic granules, section of tumor, Giemsa, ×975 (Beard); (G) LL tumors in the bursa of Fabricius (from the same bird with the liver tumor shown in (A); (H) Myeloid leukosis tumor on the surface of the skull (Peckham). (*For color detail, please see the color section*.)

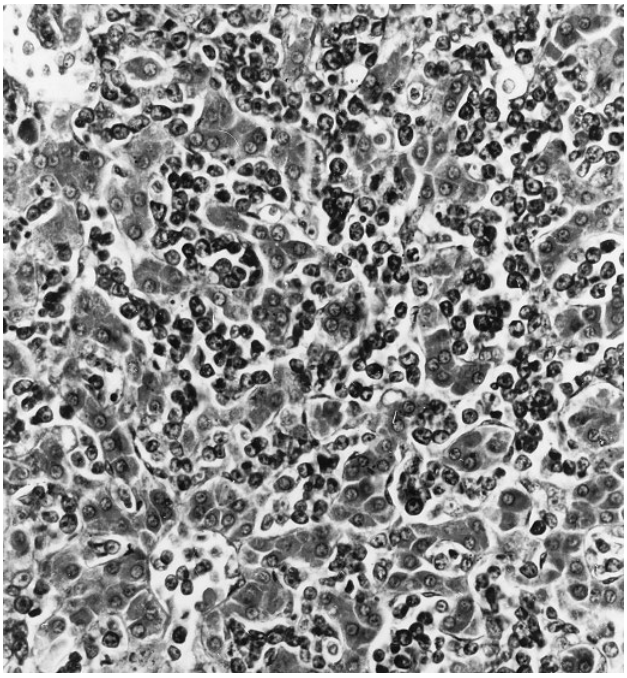


Figure 15.31 Erythroblastosis. Liver sinuses permeated with erythroblasts in bird 40 days after inoculation with strain MC29 leukosis virus. ×280. (Beard).

proliferating erythroblasts in bone marrow sinusoids. By day seven, the primitive cells reach the circulating blood, and some foci of erythroid proliferation are present in sinusoids of the liver and spleen. Erythroblasts continue to accumulate in hepatic sinusoids and elsewhere until death of the host and transplantable erythroblastosis tumors can be developed (316).

Myeloblastosis

Gross. Natural cases of myeloblastosis (myeloblastic myeloid leukosis) are uncommon and usually occur in adult chickens. The liver is greatly enlarged and firm with diffuse, grayish tumor infiltrates, which give a mottled or granular ("Morocco leather") appearance (Figure 15.30C). The spleen and kidneys are also diffusely infiltrated and moderately enlarged. The bone marrow is replaced by a solid, yellowish-gray tumor cell infiltration.

A severe leukemia exists, with myeloblasts comprising up to 75% of peripheral blood cells and forming a thick buffy coat and usually an anemia and thrombocytopenia.

Microscopic. Parenchymatous organs, notably the liver, show a massive intravascular and extravascular accumulation of myeloblasts with a variable proportion of promyelocytes (Figure 15.32). In the spleen, these tumor cells accumulate in the red pulp. In the bone marrow, myeloblastic activity is confined to extrasinusoidal areas.

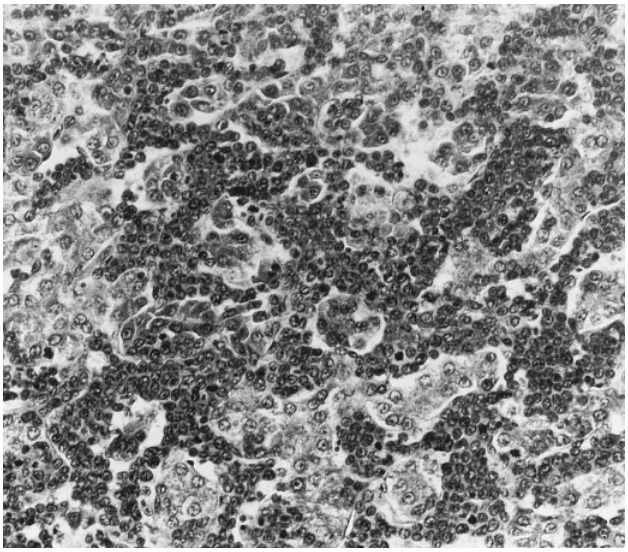


Figure 15.32 Myeloblastosis. Distribution of myeloblasts in liver of bird with myeloblastic leukemia 19 days after inoculation with BAI-A virus. ×280. (Langlois).

Myeloblasts in leukemic blood smears are large cells with slightly basophilic clear cytoplasm and a large nucleus containing 1–4 acidophilic nucleoli, which do not stain prominently (Figure 15.30E). Often, promyelocytes and myelocytes are also present; they easily can be identified by their specific granulation, which in the early forms is primarily basophilic. The disease may result in a secondary anemia, with the presence of polychrome erythrocytes and reticulocytes. Such a secondary anemia is distinguished easily from the conditions in which erythroblastosis and myeloblastosis occur together, because then blast cells of both cell series are present in the circulating blood.

Ultrastructural. In circulating myeloblasts from birds with myeloblastosis induced by BAI-A AMV, virus particles are only rarely found and then in small numbers in clear vacuoles (28, 105, 172, 173). However, reticular and phagocytic elements of the spleen and bone marrow frequently are packed with virus particles. When myeloblasts are transferred to cell culture, large numbers of lysosomes appear in the cytoplasm. After some time in cell culture, virus particles can be seen in lysosomes, in vacuoles, and budding at the cell membrane. No other changes are observed in these cells.

Pathogenesis. The v-*myb* gene of AMV is responsible for neoplastic transformation of the target myeloblasts (118). Experimental infection is followed within a few days by the appearance of multiple foci of proliferating myeloblasts in the extrasinusoidal areas of the bone marrow, followed rapidly by leukemia and infiltration of the liver, spleen, and other organs (for details of these

references, please see 266). The v-*myb* oncogene acts as a transcription factor that transforms myelomonocytic cells by deregulating the expression of specific target genes as well as through alterations of the nucleosomal organization (430).

Myelocytomatosis

Gross. Tumors of myelocytomatosis (myelocytic myeloid leukosis) are distinctive and can be recognized on gross examination with some degree of certainty. Characteristically, they occur on the surface of bones in association with the periosteum and near cartilage, although any tissue or organ can be affected. Myelocytomas often develop at the costochondral junctions of the ribs, on the inner sternum, pelvis, and on the cartilaginous bones of the mandible and nares. Flat bones of the skull are also commonly affected (Figure 15.30H). Tumors may also be seen in the oral cavity, trachea, and in and around the eye (317). The tumors are usually nodular and multiple, with a soft, friable consistency and of creamy color. In the disease caused by subgroup J ALV, myelocytomatous infiltration often causes enlargement of the liver and spleen and other organs, in addition to skeletal tumors (432). Myelocytic leukemia may also occur (299).

Microscopic. Tumors consist of masses of uniform, usually well-differentiated, myelocytes. Their nuclei are large, vesicular, and usually eccentrically located, and a distinct nucleolus is usually present. The cytoplasm is usually tightly packed with acidophilic granules, which are usually spherical. When imprint preparations of fresh tumors are stained with May–Grunwald–Giemsa, granules appear brilliant red (Figure 15.30F). Areas of less well-differentiated myelocytes are not uncommon within the myelocytomas, and areas of undifferentiated cells, which may be stem cells of the myelocyte–monocyte series, may also be found. In the liver, accumulations of neoplastic myelocytes occur around blood vessels and in the parenchyma. In the spleen, tumor cells are present in the red pulp. In the marrow, the extrasinusoidal myelopoietic areas are greatly expanded by uniform neoplastic myelocytes. A detailed description of bone and bone marrow lesions in myelocytomatosis apparently caused by subgroup J ALV is provided by Nakamura et al. (268).

Although the naturally occurring disease has been stated to be usually aleukemic, myelocytomatosis induced by subgroup J ALV frequently is accompanied by a marked leukemia of myeloid cells. Laboratory strains of myelocytomatosis-inducing virus, such as MC29, also cause leukemia (Figure 15.33).

Ultrastructure. Ultrastructural features of myelocytoma cells vary from those of well-differentiated myelocytes to those of undifferentiated, nongranulated myeloid cells (253).

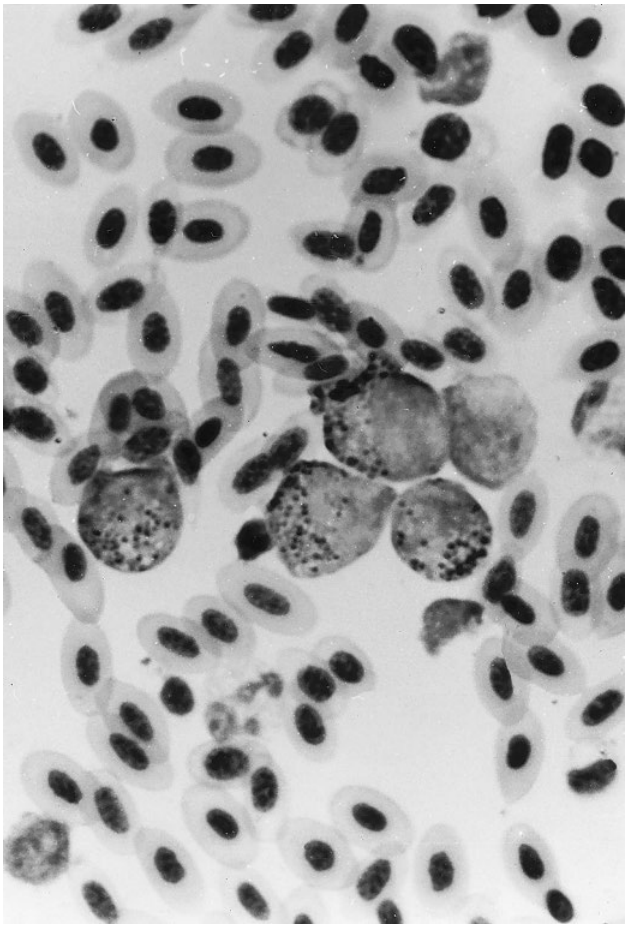


Figure 15.33 Myelocytomatosis. Granulated myelocytes in blood smear from bird 23 days after inoculation with strain MC29 leukosis virus. ×750. (Beard).

Pathogenesis. Acutely transforming strains of ALV that induce myelocytomatosis, such as MC29 and CMII, carry the v-*myc* oncogene (118, 257). Slowly transforming strains of subgroup J ALV that also induce myelocytomatosis, such as HPRS-103 and ADOL-Hc1, do not carry an oncogene, but molecular studies of HPRS-103-induced myelocytomatosis indicate that c-*myc* is activated (66–68). The acutely transforming strain 966 ALV, derived from myelocytoma and induced by strain HPRS-103 of subgroup J ALV, has been shown to carry v-*myc* (66, 302). Studies on HPRS-103 and 966 showed that they have a tropism for the myelomonocytic cell lineage, which may relate to their ability to cause myelocytomas (6, 7). A recombinant ALV with envelope of subgroup B and LTR of subgroup J (ALV-B/J) was isolated from a field outbreak of myeloid leukosis in commercial layers has been reported (159). However, inoculation of experimental and commercial strains of White Leghorn chickens with this recombinant ALV-B/J resulted in primarily LL, but not myeloid leukosis, suggesting that differences in the genetic makeup of the

commercial layers from which ALV-B/J was originally isolated and lines of chickens used in experimental inoculations studies may be responsible for the differences in pathogenicity observed (233).

The earliest alterations occur in bone marrow in which there is crowding of intrasinusoidal spaces, principally by myelocytes, and destruction of sinusoid walls. The spaces may contain two types of cell—the primitive hemocytoblast-like cell (myeloid stem cell) and the neoplastic myelocyte. The latter appears to arise directly from the stem cell, and differentiation is arrested both at the nongranulated and granulated myelocyte level (253). Myelocytes proliferate and soon overgrow the bone marrow. Tumors form by expansion of marrow growth and may crowd through the bone and periosteum. Extramedullary tumors may also arise by blood-borne metastasis.

Hemangioma

Gross. This tumor is found in the skin or in visceral organs in chickens of various ages. They appear as blood-filled cystic masses (blood blisters) (Figure 15.34) or more solid proliferative, lesions (56). They are often multiple and may rupture, causing fatal hemorrhage (372). More recently, many workers have reported the incidence hemangiomas in layer chickens infected with ALV-J in China (215, 287, 454).

Microscopic. The cavernous form is characterized by greatly distended blood spaces with thin walls composed of endothelial cells (Figure 15.35). Capillary hemangiomas are solid masses in which endothelium may proliferate into dense masses (hemangioendothelioma), leaving mere clefts for blood channels (Figure 15.36); develop into a lattice with capillary spaces; or grow into collagen-supported cords with larger interspersed blood spaces. Solid and papillary forms have also been described (264).

Ultrastructural. Hemangiomas consisted mainly of undifferentiated mesenchymal cells and had an alveolar structure (241).

Pathogenesis. Sequence analysis of an avian hemangioma-inducing virus isolated from layer hens revealed unique elements in both *env* gene and LTR



Figure 15.34 Hemangioma of gizzard serosa of RPL12 virus-inoculated bird. Note the dark circumscribed and raised tumor nodules (236).



Figure 15.35 Cavernous hemangioendothelioma of mesentery. (Feldman and Olson).

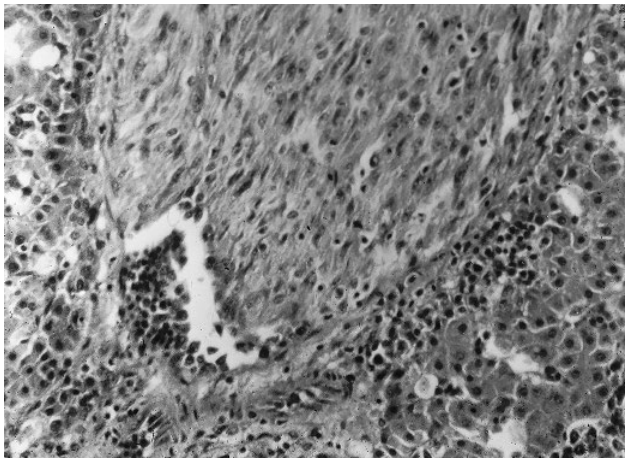


Figure 15.36 Endothelioma in liver of bird inoculated with RPL30 leukosis virus. Occlusion of portal vein by inward-growing spindle cells from blood vessel ×250. (Fredrickson).

that were thought probably responsible for its biologic and pathogenic characteristics (55). This virus was cytocidal and had an affinity for endothelial cells (330, 331). Although several sequence changes have been reported in the genomes of hemangioma-inducing ALV-J strains isolated from layers (287, 443), the precise molecular mechanisms remain unknown. Recent study has suggested MET gene as a common integration site in cases of hemangiomas induced by ALV-J (196).

Nephroma and Nephroblastoma

Gross. Two types of renal tumor occur: nephroblastomas (Wilms' tumor) and adenomas and carcinomas. Nephroblastomas vary from small, pinkish-gray nodules embedded in the kidney parenchyma to large, yellowish-gray, lobulated masses that replace most of the kidney tissue (Figure 15.37). Tumors may be pedunculated and connected to the kidney by a thin fibrous vascular stalk. Large tumors are often cystic and may involve both kidneys. Adenomas and carcinomas vary in size and appearance, similar to nephroblastomas. They are often multiple and within cysts.

Microscopic. In nephroblastomas, the histologic variation between different tumors or areas of the same tumor is striking. There is usually neoplastic proliferation of both epithelial and mesenchymal elements, although their proportion and differentiation vary widely. Epithelial structures vary from enlarged tubules with invaginated epithelium and malformed glomeruli; through irregular masses of distorted tubules; to groups of large, irregular, cuboidal, undifferentiated cells with little tubular organization (Figure 15.38). The growth may be embedded in a loose mesenchymal or sarcomatous stroma. There may be islands of keratinizing stratified

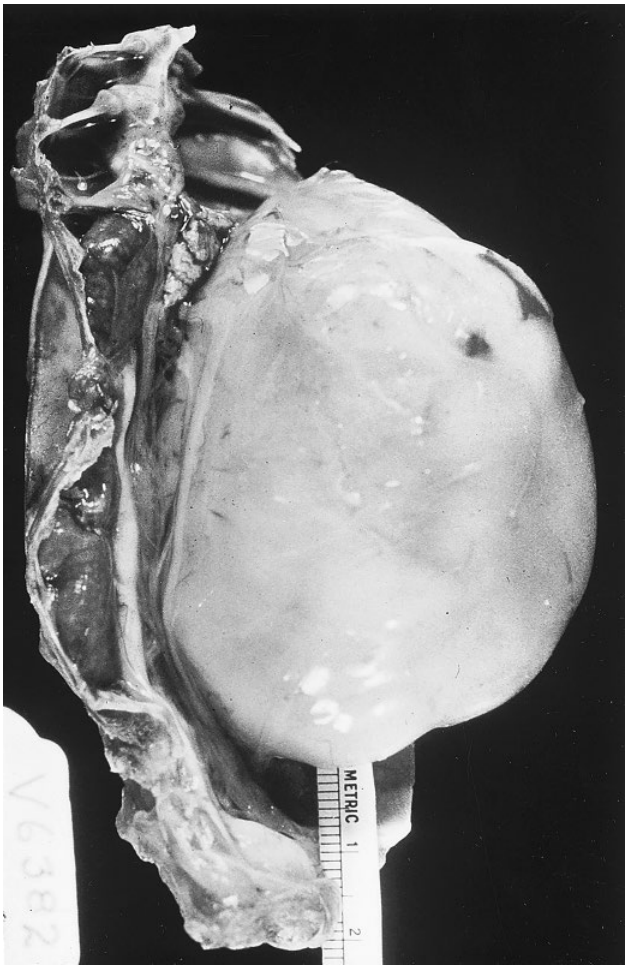


Figure 15.37 Nephroblastoma. Bird was inoculated at one day of age with avian myeloblastosis virus BAI-A strain.

squamous epithelial structures (epithelial pearls), cartilage, or bone (98, 178, 190). Primary multiplicity of tumors may occur, but metastases are rare.

Adenomatous or carcinomatous growths also vary greatly in microscopic appearance. In tubular adenocarcinomas, primitive abnormal glomeruli frequently occur in large numbers among abnormal tubules. Papillary cyst adenocarcinomas are frequent. At times, solid carcinomas with little evidence of renal tubules develop (30, 253). Rarely is there cartilage and never other mesenchymal tumor tissues. A trabecular fibrous tissue stroma may separate masses of epithelial tumor tissue.

Ultrastructural. In the epithelial nephronic elements of nephroblastomas induced by strain BAI-A of ALV (28), cytoplasmic aberrant structures occasionally are seen in large or small aggregates. Virus particles bud from cell membranes of epithelial cells, fibroblastic elements of the stroma, and chondrocytes. Sarcomatous elements consist of cells similar in morphology to those in other avian sarcomas. Virus particles have been observed



Figure 15.38 Nephroblastoma. Bird was inoculated at one day of age with cloned preparation of avian myeloblastosis virus BAI-A strain. Note primary multiplicity of tumors of two distinct types in different areas (arrows) ×20.

budding from epithelial cells in cystadenomas and adenocarcinomas induced by strain MC29 myelocytomatosis virus (255). Large accumulations of particles in spaces in the cysts and tubules probably were related to a lack of tubule and glomerular drainage.

Pathogenesis. A target oncogene for ALV-induced nephroblastomas was not consistently identified (74). More recently, a new proto-oncogenes such as *nov*, (194) and *twist* (285) were identified as common integration sites in nephroblastomas (286).

Nephroblastomas originate from nephrogenic blastema (embryonic nephrons and embryonal rests) (56, 190). This blastema tissue is present in the metanephros (functional kidney) at hatching until at least six weeks of age and appears as wedge-shaped foci of immature renal tissue particularly beneath the capsule. These epithelial structures enlarge and become neoplastic. The supporting stroma of mesenchymal elements also proliferates and, in turn, may be altered. There is extensive multiplication of tumor cells (usually convoluted tubules and/or stroma) and varying degrees of differentiation, some abnormal. In the most differentiated form, nephrogenic cells form glomeruli, tubules, or keratinized epithelium; whereas cells of the stroma form sarcomas, cartilage, and bone. Anaplasia of kidney cells can result in sheets of large epithelioid cells with almost no tubular organization. Malformed and blocked tubules result in cysts. Nephroblastomas have been induced by BAI-A strain AMV (29, 53, 419), MAV-2(N) (445), MAV-2-O (36), and subgroup J-related strain 1911 (301). Transplantable nephroblastomas have been developed (419).

Carcinomatous growths originate only from the epithelial part of the embryonal blastema and not from mesenchymal elements. Depending on the degree of anaplasia of epithelial elements, tumors formed may be adenomas, adenocarcinomas, or solid carcinomas. These tumors have been induced by MC29, ES4, and MH2 virus strains and by various field isolates. Renal adenomas and carcinomas can be caused by slowly and acutely transforming subgroup J ALV (for details of these references, please see 266).

Fibrosarcoma and Other Connective Tissue Tumors

Gross. A variety of benign and malignant connective tissue tumors occur naturally, usually sporadically, in young and mature chickens, and transmission of many of these by cell-free filtrates has been demonstrated. These tumors include fibromas and fibrosarcomas, myxomas and myxosarcomas, histiocytic sarcomas, osteomas and osteosarcomas, and chondromas and chondrosarcomas. The benign tumors grow slowly, are localized, and are noninfiltrative. The malignant counterparts grow more rapidly, infiltrate surrounding tissue, and may metastasize.

Fibromas arise as firm fibrous lumps attached to the skin, subcutaneous tissues, muscles, and occasionally other organs; fibrosarcomas are of a softer consistency. In the skin, they may ulcerate. Myxomas and myxosarcomas are softer and contain tenacious slimy material. They occur mainly in the skin and muscles. Histiocytic sarcomas are firm, fleshy tumors occurring mainly in the viscera. Osteomas and osteosarcomas are uncommon and occur as hard tumors that may arise from the periosteum of any bone. Chondromas and chondrosarcomas are rare. They occur where cartilage is present and sometimes within fibrosarcomas and myxosarcomas. Ganglioneurosarcoma was reported associated with subgroup J ALV infection (161).

Microscopic. Fibromas in their simplest forms consist of mature fibroblasts interspersed with collagen fibers arranged in wavy parallel bands or whorls. Slowly growing tumors are more differentiated and contain more collagen and fewer cells than those growing more rapidly. Some fibromas may have edematous areas and should not be confused with myxomas and myxosarcomas. If necrosis, ulceration, and secondary infection have occurred, various inflammatory and

necrotic alterations may be observed in the tumor. Inflammatory changes may be so prominent that the tumor may be confused with a granuloma.

Aggressive and destructive growth, their cellular composition, and the immaturity of constituent cells (Figure 15.39) characterize fibrosarcomas. Large irregular and hyperchromic fibroblasts are abundant, and mitosis is common. Tumors contain less collagen than fibromas, and this is concentrated in and near irregular septa that subdivide the tumor. Regions of necrosis often occur in rapidly growing tumors. Edema is sometimes present. Multiple undifferentiated pulmonary sarcomas associated with subgroup J ALV infection have been reported (170).

Myxomas consist of stellate or spindle-shaped cells surrounded by a homogeneous, slightly basophilic, mucinous matrix. In the malignant form (myxosarcoma), the mucinous matrix is less abundant, and fibroblasts are proportionally more numerous and immature than in myxomas (Figure 15.40). Myxosarcomas associated with ALV-A infection have been reported in fancy breed chickens (431).



Figure 15.39 Fibrosarcoma in musculature of breast ×120. (Feldman and Olson).

Histiocytic sarcomas are derived from cells of the monocyte and macrophage lineage, and the cellular constituents are highly variable both within a tumor and between tumors (Figure 15.41). They have been reported associated with infection by subgroup J ALV (5, 169). Further studies have shown that ALV-J-induced histiocytic sarcomatosis occurred in persistently viremic but not immunotolerized meat-type chickens (289).

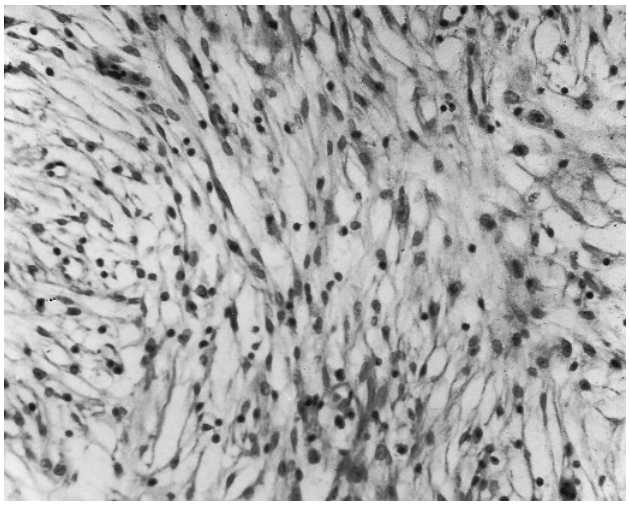


Figure 15.40 Myxosarcoma induced by Rous sarcoma virus ×240. (Helmboldt).



Figure 15.41 Histiocytic sarcoma of the heart. Note the varied character of cellular components ×240. (Helmboldt).

The cells may be spindle-shaped, usually appearing in groups or bundles as in fibrosarcomas; stellate reticulum-producing elements; and/or large phagocytic cells or macrophages. Tumors apparently derived from stem cells of the myelomonocytic lineage may also be considered to be histiocytic sarcomas. The so-called endotheliomas induced by MH2 and MC29 may be tumors of this lineage (119). In primary tumors, spindle-shaped cells usually predominate; whereas in metastatic foci, primitive histiocytic forms are more numerous.

Osteomas are structurally similar to bone except that much of the inner histologic detail is lacking. They consist of a homogenous acidophilic matrix of osseomucin containing collections of osteoblasts at irregular intervals. Osteosarcomas are usually very cellular infiltrative growths that invade and destroy surrounding tissues. The cells are spindle-shaped, ovoid, or polyhedral, and many are in mitosis. Nuclei are prominent, and cytoplasm is basophilic. Multinucleated giant cells may be quite numerous. Although very cellular and rapid growing, some areas usually have sufficient differentiation for the production of osseomucin, which is usually sufficient to identify these tumors.

Chondromas have a typical and unique structure (i.e., groups of two or more chondrocytes lying in a matrix of chondromucin). In chondrosarcomas, considerable cellular variation exists, ranging from the most immature to the fully mature chondrocyte.

Ultrastructural. Only the sarcomas produced by RSV have been examined in detail. The morphology of fibroblasts, macrophage-like cells, and mast cells, found in Rous sarcomas, have been described (28, 172).

Pathogenesis. Induction of sarcomas and other connective tissue tumors in the field is likely to be by activation of a cellular oncogene by a slowly transforming ALV, occurring up to several months after infection (388). Avian leukosis virus related to MAV-1 has been implicated in a field outbreak of sarcomas in commercial layers; inoculation of susceptible White Leghorn chickens with new isolate resulted sarcomas and myelocytomas (448).

Viral oncogenes that have been associated with sarcoma induction include *src*, *fps*, *yes*, *ros*, *eyk*, *jun*, *qin*, *maf*, *crk*, *sea*, and *erb*B (73, 118, 309, 416) (Table 15.5). These viral oncogenes reflect the cellular oncogenes that are activated by insertional mutagenesis and that may undergo mutation. These cellular oncogenes control a variety of functions in the cell (their products are generally growth factors, growth factor receptors, signal transducers, or DNA transcription factors), and it is their altered expression that results in the loss of regulation of cell proliferation or differentiation that causes neoplasia.

Osteopetrosis

Gross. The first grossly visible changes occur in the diaphysis of the tibia and/or tarsometatarsus. Alterations soon are seen in other long bones and bones of the pelvis, shoulder girdle, and ribs but not the digits. Lesions are usually bilaterally symmetric; they first appear as distinct, pale yellow foci against the gray-white, translucent, normal bone. The periosteum is thickened, and the abnormal bone is spongy and at first easily cut. The lesion is commonly circumferential and advances to the metaphysis, giving the bone a fusiform appearance (Figures 15.42 and 15.43). Occasionally, the lesion remains focal or is eccentric. Severity of the lesion varies from a slight exostosis to a massive asymmetric enlargement with almost complete obliteration of the marrow cavity. In long-standing cases, the periosteum is not as thickened as it was earlier; when it is removed, the porous irregular surface of the very hard osteopetrotic bone is revealed.



Figure 15.42 Osteopetrosis. A 24-week-old chicken, injected with RPL12 at one day of age, with advanced osteopetrotic lesions of the shanks. (Sanger).

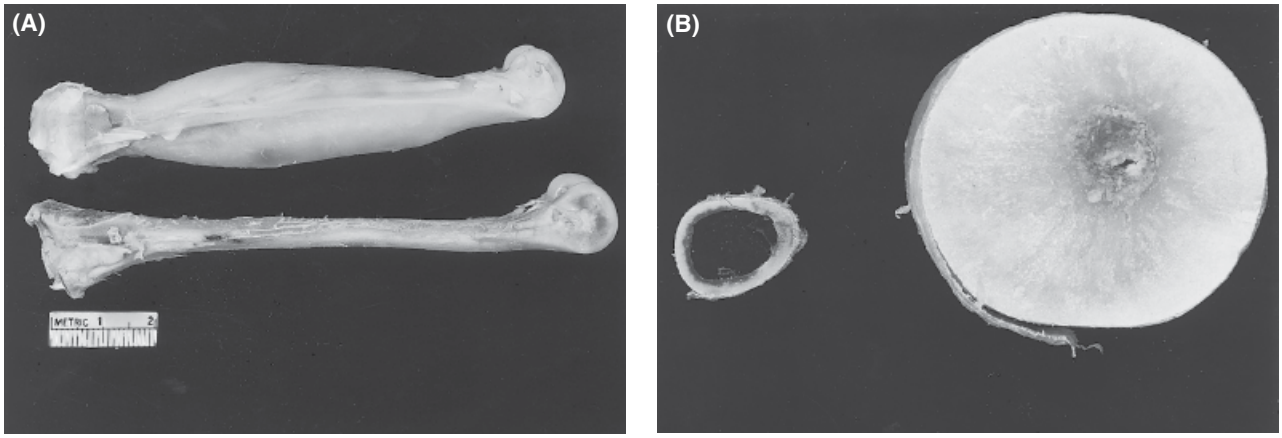


Figure 15.43 Osteopetrosis of tibia in 10-week-old chicken. (A) Shorter length of bone is due to reduced growth. Lower tibia is from control bird of same age. (B) Cross-section of middle of shaft of bones in A (451).

Early in the disease, the spleen may be slightly enlarged. Later, severe splenic atrophy occurs as well as premature bursal and thymic atrophy. Lymphoid leukosis often occurs in individual birds with osteopetrosis.

Microscopic Lesions. The periosteum over the lesion is greatly thickened from an increase in number and size of basophilic osteoblasts. The number of osteoclasts per tibia increases, but the density of osteoclasts (i.e., the number per unit volume of bone) decreases (354). Affected bones differ from normal bones in the following ways. Spongy bone converges centripetally toward the center of the shaft (Figure 15.44). An increase occurs in size and irregularity of the haversian canals, as well as an increase in number and size, and an alteration in position, of lacunae. Osteocytes are more numerous, large, and eosinophilic; the new bone is basophilic and fibrous.

The blood picture is ordinarily aleukemic, and a secondary anemia often exists. There may be active erythropoiesis in remaining bone marrow and sometimes in focal areas in the liver. Experimentally, viruses that cause osteopetrosis can induce an aplastic anemia and an increased corpuscular fragility (179, 320).

Ultrastructural. Virus particles bud transiently from osteoblasts and continuously from osteocytes and accumulate in the periosteocytic space. With calcification of the bone, the particles become incorporated in the bone trabeculae. No virus production is observed from osteoclasts (141).

Pathogenesis. Osteopetrosis is a polyclonal disease of the bone and is thought to be caused by high levels of virus infection perturbing the growth and differentiation of osteoblasts. Much higher levels of virus infection were found in diseased bones than in cultured osteoblasts infected with the Br21 strain of an osteopetrosis-inducing ALV (140). Severe cases of osteopetrosis contained 10 times more viral DNA, 30 times more gag precursor protein, and 2–3 times more env protein than the infected osteoblast cultures. The osteopetrotic lesion is basically proliferative or hypertrophic (57, 349) and may be neoplastic (39, 354). Lesions of the lymphoid organs and bone marrow are degenerative or anaplastic (179).



Figure 15.44 Osteopetrosis. Cross-section of humerus from 8-week-old chicken. Six separate osteopetrotic foci are present, two of which extend from endosteum to periosteum ×18 (451).

The propensity for certain ALVs to induce osteopetrosis depends on sequences in the *gag-pol-5'env* region of the viral genome (358). Env proteins have also been implicated in osteopetrosis induction (193). Detection of exogenous ALV sequences most similar to the envelope of myeloblastosis associated virus type 1 (MAV-1) was identified from the archived bones of an outbreak of osteopetrosis in commercial birds have been reported (23).

Other Tumors

Apart from renal tumors, epithelial tumors caused by ALV are uncommon. They mainly have been reported following experimental infections with acutely transforming viruses, although some have occurred in natural and experimental infections with subgroup J ALV. Strains BAI-A (29) and HPRS-103 (301) of ALV have induced thecomas and granulosa cell tumors of the ovary. A seminoma in the testis occurred in a bird inoculated with strain MH2 (29) and possibly in birds inoculated with subgroup J ALV isolates (308). Adenocarcinomas of the pancreas have been induced in chickens by strains MC29, MH2, and HPRS-103 of ALV (29, 254, 301). The Pts-56 strain of osteopetrosis virus produced pancreatic adenomas and adenocarcinomas and duodenal papillomas in guinea fowl (202–204). Squamous cell carcinomas have been observed in a few chicks with strains MC29 and MH2 (29). The MC29 and MH2 strains have induced hepatocarcinomas (29, 216). Other epithelial tumors induced by subgroup J ALV include cholangioma and ovarian carcinoma (301).

Strains MC29 (29) and HPRS-103 of ALV (301) have been shown to induce mesotheliomas.

Immunity

Active Immunity

Immune responses to oncogenic viruses including ALV have been reviewed (106, 137, 262, 265, 425). Under natural conditions, most chicks become infected by exogenous ALV from penmates or their surroundings and, after a transient viremia, develop virus-neutralizing antibodies directed against virus envelope antigens that rise to a high titer and persist throughout the life of the bird. The virus-neutralizing antibodies serve to restrict the amount of virus in the bird, which in turn, will limit neoplasia, but they generally are considered to have little direct influence on tumor growth. After inoculation of birds with ALV at four weeks of age or older, transient viremia was detectable at one week and was followed by antibodies at three weeks and later (237). In a study of birds naturally infected after hatching, antibodies were first detected at 9 weeks of age, with a marked increase in the proportion with antibodies between 14 and 18 weeks, when 80% were positive (340).

Antibodies against gs-antigen may also occur in ALV-infected birds, but these apparently have no influence on tumor growth (335, 359). The presence of cytotoxic lymphocytes against viral envelope antigens has been shown in birds immunized with ALV or RSV (26, 27, 212), and cell-mediated immunity and the major histocompatibility complex (MHC) complex are clearly implicated in the regression of Rous sarcomas (350, 351). Viral proteins expressed on the surface of tumor cells appear to be important targets for the cell-mediated immunity, and nonvirion transformation-specific cell surface antigens may also be implicated.

Chickens that are infected congenitally by ALV do not develop immune responses to the virus. Instead, they become immunologically tolerant to the virus and develop a persistent viremia in the absence of neutralizing antibodies (248, 340). Inoculating chickens up to two weeks of age with ALV may also induce tolerant infection. Early infection with subgroup J ALV is particularly likely to induce a tolerant infection (132, 436, 438). Birds with a tolerant viremic infection are more likely to develop neoplasms than are immune-infected birds, because of the greater virus load in viremics.

Infection by ALV can depress primary and secondary antibody responses and cell-mediated immunity (341) to unrelated antigens, although these effects have been variable in different studies. Fadly et al. (129), in a study of congenital infection with an A subgroup ALV, RAV-1, failed to detect effects on B- and T-cell function during the early and late stages of infection, and they reported no histological damage to the bursa, thymus, or spleen. In contrast, subgroup B ALVs have been reported to induce a marked suppression of the humoral immune response to several antigens and decreased responsiveness to several mitogens (426). Evidence that subgroup J ALV is immunosuppressive appears to be equivocal (136, 230, 231, 379, 380). ALV-J infection induced a strong immune response at 2-weeks-of-age, but after 4-weeks-of-age, the response decreased quickly suggesting that 3–4 weeks postinfection is the critical time at which the ALV-J virus exerts its immunosuppressive effects on the host (420). Nucleotide sequence analysis of consecutive isolates from V+A+ infection profile suggested viral evolution to escape the host immune response thereby contributing to ALV J persistence (290).

Passive immunity

Serum antibodies, which are mainly in the IgG fraction (249), are passed on by the hen to her progeny via the egg yolk and provide a passive immunity that lasts 3–4 weeks. Passive antibody delays infection by ALV (439), reduces the incidence of viremia and shedding of ALV (122) and reduces the incidence of tumors (46). Level and persistence of antibody in the chick is related to the titer of antibody in the dam's serum.

Genetic Resistance

Two levels of genetic resistance to leukosis or sarcoma virus-induced tumors are recognized: cellular resistance to virus infection and resistance to tumor development (for references to these, please see 266).

Inheritance of cellular resistance to infection is of a simple Mendelian type (Table 15.12). Independent autosomal loci control responses to infection by ALSVs of subgroups A, B, and C and are designated *tva* (tumor virus A subgroup), *tvb,* and *tvc* respectively (79). At each *tv* locus, alleles for susceptibility and resistance exist that are designated tva^s, tva^r; tvb^s, tvb^r; and tvc^s tvc^r, respectively, and the susceptibility alleles are dominant over the resistance alleles. These genes usually are abbreviated to a^s, a^r, etc. It is probable that multiple alleles occur at each locus, encoding different levels of susceptibility (see 232, 266, 393).

Mutations in the *tvb* receptor gene account for the resistance to subgroups B, D, or E infections (25, 184, 205, 329). Similarly, intronic deletions that disrupt mRNA splicing of the tva receptor gene was shown to result in decreased susceptibility to infection by subgroup A ALV (328).

Genetic resistance to infection by subgroup J virus has not been recognized in chickens, although a number of other avian species are resistant (306). There is no evidence of the segregation of the ALV-J cell receptor, Na+/H+ exchange type I molecule (59), in the chicken population (279). However differences between avian species in susceptibility to ALV-J were dependent on the polymorphism in this receptor (206, 315, 327).

Cellular susceptibility phenotypes associated with these genes are designated according to a convention that recognizes the virus subgroups to which the chicken (C) cell is resistant (/) (e.g., C/AE denotes a cell resistant to A and E subgroups but susceptible to B, C, D, and J subgroups); C/0 denotes a cell resistant to no subgroup (i.e., susceptible to A, B, C, D, E, and J). Recent studies have shown that precise editing of receptor sequences could be used to induce resistance to infection by ALV (217, 218).

Chickens with genetic resistance to infection and tumor induction by different subgroups of ALSVs usually fail to develop antibodies (78, 85). Genetic resistance to tumor development has been studied mainly with the Rous sarcoma (355, 398), regression of which is determined by a dominant gene, *R-RS-1*, that lies within the MHC locus of the chicken and located in the BBL region (75, 155, 319, 353). Conserved peptide motifs of the RSV proteins that bind to the MHC have been identified and shown to be immunoprotective against Rous sarcoma growth in chickens with Class I allele B-F12 (180) and peptide motifs of the single dominantly expressed class I molecule explain the MHC-determined responses to RSV (418). The MHC (*Ea-B*) locus also influences incidence of erythroblastosis and, to a lesser extent, LL (15). Some influence of the lymphocyte antigen *Bu-1* locus on Rous sarcoma regression and of the *Th-1* locus on LL is reported (12). Structural analysis of the MHC alleles in RSV tumor regression has ruled out the DMA1, DMA2, BRD2, TAPBP, and BLB2 genes in regression in B6 haplotype (386, 387).

Diagnosis

Isolation and Identification of Causative Agent

Because ALV is widespread among chickens, virus isolation and the demonstration of antigen or antibody have limited or no value in diagnosing field cases of lymphomas. However, assays for the detection of ALV are very useful in identification and classification of new isolates,

Table 15.12 Genes controlling cellular susceptibility to leukosis and sarcoma viruses.

	Locus		Alleles		
Virus subgroup	**Old**	**New**	**Old**	**New**	**Dominant trait**
A	*tva*	*TVA*	tva^s tva^r	TVA*S TVA*R	Susceptibility
B and D	*tvb*	*TBV*	tvb^{s1} tvb^r	TVS*S1, S3 TVB*R	Susceptibility
C	*tvc*	*TVC*	tvc^s tvc^r	TVC*S TVC*R	Susceptibility
E	*tved*	*TVE*	tve^s tve^r	TVE*S TVC*R	Susceptibility
	ie		I^e i^e		Resistance

Note: The locus designation is adapted from Crittenden (117). The new locus designation is that agreed by the Poultry Committee of the USDA National Animal Genome Research Program, 1994. The allele previously designated tvb^{s2} now is considered to be identical to tvb^{S1}. The existence of a *tve* locus is not settled. The i^e locus is now considered to be an *ev* locus, with blocking of the subgroup E virus receptor by endogenous virus ENV glycoprotein expression.

safety testing of vaccines, and in testing pathogen-free and other breeder flocks for freedom from virus infection. Samples most commonly used for detection of ALV include blood, plasma, serum, meconium, cloacal and vaginal swabs, oral washings, egg albumen, embryos, and tumors (265, 266). Virus also can be isolated from albumen of newly laid eggs or the 10-day-old embryo of eggs laid by hens that are transmitting virus vertically, from feather pulp, and from semen. All ALSVs are very thermolabile and can be preserved for long periods only at temperatures below –60°C. Thus, materials used for biological assays for infectious virus should be collected and placed on melting ice or stored at –70°C until assayed. In contrast, samples for detection of ALV gs antigens by direct assays can be stored at –20°C; reviewed in (134, 266).

Because most strains of ALV produce no visible morphologic changes in cell culture, assays for ALV are based on the following: (1) detection of specific proteins or glycoproteins coded for by one or more of the three major genes of ALV, namely *gag*, *pol*, and *env* genes (Figure 15.18), or (2) detection of specific proviral DNA or viral RNA sequences of ALV by the polymerase chain reaction (PCR) and reverse transcription (RT)-PCR, respectively.

The presence of virus is determined by the detection of ALV p27 by indirect biologic assays, such as complement fixation (CF) for avian leukosis (COFAL), ELISA for ALV, phenotypic mixing, resistance-inducing factor, and nonproducer cell activation. The biological assays require CEF with specific host range (Table 15.9). Chicken embryo fibroblasts that are resistant to infection with endogenous ALV (C/E) are desirable to use in tests for detection and isolation of exogenous ALV. Other cells, such as those resistant to subgroup A (C/A) and resistant to subgroup J ALV (C/J) (186), can also be used to confirm the subgroup of isolated ALV. Testing samples on CEFs that are susceptible to all subgroups of ALV (C/O), and those that are resistant to subgroup E (C/E) can be used in differentiating exogenous and endogenous ALV. If a positive test is obtained from using C/O but not C/E CEFs, the sample is positive for endogenous ALV. Positive tests using both C/E and C/O indicates the presence of exogenous ALV. Recently, a flow cytometry method using a highly specific alloantibody termed R2 has been described for detection of endogenous ALV envelope in chicken plasma (11, 14). It should be noted that some tests such as CF and ELISA and possibly non-producer (NP), phenotypic mixing (PM), R(-)Q cell, and FA can be suitable for all leukosis and sarcoma viruses. The resistance-inducing factor (RIF) test can be performed only on ALVs that are not rapidly cytopathogenic. Other tests are specific for certain virus strains. Rapid transformation of fibroblast cultures is produced only by certain RSV and of hematopoietic cell cultures only by defective ALV. The test for adenosine triphosphatase activity is specific for avian myeloblastosis virus. The procedures that are most widely used have been reviewed extensively in the previous edition of this book as they are not covered here (126, 134, 266). These include the RIF test (337), the COFAL test used to detect the group-specific antigens of ALV (123, 266) and tests based on phenotypic mixing of viruses (80, 266, 282, 322).

Tests for Viral-Internal, Group-Specific (gs) Antigens

Detection of the major antigen (p27) forms the basis of several diagnostic tests for virus. Highly sensitive ELISA tests for gs antigens are widely used directly for the assay of test material or indirectly using cell cultures inoculated with test material. These antigens may also be detected in cells by FA techniques (200, 294). Using indirect FA tests, monoclonal antibodies to ALV-J proved useful in the detection of ALV-J infected cell cultures (131, 324, 384, 410). A variety of samples can be tested by ELISA for the presence of ALV; however, serum has been shown to be unsuitable for the detection of exogenous ALV by direct ELISA (303). For the detection of exogenous ALV, samples are inoculated on CEFs that are genetically resistant to subgroup E ALV. Seven to nine days later, cell lysates are tested for the presence of ALV gs antigen by ELISA (123, 134, 364). Rabbit anti-p27 antibody, which is used to coat ELISA plates and rabbit anti-p27 conjugate, as well as complete kits for running ELISA for detection of ALV gs antigen, are available commercially. An indirect ELISA using a recombinant capsid protein has also been reported (326).

Comparison of Tests

In vivo and *in vitro* cell culture tests for detection or assay of exogenous ALVs are compared in Table 15.13.

All the *in vitro* tests require a standard source of chicken embryos free from exogenous ALSVs and of known phenotype for use in cell culture. The following reagents are also required: for the RIF test, stocks of challenge RSV of each subgroup; for the COFAL and ELISA tests, specific antiserum; for the NP test, quantities of NP cells; and for the PM test, stocks of RSV with endogenous helper, RSV(RAV-0). Cells obtained from embryos of unknown genetic origin should not be used in RIF, COFAL, and indirect ELISA tests because the results may be confused by genetic resistance. Both COFAL and indirect ELISA tests require either prolonged maintenance of culture or several subcultures to propagate the virus sufficiently; therefore, much more work is involved than in the NP or PM tests.

The subgroup of an infecting ALV can be determined by any of the tests. In the RIF test, only RSV belonging to the same subgroup as the ALV is subjected to interference. In COFAL and ELISA tests, genetically resistant

Table 15.13 Comparison of methods for assaying exogenous avian leukosis viruses (ALVs).

Method	Requirements	Response measured	Additional requirements for subgroup determination	Time required (days)[c]
In vivo				
Chick inoc 1 day IA	LL susceptible[a]	LL	Genetically resistant chickens	270
Chick inoc 1 day IA	Erythro susceptible[b]	Erythro	Genetically resistant chickens	63
Embryo inoc 11 days IV	Erythro susceptible	Erythro	Genetically resistant chickens	43
Cell culture RIF	RSV pseudotypes, C/E cells	Resistance to formation of RSV foci in CC[d]	Challenge virus of known subgroup	12 + 6
COFAL	Hamster antiserum, C/E cells	Complement fixation	Genetically resistant cells	14 + 1
ELISA	Enzyme-linked antisera C/E cells	Color change of substrate	Genetically resistant cells	14 + 1
NP	NP cells (chicken or quail)	RSV foci in CC	Genetically resistant cells of RIF test with leukosis virus of known subgroup	8+6
PM	RSV (RAV-0), C/O, and C/E cells	RSV foci in CC	Genetically resistant cells of RIF test with leukosis virus of known subgroup	5+6

Note: C/E, cells, genetically resistant to infection with viruses of E subgroup, but susceptible to viruses of other subgroups; C/O, cells phenotypically susceptible to infection by viruses of all subgroups; COFAL, complement fixation for avian leukosis viruses; CC, cell culture; ELISA, enzyme-linked immunosorbent assay; erythro, erythroblastosis; IA, intraabdominal; IV, intravenous; LL, lymphoid leukosis; NP, nonproducer; RIF, resistance-inducing factor; RSV, Rous sarcoma virus; RSV (RAV-0), Rous sarcoma virus with endogenous helper.
[a] Chickens susceptible to LL tumor formation (e.g., line 15I chickens).
[b] Chickens susceptible to virus infection and to development of erythroblastosis (or myeloblastosis).
[c] Approximate number of days necessary to cultivate the virus plus the number of days to indicate the presence of virus.
[d] Cell culture.

cells can be used; thus, an ALV of subgroup A will not produce CF antigens in cells of the C/A phenotype (resistant to subgroup A viruses). In the NP test, genetically resistant NP cells can be prepared, and in the PM test, genetically resistant cells can be used in the mixing phase. In NP and PM tests, supernatant from the activation or mixing phase, which contains RSV of the same subgroup as the ALV, can be placed on genetically resistant cells or embryos or used in an interference test with an ALV of known subgroup.

Immunohistochemical Tests

Direct (200) and indirect (294) FA tests as well as flow cytometry (185, 186) have been used to detect viral antigen in CEF cultures; flow cytometry has also been shown to be a very useful tool in identifying the subgroup of ALV strains contaminating commercial MD vaccines (24, 121, 361).

Enzyme Assays

Avian myeloblastosis virus has on its surface an enzyme (ATPase) that dephosphorylates adenosine triphosphate. This activity can be used as a quantitative assay to determine the amount of virus present in the plasma of infected chickens or in supernatants of myeloblast cultures (31).

Assays for RT activities have been used for the detection of oncogenic RNA viruses including all ALSVs (399). Detection of this enzyme, either directly when the correct template is used (199, 401) or indirectly when the radioimmunoassay is used (292), is an indication of presence of virus. Most recently, a highly sensitive PCR-based RT assay has been used to screen human vaccines that are produced in CEF or embryonated eggs for freedom from avian retroviruses (188, 236, 404, 413).

Detection of Viral Nucleic Acids

The PCR is the most common DNA-based test used for detection and identification of ALV including subgroup E viruses (Figure 15.45). Reverse transcriptase-PCR has also been used to detect several subgroups of ALV (176, 454). A specific PCR for ALV subgroup A can be used to detect proviral DNA and viral RNA in various tissues from ALV-infected chickens (408). Reverse transcriptase-nested PCR (RT-nested PCR) test that amplifies a fragment of the LTR of exogenous ALV subgroups A, B, C, D, and J, but not endogenous retroviral sequences has been described (151). Several primers specific for the

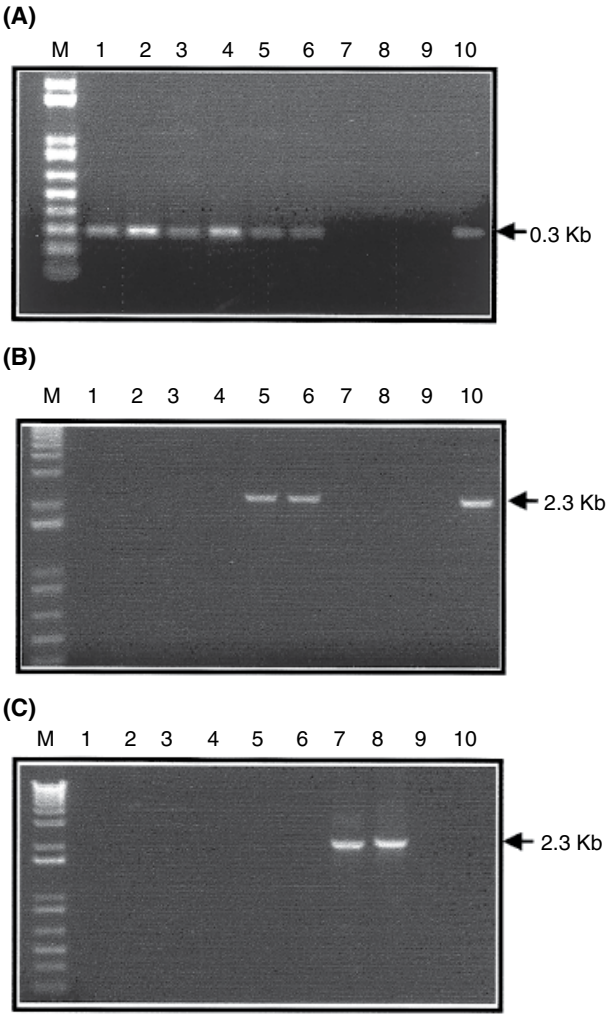


Figure 15.45 Polymerase chain reaction (PCR) analysis of DNA isolated from line 0 CEF uninfected and infected with RAV-1 (ALV-A), RAV-2 (ALV-B), RAV-49 (ALV-C), RAV-50 (ALV-D), ADOL-HC-1 (ALV-J), and ADOL-R5-4 (ALV-J), and 15B1 cells uninfected and infected with RAV-0 (ALV-E) and EV21 (ALV-E). (A). PCR analysis using primers specific for ALV-A-E. (B). PCR analysis using primers specific for ALV-E. (C). PCR analysis using primers specific for ALV-J Lanes: M, 1 kb plus DNA ladder; 1, RAV-1; 2, RAV-2; 3, RAV-49; 4, RAV-50; 5, RAV-0; 6, EV21; 7, ADOL-HC-1; 8, ADOL-R5-4; 9 line 0 CEF; 10, 15B1 CEF (481, 482). (B. Lupiani).

detection of the most commonly isolated ALVs, particularly subgroup A (234), and the new subgroup ALV-J (360, 369, 370) have been developed. Other primers specific for endogenous, subgroup E ALV can also be used to detect cell culture infected with endogenous ALV-E, but not those infected with exogenous ALV of subgroups A, B, C, D, and J (134). Recently, a sensitive and specific multiplex PCR for detecting subgroups ALV-A, ALV-B, and ALV-J has been reported (149). Taqman-based or SYBR Green-based real-time PCR tests for detection of ALV have also been used for diagnosis of ALV (94, 325). Use of a proximity ligation technique combined with PCR was used to develop a novel immune-PCR (Im-PCR) for the detection of ALV (444). As further modification of PCR, loop-mediated isothermal amplification (LAMP) method for ALV subgroups have also been developed (423, 453).

Hematopoietic Transformation

Avian myeloblastosis virus, an acutely transforming strain of ALV, harboring an oncogene, can infect and transform cultures of avian myeloblasts. Assays usually are based on a quantal response in which individual cultures are scored as positive or negative (21, 256). Focus assays for myeloblastosis, erythroblastosis, and other defective ALVs have been developed (164, 165, 258). Cultured chicken bone-marrow cells and blood monocytes are useful in isolation and propagation of acutely transforming viruses recovered from cases of myeloid leukosis induced by strain HPRS-103 ALV-J (302).

Transformation of Fibroblasts and Cytopathology

Avian sarcoma viruses transform spindle-shaped flat CEFs into spherical and refractile foci (323, 400) that can be seen microscopically after 4–5 days (Figure 15.23). Genetically susceptible cultures are inoculated with test material. The next day, medium is decanted and is replaced with an agar overlay (134). Inoculated cultures should be examined daily for RSV-induced foci, which usually develop within 4–7 days PI.

Serology

Plasma, serum, and egg yolk are suitable samples for the detection of antibodies to ALSVs.

Tests

Antibody to ALV can be measured by its reaction with RSV or ALV; a virus of one subgroup will not be neutralized by antibodies provoked by a virus of a different subgroup. Usually, a 1:5 dilution of heat-inactivated (56°C for 30 minutes) serum is mixed with an equal quantity of a standard preparation of RSV of a known pseudotype; after incubation, the residual virus is quantitated by any one of many procedures, the cell culture assay being most commonly used. A microneutralization test to assay for residual virus can be used for detection of ALV antibody (134). The test can be conducted in 96-well microtiter plates, and the neutralization of the virus is determined by an ELISA on culture fluids (16).

An indirect immunoperoxidase absorbance test (250, 251), ELISA tests (252, 367, 405, 407), and flow cytometry (185, 186) have been described for the detection of antibodies. ELISA kits for the detection of antibodies to ALV subgroups A and B are available commercially. Also, molecularly cloned, baculovirus-expressed, envelope glycoproteins of ALV-J now are being used in commercial ELISA kits specific for the detection of antibody to ALV-J (220, 410).

Serotypes

Based on host range, interference spectrum, and viral envelope antigens, viruses of L/S group occurring in chickens are divided into six subgroups A, B, C, D, E, and J. Viruses of different subgroups can be distinguished by the ability of monovalent antiserums to neutralize them. Even though some cross-neutralization usually exists between viruses belonging to the same subgroup, the kinetics of neutralization vary, and slopes of curves for heterologous systems differ from those of homologous systems. No common neutralization antigens are among the viruses of different subgroups, except for a relationship between subgroups B and D. The diagnosis of infection by serologic means requires that representatives of all serotypes be employed. Avian leukosis viruses themselves may be used, but more commonly, RSV pseudotypes are employed in the neutralization tests (134).

Differential Diagnosis

Lymphoid Leukosis

Differential diagnosis of lymphomas in chickens can be difficult. The two most common lymphoid neoplasms, namely MD and LL are particularly confusing (441). Lymphoid tumors observed in REV-infected chickens, although only infrequently in cases of use of REV-contaminated vaccines, or under experimental conditions (see Reticuloendotheliosis), may also add to the confusion. Lymphoid leukosis cannot be differentiated from REV-induced bursal lymphomas on the basis of pathology, immunohistochemistry, and molecular changes in the c-*myc* region. Virologic, serologic, or PCR tests may be helpful in establishing infection for one virus and exclusion for the other. However, such assays are not particularly helpful in the diagnosis of virus-induced lymphomas of chickens including LL, as avian oncogenic viruses are widespread, and infection in the absence of tumor formation is common. Detection of proviral DNA and integration junctions by PCR assays (67, 160, 314) has been shown to be useful for tumor diagnosis.

Because LL tumors should contain ALV proviral DNA sequences inserted near the c-*myc* gene, differentiation between LL and REV-induced bursal lymphomas can be made by southern blots and hybridization analysis of tumor DNA for clonal insertion of ALV (see previous discussion).

Lymphomas in which bursal tumors are lacking or in which the latent period is too short for that of LL can be confused primarily with MD; however, under certain circumstances, REV-induced lymphoma should also be ruled out (see Reticuloendotheliosis). In cases in which bursal tumors are lacking, LL and MD can be differentiated only with difficulty, because similar lymphoid tumors may occur in both diseases in the same visceral organs during the same age period. Visceral lesions of these two diseases cannot be distinguished by gross examination. Diagnosis is possible in most instances on careful microscopic examination; however, considerable experience is necessary. In coming to a decision, history, signs, gross and microscopic lesions, and cytology should all be considered. Ordinarily, LL does not occur before 14 weeks of age, and most of the mortality occurs between 24 and 40 weeks. Marek's disease, however, may occur as early as 4 weeks, and the mortality peak varies from 10–20 weeks. Occasionally, losses continue and may reach a peak after 20 weeks.

Nodular tumors of the bursa can often be palpated through the cloaca in birds infected with ALV. Paralysis associated with gross lesions in autonomic and peripheral nervous systems and gross lesions of the iris ("gray eye") are specific for MD.

As stated previously, the bursa of Fabricius plays a central role in development of LL. When distinct focal or nodular lymphoid tumors are present in the bursa, a diagnosis of LL can be made; however, REV-induced bursal lymphomas must be ruled out. Such tumors are sometimes quite small and may be overlooked. In some birds, MD induces a premature atrophy of the bursa. In others, the bursa may be tumorous, in which case the walls and the plica may be thickened from interfollicular infiltration with pleomorphic lymphocytes. In contrast, intrafollicular tumors of the bursa consisting of uniform large lymphocytes are usual with LL.

Microscopic lymphoid infiltration in nerves, cuffing around small arterioles in the white matter of the cerebellum, and the feather follicular pattern of lymphoid cell infiltration in the skin, which are characteristic of MD, are not seen with LL.

Cytologically, LL tumors generally are composed of a homogeneous population of lymphoblasts (Figure 15.27). In contrast, tumors of MD usually contain lymphoid cells varying in size and maturity from lymphoblasts to small lymphocytes, and plasma cells may also be present. Special stains such as methyl green pyronin are helpful for cytology. Immature lymphoblasts characteristic of LL tumors are highly pyroninophilic; whereas the medium and small lymphocytes that predominate in tumors of MD do not stain with pyronin.

Lymphoid leukosis tumors are composed almost entirely of B cells and have surface IgM markers; whereas 60–90% of MD tumor cells are T cells that lack IgM markers and only about 3–25% are B cells. In addition, from 0.5–35% of MD tumor cells have a tumor-associated cell surface antigen (MATSA), which is absent from LL tumor cells (113, 277, 278, 318). Recently, Witter et al. (441) introduced a diagnostic strategy for the differential diagnosis of viral lymphomas in chickens.

Other diseases that may be confused with LL are erythroblastosis, myeloblastosis, myelocytomatosis, pullorum disease, tuberculosis, enterohepatitis, Hjarre's disease, and fatty degeneration of the liver.

Erythroblastosis

Although gross lesions of liver, spleen, and bone marrow provide the basis for a presumptive diagnosis, a firm diagnosis must be based on finding large numbers of erythroblasts by microscopic examination of a blood smear and sections or smears of liver and bone marrow. Chickens in early stages of disease or without obvious signs may be missed easily unless microscopic examination is made.

Erythroblastosis with concurrent anemia is often difficult to differentiate from anemia resulting from nonneoplastic causes. In erythroblastosis, there is usually a defect in maturation of erythroblasts, resulting in the presence of large numbers of them and very few polychrome erythrocytes. In anemia, the reverse usually occurs. Extramedullary erythropoiesis and stasis of erythroblasts in the sinusoids are usually more prominent in erythroblastosis than in anemia.

Erythroblastosis can be distinguished from myeloblastosis on the following grounds. In myeloblastosis, the liver is usually pale red and the marrow is whitish; whereas in erythroblastosis, the liver and marrow are usually cherry red (Figures 15.30B and 15.30C). In myeloblastosis, the cells accumulate intravascularly and extravascularly, whereas in erythroblastosis they are always intravascular. The erythroblast and myeloblast may be difficult to distinguish. Erythroblasts have a basophilic cytoplasm and perinuclear halo; myeloblasts often have some granules (Figures 15.30D and 15.30E).

Erythroblasts are cells of the erythropoietic system and can be differentiated from cells of the myelopoietic system on the basis of the presence of certain markers. Thus, erythroblasts have erythroid markers including hemoglobin, chicken erythrocyte-specific histone H5, and chicken erythrocyte-specific cell surface antigens detected by immunofluorescence. Myeloblasts and myelocytes have myeloid markers including adherence and phagocytic capacity, Fc receptors as determined by rosette formation, macrophage- and granulocyte-specific cell surface antigen as detected by immunofluorescence, and dependence of colony formation on colony-stimulating factor (165, 259).

Erythroblastosis can be distinguished from LL by the nature and distribution of lesions. Microscopically, the cytoplasm of lymphoblasts is somewhat less basophilic than that of erythroblasts, and there is also a larger nuclear<thin:cytoplasmic ratio than in the latter cells. Lymphoblasts are more variable in size and shape than erythroblasts, but they are all at the same primitive developmental stage. Lymphoblasts tend to have an ovoid rather than spherical nucleus and a finer, more delicate-looking chromatin network. Myelocytomas and erythroblastosis can be distinguished histologically.

Myeloblastosis

As in erythroblastosis, a tentative diagnosis may be based on gross lesions; however, these are often so similar to those of LL that specific diagnosis cannot be made without examination of a blood smear. Examination of liver or bone marrow sections is helpful when identity of the cell type is in doubt. The myeloblast is, on the average, smaller than the erythroblast or lymphoblast; its cytoplasm is more acidophilic and is polygonal or angular. The nucleus is less vesicular; the nucleolus, while present, is not nearly so frequently seen or conspicuous as in the other two leukoses. Myeloblasts also have physiologic markers that identify them as members of the myeloid series.

Myelocytomatosis

The distinctive character and location of tumor (see the previous discussion) provide the basis for diagnosis, which can be verified by examination of a stained smear or tumor section. Gross tumors must be differentiated from myeloblastosis, LL, osteopetrosis, and necrotic and/or purulent processes occurring in tuberculosis, pullorum disease, and mycotic infections. In recent outbreaks of ALV-J-induced tumors, myelocytomatosis was diagnosed primarily on the basis of presence of characteristic microscopic feature of tumor cells (see 266).

Hemangioma

Hemangiomas on the skin should be differentiated from wounds, bleeding feather follicles, and cannibalism. Those in the visceral organs should be differentiated from hemorrhages and sarcomas.

Renal Tumors

Renal tumors should be suspected when tumor nodules or large masses are found only in the kidney or are encountered suspended from the lumbar region. Diagnosis can be verified by microscopic examination. Tumors should be differentiated from other causes of kidney enlargement including hematomata, LL, and accumulation of urates.

Osteopetrosis

Bone lesions of advanced cases are sufficiently distinctive to present no difficulty in diagnosis. Cross- and longitudinal-sectioning of long bones is helpful in detecting slight exostoses and endostoses, particularly in early stages.

Intervention Strategies

Vaccination

No commercial vaccine is available for the protection of chickens from infection with ALV. However, the idea of using vaccines to increase host resistance to ALV infection is very attractive (347). In a series of attempts to

inactivate ALV by various means, however, Burmester (47) demonstrated that ability of these virus preparations to induce antibody was destroyed almost concurrently with inactivation. Attempts to produce attenuated strains of ALV that do not induce disease have also failed (283). Results of experimental vaccination with live ALV on shedding and congenital transmission of the virus are equivocal. Some success has been obtained in attempts to increase the resistance of the host to RSV by immunization with viral or cellular antigens (33, 295). Recombinant ALV-J gp85 protein vaccine with either a liposomal, a cytosine–phosphate–guanine oligodeoxynucleotide (CpG-ODN), or silica nanoparticles adjuvant provided partial protection and elicited high antibody titers (65, 110, 449, 452). Recombinant ALVs expressing subgroup A (60, 125, 243, 442) and ALV-J (220, 410) envelope glycoproteins have been produced that could have potential as vaccines to protect against horizontal transmission. It is worth noting that congenitally infected chicks are immunologically tolerant and, thus, cannot be immunized even if a suitable vaccine was available. These chickens constitute the major source of ALV transmission and are the most likely to develop neoplasms.

Treatment

No practical measures have been found for treatment of the various forms of the avian leukosis complex. In general, all attempts to treat virus-induced neoplasia have resulted in negative or non-reproducible results. RNA interference (RNAi)-based methods of inhibiting ALV replication have been demonstrated experimentally (62, 247) although its value in future treatment cannot be predicted. Recombinant chicken interferon-alpha can inhibit ALV replication in DF-1 cells and could be useful for antiviral approaches (95).

Prevention and Control Procedures

Eradication

Eradication of ALV from primary breeding stocks is the most effective means for controlling ALV infection in chickens. Primary breeding companies of layer-type and meat-type stock have made significant progress in reducing or eradicating ALV of subgroups A, B, and J from their elite breeding lines (306).

Programs for eradication of ALV infection depends on breaking the vertical transmission of virus from dam to progeny. Breeder hens are tested by various methods for the presence of ALV, and those that test positive are discarded. In order to establish an ALV-free flock, it is necessary to hatch, rear, and maintain in isolation a group of chickens free from congenital infection. To achieve this, embryos must be obtained from dams that are not transmitting virus to their progeny. In earlier work on development of ALV-free flocks, several methods for selecting dams were used. The dams selected to produce the next generation and hoped to be a virus-free generation were:

1) immune, non-virus shedders. Hens with antibody were selected on the assumption that they were less likely to shed virus than hens without antibody. Chicks were hatched from those that did not transmit virus to their embryos, based on tests on at least three embryos per hen.
2) Nonimmune, nonvirus shedders. Hens without antibody were selected on the assumption that they never been infected and were less likely to become intermittent shedders.
3) Nonviremic hens regardless of immune status. These were used to provide replacements; however, up to four generations of testing were needed to establish freedom, and even then infection could not be ruled out (446).

Application of eradication programs of ALV to commercial flocks has depended on associations between virus infections in hens, eggs, embryos, and chicks (375): (1) egg albumen may contain exogenous ALV and gs antigen, and both are usually present together; (2) a strong association exists between ALV or gs antigen in egg albumen and ALV in vaginal swabs; (3) an association exists between ALV in vaginal swabs or egg albumen and ALV in chicken embryos and newly hatched chicks. Consequently, hens with a low probability of producing infected embryos are hens negative for virus (or gs antigen) by the vaginal swab test, or hens that produce eggs with albumen free from virus or gs antigen. Commonly, virus in vaginal or cloacal swabs may be detected by ELISA, NP, or PM tests and in egg albumen by ELISA or direct COFAL tests. It is unlikely that a single test will detect all potential shedder hens. A problem that arises in applying the ELISA test to albumen or swabs is the need to differentiate positive reactions due to the presence of gs antigen derived from endogenous ALV or loci from the reactions due to the presence of exogenous ALV infection. Reactions due to the latter are usually markedly higher, but the setting of the boundary between endogenous and exogenous virus infections is sometimes difficult and somewhat arbitrary. High reactions due to exogenous virus are clearer with albumen samples than with swabs (89).

A procedure for eradication of ALV involves: (1) selection of fertile eggs from hens negative in the egg albumen or vaginal swab test (reviewed in 266); (2) hatching of chicks in isolation in small groups (25–50) in wire-floored cages, avoidance of manual vent sexing and vaccination with a common needle to prevent mechanical spread of any residual infection; (3) testing of chicks for ALV by a biologic assay or PCR on blood, discarding

reactors and contact chicks; and (4) rearing ALV-free groups in isolation (130, 440). In practice, selection of hens with a low shedding rate is a simpler requirement to fulfil than the subsequent chick testing and isolation rearing needed to achieve complete eradication. Consequently, some commercial breeder organizations are concentrating only on reduction of infection rate by hen testing. Small group hatching and rearing procedures allowed identification and removal of groups containing chickens infected prior to hatching and prevented horizontal transmission of ALV-A in egg-type chickens and ALV-J in meat-type chickens (440).

Chicks are most susceptible to contact infection by ALVs during the period immediately after hatching. Although congenitally infected hatchmates are likely to be the main source of such infection, several procedures can reduce or eliminate infection remaining from previous populations. Incubators, hatchers, brooding houses, and all equipment should be thoroughly cleaned and disinfected between each use. Chick boxes should not be reused, and each farm ideally should have only one age group of chickens. Demonstration of natural infection and transmission of MAV-1 in egg-type chickens stress the importance of testing of birds to prevent introduction of infection (447). The danger of introducing strains of virus not already present in the population can be eliminated if eggs or chicks from different sources are not mixed, and if chicks are reared under isolation, conditions that will prevent cross-contamination of flocks.

Selection for Genetic Resistance

The frequencies of the alleles that encode cellular susceptibility and resistance to infection by exogenous ALSVs (see Genetic Resistance) vary greatly among commercial lines of chickens (84, 260). In some lines, high frequencies of a resistant allele may be found naturally. In others, frequencies of the resistant alleles can be increased by artificial selection.

In artificial selection, genotypes of unknown parents may be determined in a progeny test by mating them to recessive tester birds of the subgroup in question (e.g., $a^r a^r$ for A subgroup virus) (293). Depending on the segregation of susceptible and resistant progeny in a particular mating, the genotype of the unknown parent may be determined. The phenotypic identification of progeny in the test may be determined by inoculation of RSV onto the CAM, the embryo being scored as susceptible or resistant on the basis of pock count (86) or intracranial inoculation of RSV into hatched chicks, chicks being scored on the basis of death or survival. The former method is preferable and has many advantages.

There are concerns whether genetic selection approach could cause problems with generation of mutant viruses that may overcome the restriction imposed by this selection (78). However, this type of resistance is poorly defined but may be controlled by a number of genes and is, consequently, more difficult to overcome by viral mutation.

Recent technological advances in genome editing have already demonstrated the feasibility of this approach in generating cell lines resistant to infections with different ALV subgroups (217, 218). Success in the application of new methodologies of avian transgenesis using genetically modified primordial germ cells (174, 271) point toward the feasibility of using these approaches for experimental generation of resistant stock as another tool for the control of avian retroviral diseases in poultry.

Reticuloendotheliosis

Guillermo Zavala and Venugopal Nair

Summary

Agent and Disease. Reticuloendotheliosis (RE) represents a group of syndromes associated with a common, but not ubiquitous retrovirus designated reticuloendotheliosis virus (REV). The syndromes include chronic lymphoid neoplasia, runting disease syndrome or acute reticulum cell neoplasia. Reticuloendotheliosis virus also poses significant danger as a contaminating pathogen in avian vaccines.

Diagnosis. Clinical diagnosis of the disease is challenging because of the difficulty in differentiating from other avian neoplastic diseases. Hence virological, molecular, and serological diagnostic tests are needed for confirmation.

Intervention. Eradication of the pathogen by testing and eliminating infected birds is the best intervention strategy. However, vaccination has very little value, except perhaps in endangered avian species.

Introduction

Definition and Synonyms

Reticuloendotheliosis (RE) designates a group of syndromes associated with chronic and acute neoplasia, immunosuppression, runting disease, and acute death in several avian species caused by retroviruses of the reticuloendotheliosis virus (REV) group. Clinical disease is infrequent but infection appears to be widespread.

The REV group includes the laboratory strain T (REV-T), chick syncytial virus (52), duck infectious anemia virus (140), and spleen necrosis virus (230). Many other nondefective strains have been isolated from avian species including turkeys, chickens, ducks, pheasants, partridges, geese, prairie chickens, sparrows, and pigeons (87, 166, 231, 271). Nondefective strains associated with runting disease and chronic neoplasia belong to a single serotype, but three antigenic subtypes have been identified (46). The acute reticulum cell neoplasia is induced only by the laboratory-derived defective strain T (REV-T) not known to occur in nature. REV-T carries a unique oncogene of cellular origin (v-rel) that is responsible for its acute oncogenicity (101, 102). Stocks of REV-T also contain a nondefective helper REV designated as REV-A that replicates in chicken fibroblasts but lacks acute oncogenic properties (101).

Economic Significance

Severe runting syndrome, feathering abnormalities, chronic neoplasia, or immunosuppression have occurred when REV-contaminated vaccines were administered to embryos or very young chickens (85, 112, 127–129, 267). Clinical disease from natural infection is relatively rare, but exports of seropositive breeding stock may be prohibited. Vaccine and specific pathogen free companies must routinely monitor their products for REV contamination. Significant immunosuppression may result from environmental exposure, administration of contaminated vaccines, or congenital transmission (250, 267).

Public Health Significance

The extended host range of REVs, which includes certain mammalian cells (2, 171, 243) and other characteristics of REV suggesting an evolutionary linkage with mammalian retroviruses (15, 126, 171, 189), raised the possibility of human infection (113). Reticuloendotheliosis virus antibodies have been detected in human sera (49, 114, 115), but such findings have been considered insufficient to warrant concern (68, 70, 71, 94, 200).

Scientific Significance

Reticuloendotheliosis virus has received considerable attention as an oncogenic and immunosuppressive virus with a wide host range (171). Reticuloendotheliosis virus can infect or transform various cell types (2, 15) and has been used in comparative retrovirology models. Some REV subgenomic sequences display high similarity to those of some mammalian retroviruses (17, 171). Reticuloendotheliosis virus can integrate into the genome of cells and of large DNA viruses, including Marek's disease (MD) and fowl pox viruses (100, 110). Recent historical, phylogenetic, and paleovirological evidence suggest the origin of REV as a mammalian retrovirus that was iatrogenically introduced into the avian hosts which subsequently spread through herpesviruses and poxviruses (77, 171).

History

The the defective strain T (REV-T), was obtained in 1957 from turkey visceral lymphomas (191). REV-T is acutely oncogenic, causing death of young chicks 6–21 days postinoculation (207). Theilen et al. confirmed the acute oncogenicity of REV-T for young chickens, turkeys, and Japanese quail; and designated the disease as reticuloendotheliosis on the basis of the prominent cell type in tumors (227), now termed acute reticulum cell neoplasia. Importantly, Theilen et al. (227) propagated the strain in cell culture, determined it to be a retrovirus unrelated to avian leukosis virus, and named it "reticuloendotheliosis" virus (strain T).

Purchase (182, 183) recognized the antigenic relationships between REV-T, the nononcogenic chick syncytial spleen necrosis and duck infectious anemia viruses, all within the reticuloendotheliosis virus group. Thus, nomenclature for the disease and the virus originated from the atypical pathology induced by REV-T and was extended to all viruses in the group.

Additional reviews on the history of REV are available for consultation (147, 166, 171, 177, 183, 249).

Etiology

Classification

Reticuloendotheliosis viruses are retroviruses that are antigenically, morphologically, and structurally distinct from the leukosis/sarcoma (L/S) group of avian retroviruses (ALSV) (183). Lack of nucleic acid sequence homology between REV and members of the ALSV group has been long recognized (118). The International Committee on Taxonomy of Viruses (ICTV 9th Report, 2011) has classified REVs within the family Retroviridae, subfamily Orthoretrovirinae, genus *Gammaretrovirus*, with no endogenous counterpart. Reticuloendotheliosis virus is phylogenetically related to mammalian C-type retroviruses based on virion morphology, nucleic acid and major polypeptide amino acid sequences (171), as well as immunologic determinants and receptor interference patterns (122).

Morphology

Viral particles are typical of retroviruses, with about 100 nm in diameter (270); display surface projections

about 6 nm long and 10 nm in diameter (116). Virions have a density of 1.16–1.18 g/mL in sucrose density gradients (23), and can be differentiated from avian L/S viruses by morphology in thin sections (153, 272). The morphology of the viral particles is shown in Figure 15.46.

Chemical Composition

Nucleic Acid

The genomic single-stranded, positive sense RNA consists of a 60–70 S complex containing two 30–40 S RNA subunits, each having a size of about 3.9×10^6 d (25, 142). The nondefective REV has a genome of about 9.0 kb (17), while the replication-defective REV-T genome is only about 5.7 kb due principally to a large deletion in the gag-pol region and a smaller deletion in the env region (50). Moreover, the REV-T genome contains a substitution of 0.8–1.5 kb in the env region that represents the transforming gene v-*rel* (44, 51, 261), which is not present in nondefective REVs or other avian or mammalian retroviruses. Related sequences (c-*rel* proto-oncogene) are present in the DNA of normal avian cells, including turkey cells from where the oncogene was most likely transduced (44, 45, 247, 248). No endogenous REV sequences in host DNA have been recognized. The long terminal repeats (LTRs), 569 base pairs (bp) in length (17) are efficient promoters in a variety of cell types (14, 17, 138). Several complete genome sequences of various field and vaccine-contaminant isolates from China and the United States have been resolved (14, 17, 138).

Oncogene

The v-*rel* oncogene is transcribed in REV-T-transformed lymphoid cells and produces a phosphoprotein product identified as $pp59^{v\text{-}rel}$. The v-rel protein is a member of the rel/dorsal family of proteins, related to nuclear factor kappa B, which function as DNA-binding transcription factors (26, 194). It differs from the c-rel protein both in structure and transforming ability and, unlike most other oncogene products, can be detected in both the cytoplasm and nucleus of transformed cells (29). The v-rel protein is usually complexed with cellular proteins (124, 136, 216) and is responsible for the acute oncogenicity of REV-T (29). REV-T-induced transformation is associated with several changes in gene expression, including induction of miRNAs (264). However, REV isolates other than REV-T have induced neoplastic disease within very short latent periods (18, 72, 73, 184, 186).

Proteins

Reticuloendotheliosis virus genes encode various structural proteins, a protease, a polymerase and an integrase (17). The protein encoded by the v-rel gene is only present in the defective REV-T. The RNA-dependent DNA polymerase (reverse transcriptase) differs structurally and immunologically from comparable enzymes of leukosis/sarcoma viruses (22, 153). The preference of the REV polymerase for Mn^{2+} ions is a differentiating factor from enzymes of other avian retroviruses (153, 203, 263).

The envelope protein is composed of two peptides, the gp90 surface unit (SU) and the gp20 transmembrane unit (TU) (233, 234). These glycoproteins are located on the surface of the virions (156). The C-terminal epitope of gp90 locates on the surface of infected cells (234).

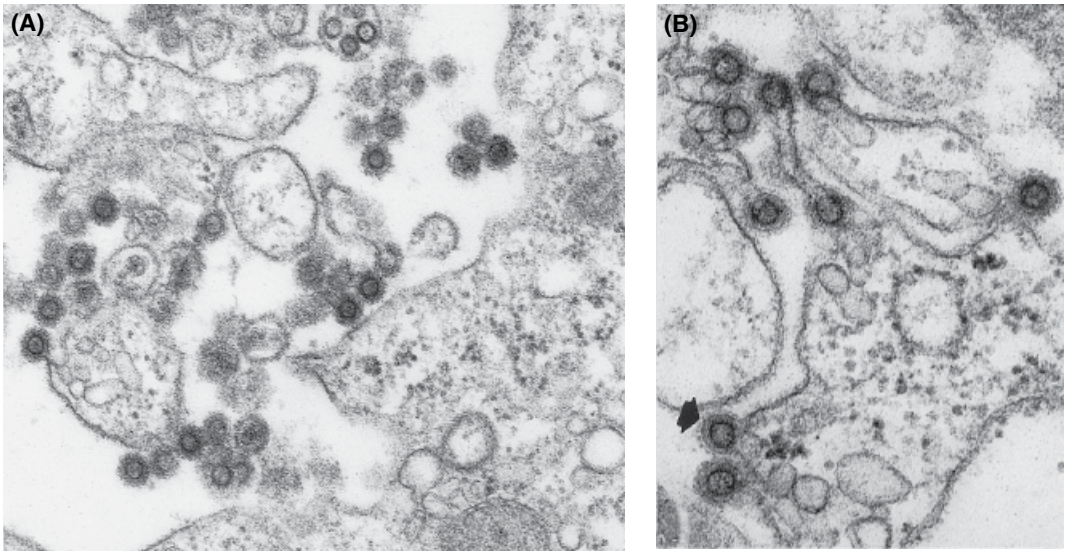


Figure 15.46 Electron micrographs of thin sections of chicken embryo fibroblasts infected with reticuloendotheliosis virus (REV). (A) Typical virus particles in the extracellular spaces ×40,000. (B) REV particles budding from the plasma membrane of infected cells (arrow). ×60,000. (Nazerian).

The gp90 protein is considered the immunodominant protein of the virus (64). The receptor binding regions have been mapped and display structural differences relative to other retroviruses (144).

There are five gag gene-encoded structural proteins, p12, pp18, pp20, p30, and p10 (232). Antiserum to the 30 kDa (p30) protein cross-reacted with p30 of several other REVs, thus establishing this protein as a group-specific antigen (143) that also plays a role in viral particle assembly and encapsidation (244).

Replication

Non-Defective Strains

In vitro virus replication is similar to that of other retroviruses (70). The virus envelope glycoprotein binds to an unidentified cell surface receptor, resulting in interference with superinfection (86). Viral entry, RNA reverse transcription and proviral DNA integration into the cellular chromosome proceed through mechanisms typical of simple retroviruses. Viral RNA transcription and translation are initiated through promoter and enhancer sequences in the LTR. Two polyproteins are encoded, gag-pro-pol and env; the gag precursor protein is myristylated. The encapsidation sequence is located in the *gag* gene. The final stage is budding of viral particles from the cell membrane. Virus particle production is first noted at 24 hours (116), and peaks 2–4 (101) days after infection in chicken cells (30, 90, 223, 224).

Defective Strain

The defective REV-T requires a nondefective RE helper virus for replication (101). Oncogenicity of this strain is maintained during passage *in vivo* (191) or during culture of infected hematopoietic cells (101), but is rapidly lost during passage in fibroblast (227, 257) and dog thymus cell cultures (2), possibly due to the loss of the replication-defective, acutely oncogenic REV after serial passages (31).

Cytopathology

Replication of REV in avian fibroblasts may induce subtle cytopathic changes (227), such as syncytia (52) but degenerative changes are more commonly seen (223, 225). Accumulation of unintegrated viral linear DNA in infected cells possibly causes cell death. Cells that are able to prevent early superinfection have few copies of unintegrated viral DNA and survive (225).

The acute cell death phase (Figures 15.47A and 15.47B) lasts 2–10 days postinfection and is followed by a chronic infection state without cytopathology but with continued virus production (Figure 15.47C) (223, 224). This cytopathology is the basis of plaque assays (35, 47, 48, 155, 223), but the methods have not been widely used due to inconsistency in cytopathology.

Host Range

Cell cultures from many avian species and certain mammalian cells are susceptible to infection and at least limited viral replication. Nondefective REV has been grown in D17 dog sarcoma cells (15, 242, 243), Cf2th dog thymus cells (2, 209), normal rat kidney cells (121), mink lung cells (2), and other mammalian cells. Rat and mouse cells were only semi-permissive for replication of REV (75, 76). Chimeric vector particles containing the REV-A matrix protein infected mammalian cells more efficiently than those containing the matrix protein of spleen necrosis virus (43). A wide range of avian species support REV replication *in vivo* but there is little evidence for *in vivo* replication of REV in non-avian species. Presence of REV antibodies in humans has been reported (114) but the significance is unknown at best (200). Although REV-A-based vectors could infect human cells (125), REVs did not infect human cells due to inability to bind to specific receptor (94).

Pseudotypes

The envelope of nondefective REV forms pseudotypes with Rous sarcoma virus (198, 235) and with vesicular stomatitis virus (117). Pseudotypes can be neutralized by REV antiserum, and thus this assay was once used for antibody detection (56).

Insertional Mutagenesis

Replication of REV requires integration of proviral DNA into the host cell genome. REV proviral DNA can also integrate into the genomes of high molecular weight DNA viruses including MD virus (111) and fowl poxvirus (92, 100). Insertions occur both *in vitro* and *in vivo* (60) and may result from coinfections of REV and a recipient DNA virus. Most insertions consist of a solitary, partially-deleted LTR (60, 154). However, full-length, infectious REV genomes have been detected in turkey herpesvirus (110). A nearly full-length, infectious REV provirus has been detected (92, 100, 123, 210) in certain strains of fowl poxvirus, even in fowl poxvirus stocks lyophilized for over 50 years (123). Such insertions could alter the biological properties of the recipient organism and also represent a distinct mechanism of infection.

Strain Classification

The antigenic properties of different REV isolates are remarkably uniform (33, 182) and, except for defective REV-T, REV isolates have similar genetic, structural, and chemical properties (22, 119). Although REVs belong to a single serotype (46), three subtypes were identified on the basis of neutralization tests and differential reactivity with monoclonal antibodies (46, 57), although subtypes 1 and 2 could not be differentiated by receptor interference (86), confirming the absence of major differences between subtypes. Reticuloendotheliosis virus isolates

differ also in certain biologic properties, including pathogenicity (183) and replication *in vivo*.

Laboratory Host Systems

Cell Cultures

Fibroblasts from several avian species and certain cell lines, such as QT35 quail sarcoma cells (48, 54) and D17 dog osteosarcoma cells (15, 243), are susceptible to infection with nondefective REVs. In infected cultures, antigens (Figures 15.47B and 15.47C), virus particles, proviral DNA, cytopathology, and reverse transcriptase may be detected and serve as criteria for virus assays. A quantitative fluorescent focus assay has been developed for infected cultured cells overlaid with agar (182). Duck embryo fibroblasts are preferred for demonstration of cytopathic effects (13). However, virus cultures in chicken embryo fibroblasts or DF-1 cells combined with molecular detection of REV or identification of viral proteins by direct or indirect immunofluorescence appear to be most practical and efficient (17, 18).

Embryos and Birds

Laboratory host systems for REV that are now seldom used include chicken embryos (208) and a variety of avian species including young chickens, Japanese quail, ducks, geese, turkeys, pheasants, and guinea fowl (18, 186, 227).

Cell Lines

Hematopoietic cells transformed *in vivo* or *in vitro* by REV-T have been developed into continuous cell lines; the cell types and surface markers vary based on the strain of helper virus and on whether transformation occurred *in vivo* or *in vitro* (29, 105). A line of transformed chicken embryo fibroblasts has also been developed (89). Cell lines have also been derived from chronic lymphomas induced by nondefective REV strains (168, 184). Cell lines induced by *in vitro* transformation of spleen cells with defective REV are useful expression systems for transfected foreign genes (181, 202) or as substrates for the propagation of other viruses (187). Some of these transformed cell lines produce growth factors or cytokines (69, 93, 95).

Pathobiology and Epidemiology

Incidence and Distribution

Reticuloendotheliosis virus infection is common but not ubiquitous in turkeys, ducks, and chickens. The prevalence of seropositive flocks and of seropositive birds within an infected flock both increase with the age of the flock. A high prevalence of virus or antibodies has been detected in the United States, Japan, Korea, and Egypt (4, 7, 13, 206, 237, 262). Seropositive commercial chicken

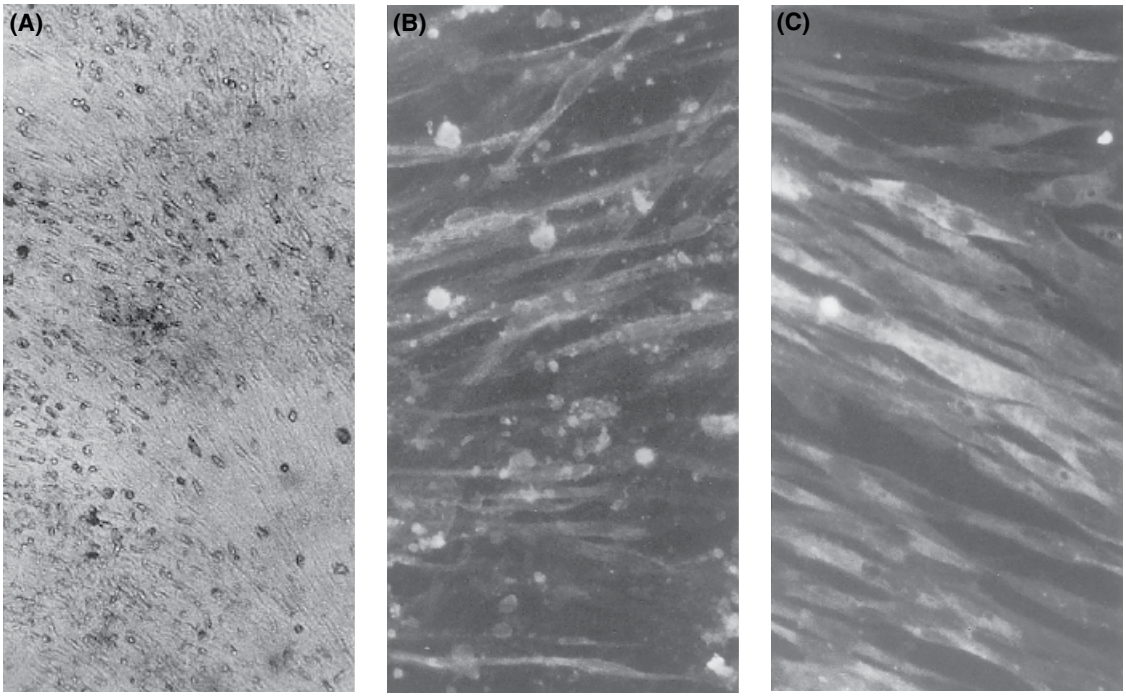


Figure 15.47 Acute (cytopathic) and chronic (noncytopathic) infection of chicken embryo fibroblasts inoculated with nondefective reticuloendotheliosis virus (REV) strain. (A) Mild cytopathic changes 13 days after infection. Unstained, ×55. (B) Cytopathic changes and viral antigens 13 days after infection demonstrated by indirect immunofluorescent staining. (C) Chronically infected cultures 48 days after infection showing relatively normal-appearing cells, most of which contain cytoplasmic viral antigens. ×360.

flocks are still common in the United States, with sporadic to negligible clinical disease. Reticuloendotheliosis virus infection is a significant clinical problem in wild endangered avian species (17, 74, 87, 268). Reticuloendotheliosis virus is frequently detected alone or in combination with fowl poxvirus or lymphoproliferative disease virus in wild turkeys of the United States (1, 3).

Runting disease syndrome and chronic neoplasia have occurred following vaccination with REV-contaminated vaccines (13, 85, 112, 120, 185, 265, 266). Immunosuppressive disease has been identified after natural infection in Korea (206). Field cases of RE-related lymphomas in turkeys have been described in the United States (55, 175, 215, 252, 258, 259), England (147), and Israel (107). Losses from mortality and condemnation at slaughter in affected flocks could be as high as 16–20% (146, 175). Lymphomas associated with natural REV infection are sometimes reported in wild turkeys (99, 132) and less often in chickens (61, 107, 150, 173). Chronic REV-induced neoplasia has also been occasionally observed in ducks (97, 176), quail (39, 201), pheasants (73), geese (72), peafowl (152), and prairie chickens (74, 268).

Natural and Experimental Hosts

Natural hosts for REV infection include turkeys, chickens, ducks, geese, pheasants, Japanese quail, peafowl, and prairie chickens. Experimental hosts include most of the above species, as well as guinea fowl, chickens, turkeys, and Japanese quail (18).

Transmission, Vectors, Carriers

Horizontal Transmission

Experimentally, REV can be transmitted by close contact with infected chickens, turkeys, and ducks (130, 158, 174). Horizontal transmission may be influenced by the host species (182, 254) and the virus strain (254, 266). Reticuloendotheliosis virus transmission was not detected when chickens were separated by wire mesh (12).

Many flocks become infected at older ages (254) presumably via contaminated environment, insects, and other biological reservoirs. Reticuloendotheliosis virus has been detected in feces and cloacal swabs (10, 179, 260, 266), body fluids (12), and litter (241). Horizontal transmission was limited amongst experimentally infected Japanese quail housed on litter (18). Flocks infected experimentally (Zavala, unpublished) or naturally at later ages seroconvert, making virus detection difficult (255). Furthermore, REVs are quickly degraded outside the host at ambient temperatures (37).

Insect transmission represents another form of horizontal spread. Virus could be recovered from *Triatoma infestans* and *Ornithodoros moubata* after feeding on infected chickens (62, 228, 229) and for up to five hours post-feeding on contaminated blood in some species of mosquitoes (62). Reticuloendotheliosis virus has also been isolated from mosquitoes (*Culex annulirostris*) (164) in contact with viremic chickens, demonstrating the possibility of mechanical transmission by insects, which may explain seasonal seroconversion (63, 164) and a higher prevalence of infection in Southern states (254, 256). Fowl poxvirus, which is also transmitted by mosquitoes, may function as a vector containing infectious clones of REV (92, 100, 211).

Vertical Transmission

Chickens, turkeys, ducks, and quail with persistent viremia may transmit infectious REV to progeny, although usually at low frequency or not at all (10, 12, 18, 148, 236, 260). However, some studies have reported experimental vertical transmission to over 50% of chicks from infected dams (163). Albumen samples from tolerant hens frequently contained RE viral gs antigen, although infectious virus was rarely isolated (260). Vertical transmission may occur at higher rates in ducks, since virus was isolated from 87% of embryos derived from tolerant females (158). Individual antibody-positive, virus-positive turkey hens may still transmit virus to progeny at a high rate (258).

Semen from tolerant turkeys contains infectious virus (149, 257). Reticuloendotheliosis virus-free turkey hens inseminated with contaminated semen have produced infected progeny (148). There is evidence of limited vertical transmission after mating infected males with noninfected female chickens (195). Genetic transmission is unlikely based on lack of clonal insertions of proviral DNA in infected hens or their progeny (258). Iatrogenic infection is possible by using accidentally contaminated needles or vaccines for embryos or very young chickens (13).

Contaminated Biological Materials

Accidental use of REV-contaminated fowl pox (85, 138) or MD (112, 120, 134, 135, 138, 245, 266) vaccines has been documented. Reticuloendotheliosis virus has also been detected in contaminated vaccines against Newcastle disease and infectious bronchitis (245). Certain stocks of avian myeloblastosis virus, for many years distributed as a source of reverse transcriptase for biochemical purposes, contained a low level of REV (256). Quality control procedures to exclude REV from licensed poultry biologics have not always been uniformly effective for detection of REV contamination in vaccines such as fowl pox (78, 84). Subgenomic REV sequences continue to be found in some (67, 92, 100, 210), but not all (154), commercially produced fowl pox virus vaccines. Reticuloendotheliosis virus has also been

detected in stocks of *Plasmodium lophurae* (140, 230), further illustrating the diversity of possible transmission mechanisms for REV.

Incubation Period

The runting disease syndrome represents a non-neoplastic disease process with an outcome depending on virus strain and other factors. Atrophic changes in the bursa and thymus can be seen as early as three days postinfection (259). Persistent weight depression in infected chicks can be detected as early as six days of age (159). By the second week postinoculation, chickens developed microscopic nerve lesions (257) and had depressed immune responses (255). Japanese quail infected experimentally displayed severe weight depression as early as 14 days of age and lymphomas were detected as early as 35 days of age (16).

Chronic neoplastic responses occur after moderate or long incubation periods. Chickens developed bursal-derived B-cell lymphomas 17–43 weeks after inoculation (260). Reticuloendotheliosis virus-associated lymphomas in turkeys occurred between 15 and 20 weeks of age in some trials (146, 175) and as early as 9 weeks post-infection in SPF turkeys (Zavala, unpublished). In transmission studies, lymphomas were induced after 8–11 weeks (174) or 11–12 weeks (146). Chronic lymphomas occur between 20 and 30 weeks in the domestic goose (72), and at 4–24 weeks in ducks (97, 176, 178). Experimental inoculation of newly hatched ducks induced lymphomas and other neoplasms between 8 and 30 weeks (133, 158).

For acute reticulum cell neoplasia, the incubation period can be as short as 3 days, but death occurs more commonly 6–21 days after inoculation (207). Because of the short latent period and high mortality induced by REV-T, this virus has been regarded as the most virulent of all retroviruses (29).

Clinical Signs

Chickens and quail with runting disease syndrome may be notably stunted and pale (16, 165). Weights of infected chickens and quail may be 20–50% lower than controls by 3–5 weeks after infection (16, 255, 257). Weight depression has also been seen in infected ducks (182). Some chickens may display abnormal feather development, termed Nakanuke, that is, wing feathers with focal adhesion of the barbs to the shaft (127, 128). Lameness or paralysis is rare even in birds with gross nerve lesions. Mortality might be rare in chickens (255) but affected birds in commercial flocks may be culled prior to death; a culling loss of over 50% between 5 and 8 weeks was described in one flock iatrogenically infected with a contaminated MD vaccine (221).

Birds developing chronic lymphomas or acute reticulum cell neoplasia after REV-T infection show few clinical signs due to the rapid onset of the disease, and mortality rates often reach 100% (208, 227).

Pathology

Runting Disease Syndrome

In chickens, the principal changes include runting (165, 257), thymic and bursal atrophy (165), enlarged peripheral nerves (257), abnormal feathering (112, 127–129), proventriculitis (112), enteritis (146), anemia (120, 140), and hepatic and splenic necrosis (183, 230), often accompanied by depression of cellular and humoral immune responses (17, 32, 40–42, 107, 120, 255). The acute hemorrhagic or chronic ulcerative proventriculitis observed in field cases (112) could not be reproduced (11) with a similar isolate. The proliferative lesions in enlarged peripheral nerves often occur in the absence of other neoplasms and it is not known whether the changes are neoplastic or inflammatory (257). The infiltrating cells, which include lymphocytes and plasma cells, are shown in Figure 15.48.

Ducks inoculated with the spleen necrosis or duck infectious anemia strains of REV may display some features of the runting disease syndrome (140, 230). Hematocrit values in ducks inoculated with spleen necrosis strain can be as low as 20%, compared to 35% for control ducks (230). Enlarged nerves (174, 175) or enteritis (146) have been observed in turkeys with RE-related chronic lymphomas.

Genetic differences in susceptibility have not yet been described; chicks from lines of different susceptibility to MD were equally susceptible to the development of nerve lesions following inoculation with REV (257). Most nondefective REV strains, when inoculated at hatch, induce high frequencies of gross lesions (182, 255)

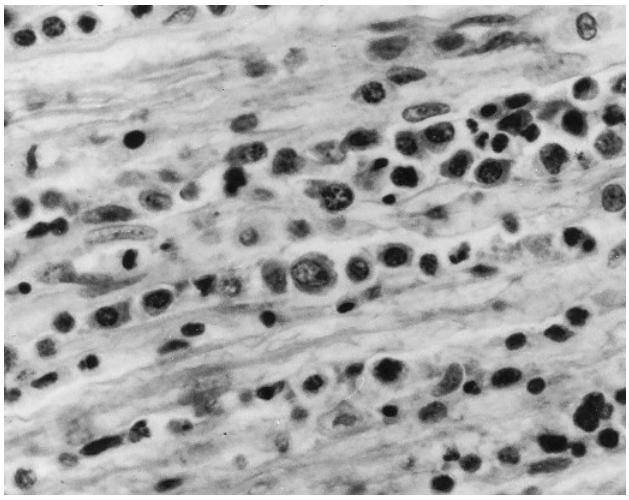


Figure 15.48 Microscopic lesions in a peripheral nerve of a chicken inoculated with nondefective reticuloendotheliosis virus (REV) strain. Infiltrating cells consist of mature and immature lymphocytes and plasma cells.

but others, such as chick syncytial strain, may induce few, if any, lesions (255).

Chicken Bursal Lymphoma

Chickens inoculated with the nondefective chick syncytial or T strains developed B-cell lymphomas, involving principally the liver and bursa of Fabricius (251, 260). The gross lesions were nodular or diffuse lymphoid lesions in the liver, other visceral organs and the bursa of Fabricius, all indistinguishable from lymphoid leukosis (Figure 15.49). A few birds may develop sarcomas or adenocarcinomas. The frequency of lymphomas was influenced by virus strain and whether a tolerant infection had been induced (260). Interestingly, coinfection of chickens with serotype 2 MDV enhanced the incidence of REV bursal lymphomas (6) as had also been reported for lymphoid leukosis (8).

The tumor cells, which are uniform populations of lymphoblasts, were identified as B cells by IgM and other B-cell specific markers (168, 260). The bursal dependency of this tumor was confirmed in chemically or surgically bursectomized chickens, which were refractory to tumor development (82). Bursal lymphomas may not always be present in field cases. Grimes et al. (96) observed what may be lymphomas in two chickens at 22 and 24 week after inoculation with a field strain of REV, but no bursal involvement was reported. However, typical bursal lymphomas were observed in two chicken flocks following administration of a REV-contaminated fowl pox vaccine (85).

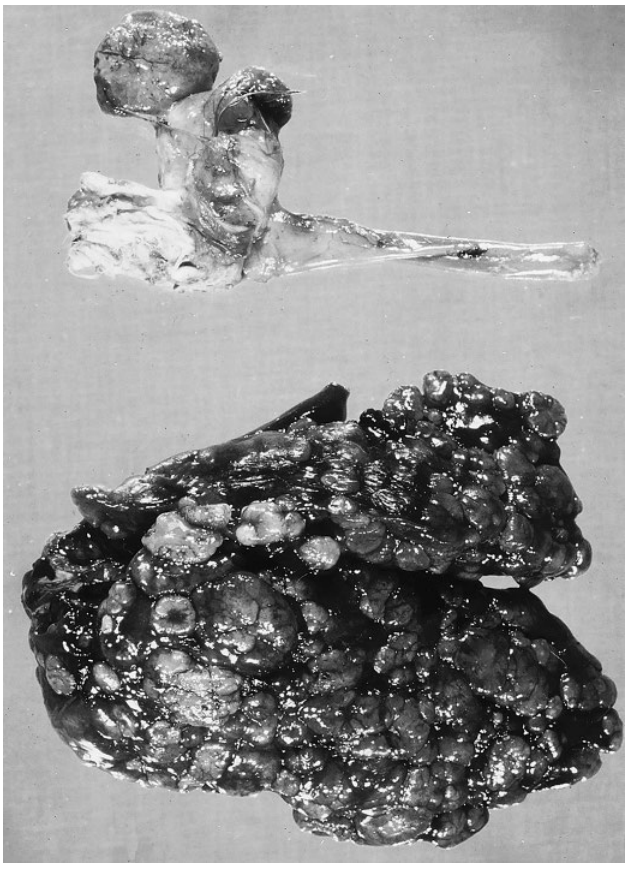


Figure 15.49 Bursal lymphoma in a chicken. Gross lymphomas in the liver and bursa of a chicken 25 weeks after inoculation with the nondefective chick syncytial strain of REV.

Chicken Non-Bursal Lymphoma

Chronic non-bursal lymphomas have been described in chickens following experimental infection with the spleen necrosis or chick syncytial strains of REV (259). Grossly, these lymphomas are focal or diffuse lymphoid infiltrations, with enlargements of the thymus, liver, and spleen or focal lesions of the myocardium (Figure 15.50). The bursa of Fabricius is not involved. Nerve enlargements may be seen. Histologically, the tumors appear to be a uniform, immature lymphoreticular cell that lacked B-cell markers and did not express MATSA, a cellular antigen associated with MD tumors (259) and also expressed on activated T lymphocytes (145). The principal tumor cell type is a CD8+ T cell but Ia antigens are not expressed (53).

Turkey Lymphoma

Chronic lymphomas in turkeys and other avian species consist of gross lymphoid infiltrations in the liver, intestine, spleen, and other viscera. Lymphomatous lesions in the bursa have been reported (146, 174), but this lesion was not common. Histologically, the lesions were composed of uniform populations of lymphoreticular cells

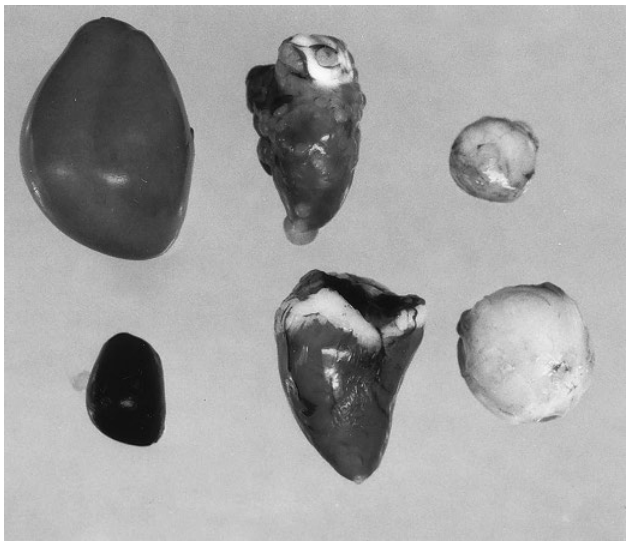


Figure 15.50 Nonbursal lymphoma in a chicken 48 days postinoculation with the nondefective spleen necrosis strain of reticuloendotheliosis virus (REV). Note enlargement of spleen, nodular lymphomas on heart, and bursal atrophy of infected chicken (top row). Organs from age-matched controls in the bottom row (262).

(146, 174). Crespo et al. (55) described T-cell lymphomas associated with a natural outbreak of reticuloendotheliosis in turkeys.

Lymphomas of Other Species

Various other species develop chronic gross lymphomatous lesions that are similar to those described for chickens and turkeys. Lesions reported in ducks include enlarged livers and spleens with diffuse or focal involvement, intestinal lesions, and infiltrations in skeletal muscle, pancreas, kidneys, heart, and other tissues (97, 133, 158). Generalized leukemia in addition to visceral lymphomas in ducks has been described (178). Similar tumors were described in geese, with occasional lymphoproliferative lesions in the bursa of Fabricius (72). Pheasants and prairie chickens displayed proliferative cutaneous lesions on the head and mouth in addition to nodular visceral lymphomas (73, 74, 268). Outbreaks in quail were characterized by liver and spleen enlargements (16, 18, 39, 201, 268) or intestinal lesions (268). Histologically, tumors from all these species generally resembled those described for chickens and turkeys.

Acute Reticulum Cell Neoplasia

The pathology of acute reticulum cell neoplasia has been well described (191, 227). Grossly, affected birds develop infiltrative focal or diffuse lesions in the livers, spleens, pancreas, gonads, heart, and kidney. The blood shows a decrease in heterophils and an increase in lymphocytes (222), leading to leukemia a few hours before death. Histologically there is infiltration and proliferation of cells described either as large mononuclear cells of the reticuloendothelial system (227) or primitive mesenchymal cells (191, 208). Some lesions are composed almost solely of such cells, whereas others include also smaller lymphoid elements, probably indicating a host immunologic response. Areas of necrosis are also frequent. A typical liver lesion is shown in Figure 15.51.

Multiple Syndromes

Lesions of different types can be observed in the same flock, experiment, or even in individual birds. Nondefective REV strains may first induce runting disease syndrome and lymphomas may occur later in the survivors, sometimes accompanied by peripheral nerve enlargement.

Pathogenesis

Virus Infection

Tolerant infection with persistent viremia and absence of antibodies is readily induced in turkeys (148, 258) and chickens by embryo inoculation (107, 260); and by vertical transmission from infected dams (12).

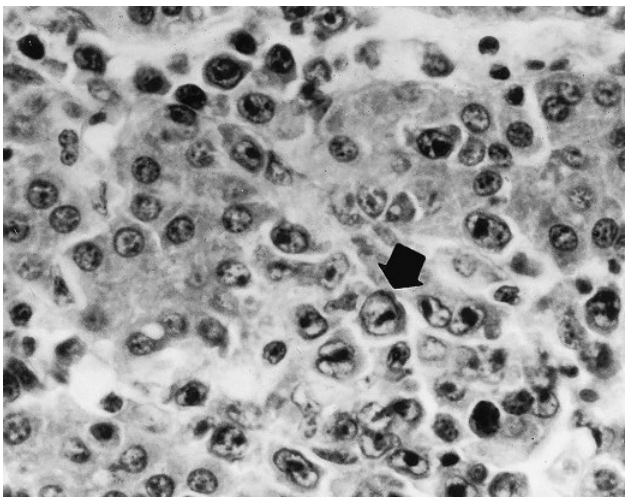


Figure 15.51 Microscopic lesions of acute reticulum cell neoplasia (reticuloendotheliosis) in the liver of a chicken inoculated with replication-defective, acutely transforming strain T REV. The liver is infiltrated with large primitive reticular cells (arrow).

Persistent infections occur rarely following inoculation at hatch (10, 148, 260) depending on the strain of chicken (83); and are unlikely to occur if exposure occurs at later ages. Some birds with persistent infections develop antibody responses. Tolerant infection is associated with higher rates of vertical transmission and tumor development, and birds are typically stunted and immunodepressed. Birds exposed after hatch most commonly develop transient viremia followed by antibody production (10, 255). Persistence of noninfectious RE viral antigens in the blood for several weeks following the disappearance of infectious virus has been reported (10). Transient infection rarely results in vertical transmission, immunosuppression, or tumor development. Infection of older birds rarely results in clinical disease (179, 182, 254, 266) except, perhaps, in turkeys in which lymphomas have been observed following contact exposure (148, 149, 174).

Various other factors influence susceptibility to infection or disease. No genetic cellular resistance has been recognized. However, some differences in the pathologic response of genetic lines or families has been recognized in chickens (83, 205, 259) and quail (226). Nevertheless, differences were not apparent when two different chicken lines were challenged with serial dilutions of REV-T (207). Maternal antibodies appear to limit susceptibility to infection (213).

Runting Disease Syndrome

The pathogenesis of runting disease syndrome has not been elucidated. Stunted chickens did not consume less feed, but had marked reduction of phosphoenolpyruvate carboxykinase, a key gluconeogenic enzyme in the liver (93). The adherence of feather barbules to the shaft

(Nakanuke) is apparently due to REV-induced necrosis of feather-forming cells (219). The microscopic lesions of chicks with runting syndrome resemble a graft versus host reaction (165), but a specific autoimmune component has not been identified.

Chronic Lymphomas

The integration site for the REV DNA provirus in the cellular genome in bursal lymphomas is located adjacent to c-*myc*, a cellular oncogene important in the induction of lymphoid leukosis by avian leukosis virus (ALV) (172). The molecular mechanism by which c-*myc* is activated by insertion of REV proviral DNA has been described (91, 190, 218). The proviral insert often contains major deletions that prevent the expression of infectious virus (217). Based on pathology, proviral insertional activation of c-*myc*, and enhancement by serotype 2 MDVs, bursal lymphomas induced by REV, and ALV appear indistinguishable. However, some subtle differences have been noted. For example, chickens of lines resistant and susceptible to lymphoid leukosis were uniformly less susceptible to lymphoma induction by REV than by ALV (83), and the REV lymphomas frequently require longer latent periods than those induced by ALV.

For nonbursal lymphomas, the molecular mechanism of oncogenesis also involves insertional activation of c-*myc*, but the strong tendency for the provirus to be oriented in the same direction as c-*myc* in bursal lymphomas was not observed in nonbursal lymphomas (109).

It is not known whether a common mechanism exists for oncogenesis in chronic lymphomas of chickens and turkeys. In ducks, the frequency of experimentally-induced REV lymphomas was not affected by embryonal bursectomy (133), indicating that these tumors may not necessarily be of B-cell origin.

Acute Reticulum Cell Neoplasia

The target cell transformed *in vivo* by replication-defective REV-T (with REV-A helper virus) expresses T lymphoid and myeloid markers (19). These cells also express surface MHC class I and II antigens, as well as interleukin-2 receptor (103), and are immunoglobulin M (IgM) negative but vary in expression of CT3 (19). Similar tumors were induced in chemically bursectomized chickens (19). Inoculation directly into the thymus induced thymomas composed of T and B cells (29). On the other hand, REV-T, when associated with chick syncytial virus helper virus instead of REV-A, induces IgM-positive B-cell lymphomas with rearrangements of the heavy- and light-chain immunoglobulin loci (20, 21). Thus, cell tropism appears to be determined, in part, by differential effects of the helper viruses on lymphoid populations.

Neoplastic transformation in acute reticulum cell neoplasia is mediated by the oncogene v-*rel*, present in REV-T. Transformation does not require the presence of a helper virus (131). Lymphoid cells transformed by REV-T *in vitro*, but which produce no infectious virus, will produce typical RE when transplanted into syngeneic recipients (131, 193).

Immunity

Humoral Responses

Birds with nontolerant infections develop robust antibody responses, detected as early as 16–21 days after inoculation in chickens (32, 157), but 6–10 weeks may be required in contact-exposed birds (107, 130, 148). Antibody titers may decline with age (10, 32, 260), but neutralizing antibodies have been detected in experimentally-infected turkeys through 40 weeks (148). Most birds that develop tolerant infections do not develop humoral immune responses, although a few tolerant chickens ultimately develop antibodies (159). The presence of antibodies may influence tumor susceptibility as chemically bursectomized quail were more susceptible to tumors than controls (186).

Cellular Responses

Major histocompatibility complex-restricted cytotoxicity against lymphoblastoid cell lines transformed with defective REV has been described in chickens within seven days after inoculation with defective or nondefective RE viral strains (141, 246). This response appears to be mediated by activated (MHC class II+) CD8+ T cells (126). However, natural killer (NK) cells were not activated (199). The induction of cytotoxic T cells by REV has been used as a general indicator of immune response in the study of other avian viruses (187).

Immunodepression

Humoral and cellular immune responses are frequently depressed in chickens infected with nondefective REV strains. Depressed antibody responses to MDC and turkey herpesvirus (HVT) (32, 120), Newcastle disease virus (107, 265), sheep erythrocytes, and *Brucella abortus* (255) are documented. The magnitude of antibody depression is influenced by the dose and strain of virus, and primary responses are more severely affected than secondary (255). Depressed responses against *Pasteurella multocida* in turkeys infected with nondefective REV isolated from Attwater's prairie chickens have been documented (17).

Different strains of nondefective REV varied in their ability to induce bursal atrophy and suppression of B cell populations available for transformation by v-rel (20). Studies on chimeric viruses derived from REV-A and chick syncytial virus showed that regions in both gag and env genes were associated with the strong immunodepressive ability of REV-A (88).

Spleen cells from chickens infected with REV-T displayed suppressed responses to the mitogen phytohemagglutinin (40, 204). This effect is associated with the nondefective helper virus in strain T stocks (34, 42) and is mediated through a population of suppressor cells (41, 192), which could be demonstrated only through the third week after infection (193). Other cellular immune responses inhibited by REV infection include mixed lymphocyte reaction and allograft rejection (240).

Depression of humoral responses and a transient mitogen responsiveness have been identified following infection with the chick syncytial strain, but persisted through 10–19 weeks in tolerant chickens infected with nondefective REV (255, 260). Infected chickens were more susceptible to the development of a MD tumor transplant (34); to reactions from infectious laryngotracheitis vaccine (160, 212); to natural fowl pox virus infection (162); to infectious bronchitis virus (212); to mortality induced by *Eimeria tenella* (161); and to *Salmonella typhimurium* infection (160). No increase in susceptibility to MDV was noted (33), but there was interference by REV infection with immunity induced by turkey herpesvirus against MD in chickens (255). Humoral immunodepression was also seen in ducks infected with a field isolate of REV (133). In the field, immunodepression is probably the most important consequence of embryo- or vaccine-derived REV infections but is less likely to result from contact infection (254) and has not commonly been associated with seropositive flocks. Reticuloendotheliosis virus-induced immunosuppression has been reviewed (267).

Tumor Immunity

Regression of strain T-induced wing-web tumors was partially abrogated by bursectomy, thymectomy, and bursectomy–thymectomy (137). Serum from hyperimmunized (104) chickens was protective against tumor development even after absorption to remove antiviral antibodies (104), suggesting the existence of tumor-specific transplantation antigens on RE tumor cells. Chickens immunized with purified or inactivated preparations of nondefective strain T helper virus were resistant to challenge with acutely transforming REV-T preparations (24). However, immunization with empty virions (151) did not provide protection.

Diagnosis

A diagnosis of RE requires not only the presence of typical gross and microscopic lesions, but also the demonstration of REV, REV antibodies and the exclusion of other oncogenic agents. Because REV, unlike avian leukosis and MD viruses, is not yet as ubiquitous, the demonstration of infectious virus, viral antigens, and proviral DNA in tumor cells has diagnostic value. Diagnostic techniques have been reviewed by Zavala et al. (269).

Isolation and Identification

Reticuloendotheliosis virus viremia is typically low titered and transient, except in tolerant birds. Birds with lesions are normally a good source of virus, which may be isolated by inoculation of susceptible tissue cultures with tissue suspensions, blood, plasma, splenocytes, white blood cells, or other inocula. Cellular inocula are preferred over cell-free inocula, because of higher titers. Cytopathic effects in cell cultures may not be evident, thus, cultures should be maintained through at least two blind 4–7-day passages of infected cells. Replication of REV may be confirmed by demonstration of viral antigen in cell cultures using fluorescent polyclonal or monoclonal antibodies (17, 18, 57), immunoperoxidase (35), complement fixation (214), enzyme immunoassay (58, 108, 170), or molecular detection of RNA or DNA (5, 17, 38, 74, 78, 87, 92, 196, 268). In comparative studies, enzyme immunoassays were more sensitive than complement fixation tests (58) and indirect immunofluorescence was more sensitive than indirect immunoperoxidase or immunoelectron microscopy (169). A convenient and sensitive indirect immunofluorescent assay conducted in 96-well plates (46) has been used for virus isolation from field samples (258).

Virus isolated by any of these procedures may be used for reproduction of the disease or for further tests. Yolk inoculations in 5–7-day-old chicken or quail embryos are useful for disease reproduction and virus replication (16, 18). Virus isolates may be assigned to antigenic subtypes by the differential reactivity to fluorescent mAbs (46).

Detection of proviral DNA by PCR assays has been shown to be a sensitive and specific method for detection of REV in chicken embryo fibroblasts or DF-1 cells, paraffin-embedded tissues, as well as in blood and tumors of infected birds (5, 17, 38, 74, 78, 87, 92, 196, 268). Polymerase chain reaction is useful for tumor diagnosis (59, 61, 63, 65) and for evaluating vaccines for possible REV contamination (78, 84, 85, 100, 139, 220). Veterinary Services Memorandum 800.88 by USDA/APHIS describes PCR, *in vivo* virus amplification, and serology as suitable direct or indirect tests for detection of REV in master seed viruses for the production of commercial poultry vaccines. Assays amplifying REV envelope and REV 3' LTR sequences provided a more accurate assessment of REV provirus than PCR assays that amplify the REV 5' LTR region (92). Although PCR assays are sensitive and specific they may not be as well suited as enzyme immunoassays for large-scale testing. A loop-mediated isothermal amplification (LAMP) method for rapid detection of REV with high sensitivity and specificity has also been reported (66).

Serology

Serological confirmation of REV exposure involves the detection of antibodies in sera from experimentally inoculated chickens or from clinically affected chickens. The most sensitive test for detection of antibodies to REV is virus neutralization, although ELISA is the most commonly used serological assay worldwide. Immunoperoxidase plaque assay (35) was once shown to be a sensitive and reliable method for detection of REV antibody. Antibodies may be detected in serum or egg yolk from suspect birds by indirect immunofluorescence (7, 257) or virus neutralization (148, 182). The agar gel precipitin test may detect viral antigen as well as antibody in sera (106, 107). Reticuloendotheliosis virus ELISA antibody kits are commercially available. Antibody tests are particularly useful in ascertaining lack of exposure in specific pathogen free flocks or breeding flocks producing progeny for export. Complementary assays that are now very seldom used are reviewed in previous editions of this book.

Differential Diagnosis

The pathology of REV-induced lymphoproliferative tumors can be confused with that of tumors seen in MD and lymphoid leukosis (81, 238, 253). Thus, neoplastic diseases must be diagnosed by exclusion; that is, by confirming specific agents involved and excluding other possible etiologies. Because avian tumor viruses are widespread and infection in the absence of tumor formation is common, in many cases virological and serological criteria do not always provide a definitive diagnosis. However, diagnosis of RE should be supported by virological evidence of REV infection, as REV is not as ubiquitous as MDVs and ALVs. Techniques based on immunocytochemistry with mAbs to cellular, tumor, and viral antigens, or molecular hybridization can be used in the differential diagnosis of avian viral lymphomas including RE, but are seldom used in diagnostic laboratories.

Retroviral lymphomas in chickens originate from either B cells (RE, lymphoid leukosis) or T cells (RE), whereas MD lymphomas are of T-cell origin. The characteristics of target cells provide the basis for tests that distinguish among B- and T-cell lymphomas using mAbs specific for cell surface antigens of B- and T-lymphocytes. Nondefective strains of REV have been shown to transform chicken B or T cells (109, 218).

The PCR assays for RE, MD, and exogenous ALV can be helpful in the differential diagnosis of RE. For instance, because MD lymphomas should contain a significant proportion of MD virus-infected cells, compared to latently infected tissues, MD lymphomas should have more infected cells, each with greater number of viral copies thus resulting in higher total estimates of viral load by quantitative PCR analysis (188) or real-time PCR. Non-quantitative PCR assays are probably of little value for diagnosis of MD because of the potential to detect MDV DNA in the absence of lymphomas. Polymerase chain reaction has been shown to detect REV-LTR sequences from lymphomas and brains of REV-infected chickens, but not from DNA from MD or lymphoid leukosis lymphomas (5).

Chronic neoplasia in the chicken where the tumors are of bursal origin cannot easily be differentiated from lymphoid leukosis on pathologic criteria (251). Virological, serological, or PCR tests should be performed to confirm one oncogenic virus and exclude another.

Chronic neoplasia in the chicken in which bursal tumors are lacking or in which the latent period is too short for lymphoid leukosis must be differentiated from MD. Here too, pathological criteria are insufficient and virological assays (including PCR) may be helpful. The pp38 antigen of MD virus, occasionally expressed in MD lymphomas, is not present in RE lymphomas. Also, MHC class II (Ia) antigens are reported to be present on MD lymphoma cells (199) but absent on RE nonbursal lymphoma cells (53). A comprehensive diagnostic strategy for the differential diagnosis of virus-induced lymphomas in chickens has been introduced (253).

The acute reticulum cell neoplasia syndrome is not known to occur in the field. A syndrome of broiler chickens characterized by reticuloendothelial proliferation in the spleen and liver, and resulting in condemnation losses at processing, has been confused with RE (98, 239), but can be distinguished by the absence of RE antigens and proviral DNA in the lesions. Virus detection tests including PCR have been used to detect REV in a lymphosarcoma in an Indian peafowl (152) and lymphomas in captive greater and Attwater's prairie chickens (74, 80, 268).

The runting disease syndrome must be distinguished from MD, especially when nerve lesions are present. Differences between REV-induced and MDV-induced nerve lesions have been discussed (255, 257), but are not consistent. Both types of nerve lesions must be distinguished from spontaneous neuropathy, possibly an autoimmune lesion of peripheral nerves (9).

Lymphoproliferative disease (LPD) of turkeys can be confirmed or excluded by a combination of histopathology and molecular detection methods for REV and LPDV (1, 3). The PCR assays for LPD (1, 3, 197) and RE (5, 17, 38, 74, 78, 87, 92, 196, 268) should be useful, albeit wild turkeys could bear dual infections with both viruses. Marek's disease has been diagnosed in turkeys in France, Israel, Germany, and Ukraine and should be ruled out in the differential diagnosis.

In summary, naturally occurring RE lesions can be confused in the chicken with MD, LL, and various other lymphoproliferative or immunodepressive conditions, and in the turkey, with LPD and MD.

Intervention Strategies

Vaccination

Reticuloendotheliosis in commercial poultry is typically controlled by testing and elimination of infected shedder breeding stock and thus vaccination is never considered. However, vaccines could be of use for immunization of endangered avian species. Vaccination of chickens with a recombinant fowl pox virus expressing the *env* gene of REV (36, 167), or empty REV particles (151), provided some protection against REV infection. Defective REV particles (243) have been shown to induce neutralizing antibodies (79). A baculovirus construct expressing the *env* gene of REV has also induced REV antibody in chickens (241).

Treatment

No treatment for RE is known. Since immune responses are mounted to infection, it is possible that some affected birds may recover.

Prevention and Control Procedures

Prevention of RE is currently accomplished through quality assurance of poultry biologics and, in SPF flocks, by strict biosecurity (250). It is desirable to prevent environmental exposure and seroconversion of breeder flocks where progeny is destined for export, but this is difficult to accomplish because it may be impractical to truly prevent exposure in the field. Control of insect vectors and fowl poxvirus infection could be important in prevention programs (250).

Procedures for the control of RE have rarely been applied in commercial practice, mainly because the disease has been sporadic and self-limiting. Enzyme immunoassay to detect RE viral antigen in albumen samples seems to be the procedure of choice in commercial situations (108, 258). Presumably, it would be necessary to eliminate vertical transmission through removal of potential transmitter hens, and to rear progeny under isolated conditions whereby horizontal infection could be precluded, as it has been done with ALV in chickens. Such control procedures could be considered if REV infection becomes endemic in especially valuable breeding stock, as is the case with the endangered Attwater's prairie chickens (27, 28, 74, 80, 87, 180, 250, 268).

Other Tumors

Susan M. Williams, Rodney L. Reece, and Scott Hafner

Summary

Agent, Infection, and Disease. Other tumors are in the category where a known etiology does not exist or is uncertain. The prevalence of these types of tumors will depend on species, breed, age, and sex along with various intrinsic and extrinsic factors.

Diagnosis. Avian tumors often exhibit distinctive microscopic appearances that may be difficult to extrapolate from similar mammalian tumors. Information on the classification and histologic appearance of some of the less common tumors of poultry is available from detailed poultry pathologist reports, zoo surveys, and noncommercial poultry reports. Immunohistochemistry can aid in identification of tumor cells in poultry and other avian species with careful interpretation of the positive and negative controls.

Intervention. There is no intervention strategy due to nature of these types of tumors.

Introduction

Other tumors refers to tumors of poultry (chickens, turkeys, quail, pigeons, ducks, geese, guinea fowl, pheasants, and ratites) in which a known etiology does not exist or is uncertain. Avian tumors often exhibit distinctive microscopic appearances that may be difficult to extrapolate from similar mammalian tumors. The most detailed examination of chicken tumors remains that of Campbell (20) whose definition of a tumor as "an abnormal tissue mass … which usually persists independent of initiating factors … whose excessive, often uncoordinated growth threatens the host through compression, infiltration, or remote spread" still suits our purposes. Detailed information on the prevalence of different tumor types in poultry exists, but earliest reports are skewed by a large number of virally-induced "leukotic" tumors and the reproductive tumors that are common in adult hens. Tumor prevalence in poultry not only depends on species, breed, age, and sex, but also various intrinsic (hormonal, genetic/developmental) and extrinsic (viral, chemical, and other environmental) factors (20). Some indications of the proportion of different tumors to be expected in aged chickens unaffected by exogenous viruses are available in the surveys of tumors in specific pathogen free (SPF) flocks (38, 99). Information on the classification and histologic appearance of some of the less common tumors of poultry is available from detailed reports by poultry pathologists, zoo surveys, and examinations of the tumors of noncommercial birds.

Examinations of tumors present in poultry at slaughter are particularly useful as the sheer numbers of poultry slaughtered may provide multiple examples of even very rare tumors, and slaughtered birds are conveniently separated by age and species. Radical deviations from the "normal" prevalence, distribution, and/or type of these tumors may indicate changes in etiology or other factors that require further elucidation. Immunohistochemical techniques that utilize antibodies to lineage-specific cell markers have found increasing applications as an aid to identification of tumor cells in both poultry and other avian species. Extended formalin fixation may reduce the avidity of some antibodies for their designated tissue epitopes; this at times may be reversed by heated acid solutions or applications of proteases. Some studies have utilized antibodies to vimentin for detecting mesenchymal cells, cytokeratin for epithelial cells, actin for smooth and skeletal muscles, and neurofilament for nerves. Some other antibodies may exhibit different avidity in avian tumors compared to that in mammalian species, for instance S100 proteins may stain normal nerves, but not label tumor cells derived from those tissues (72). Neuron-specific enolase often will intensely stain neural tissues, but may also stain other tissues, particularly muscle. Careful evaluation of the particular antibody in the both normal and neoplastic tissues of the affected species including both positive and negative controls is important for accurate interpretation of immunohistochemical findings.

Public Health Significance

There is no known public health significance.

Urogenital System

Ovary

Despite the considerable differences between avian and mammalian ovaries, particularly with regard to histologic appearance and physiology, attempts are made to characterize avian ovarian tumors using criteria developed for mammals, particularly humans. Even with the difficulty of categorizing avian neoplasms by mammalian criteria, classifying ovarian tumors as derived from surface mesothelium (adenocarcinomas), sex cord tissues (granulosa cell tumors and arrhenoblastomas), germ cells (teratomas and dysgerminomas), or other supportive tissues provides clues to both expected behavior and possible etiology, in addition to differentiating these tumors from metastatic disease. In particular, ovarian adenocarcinomas of White Leghorn hens have recently been investigated as an animal model for ovarian cancer of women, with potential for this research to yield insight into the development of this neoplasm in both species (7, 13, 57, 60).

Adenocarcinoma

Early tumors are small, firm, white nodules that occur on the surface of the ovary; these may be mistaken for atretic follicles, but are less symmetric (37). Nodules coalesce into a gray-white, cauliflower-like mass that commonly seeds serosal surfaces with transcoelomic metastases. Ascitic fluid often develops with growth of the metastases and terminally affected hens may assume an upright, penguin-like posture. Ovarian adenocarcinomas must be differentiated from oviductal adenocarcinomas as both may be widely metastatic and oviductal tumors may metastasize to the ovary. Failure to detect tumors in the lining of the oviduct suggests tumors are of ovarian rather than oviductal origin. Tumors are assumed to arise from the mesothelial covering of the ovary (germinal epithelium), including its invaginations into the ovary, but other suggested tissues include sex cord remnants, thecal cells, interstitial cells, or even the mesonephros. Small tumors appear to be located in the theca externa of small follicles (Figure 15.52) or in the ovarian stroma; a few may originate in the ovarian stalk. Multiple tumors may be present. Smaller tumors contain predominantly epithelial cells and little stroma, while larger tumors are often scirrhous. In advanced cases, ovarian follicles fail to mature and oviducts are often atrophied. Ovarian adenocarcinomas are not associated with increased production of steroid hormones (37, 38). Occasionally, ovaries affected by adenocarcinomas are covered with small cysts filled with yellow fluid; these may be dilated lymphatics or ovarian stromal lacunae (83). A survey of 400 hens revealed approximate 2% incidence rate of tumor restricted to ovary (60).

Histologically, ovarian adenocarcinomas are often composed of acini lined by cuboidal to low columnar, non-ciliated, epithelial cells. These epithelial cells are often arranged around lumina that are at times filled with intensely eosinophilic periodic acid-Schiff (PAS) positive material (Figure 15.53). In other, more densely cellular tumors, acini may be compressed into cords or islands of epithelial cells or papillary proliferations of the lining epithelium may be invaginated into dilated acini (Figure 15.54). The mitotic rate is variable, and in many cases mitoses are not prominent. In larger tumors, acini are often surrounded by a marked scirrhous response (Figure 15.55), and in some serosal implants there may be a proliferative response of the underlying muscularis (84). Similar ovarian adenocarcinomas have been described in turkey hens (119).

Granulosa-Theca Cell Tumor

Granulosa-theca cell tumors are irregularly round to multilobular and encapsulated by a smooth, glistening

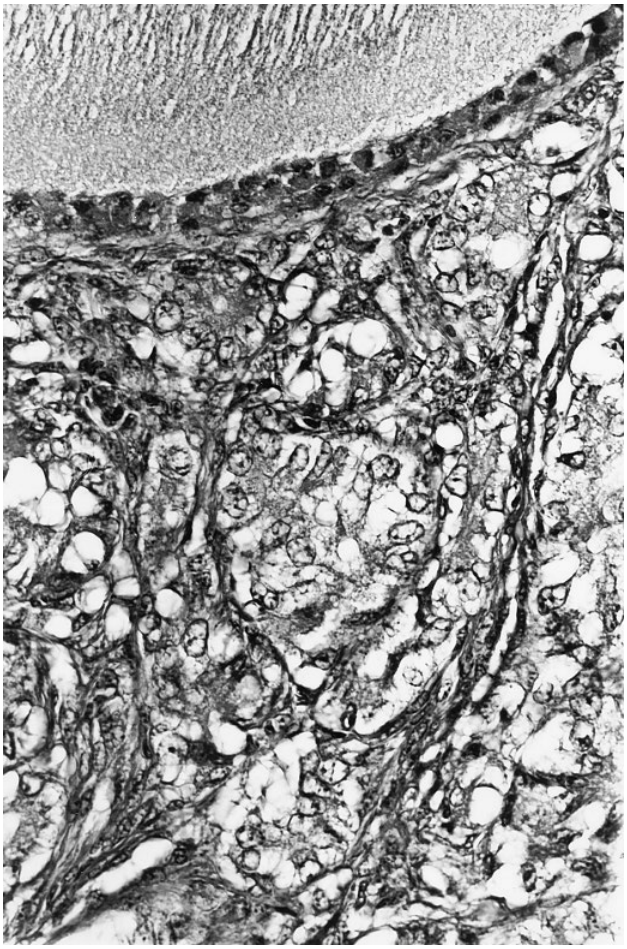


Figure 15.52 Ovarian adenocarcinoma in the theca region demonstrating delicate trabeculae and round nuclei; note the granulosa cells and yolk of the developing ova (top). H&E, ×360.

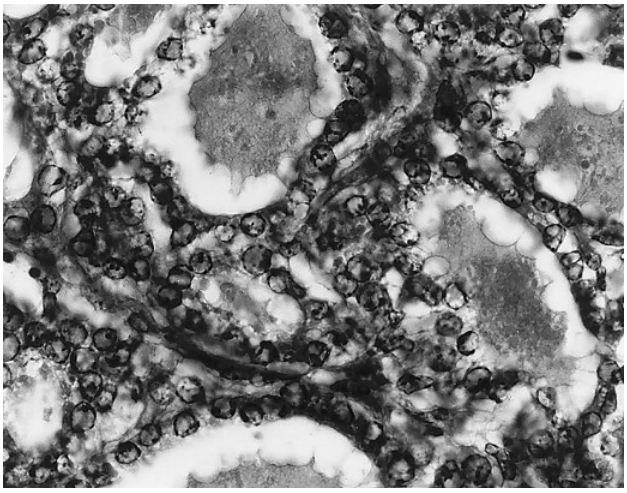


Figure 15.53 Acinar structures, typical of ovarian adenocarcinoma filled with eosinophilic material and lined by cuboidal cells containing round nuclei with condensed chromatin and sparse eosinophilic cytoplasm. H&E, ×600.

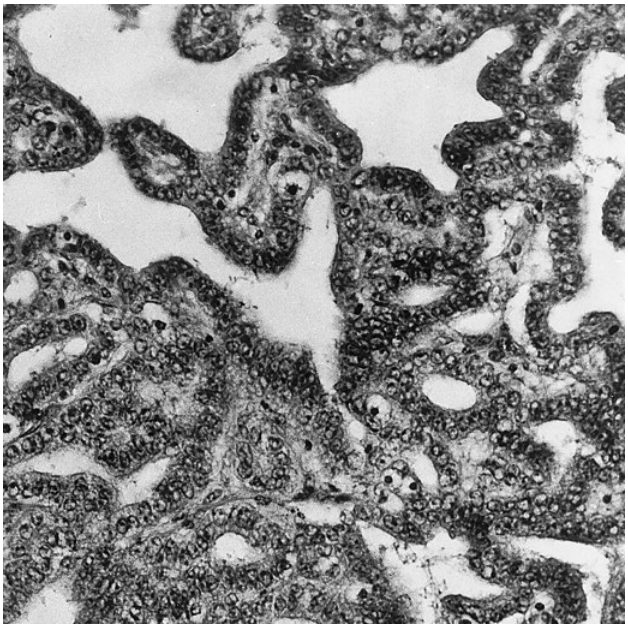


Figure 15.54 Ovarian adenocarcinoma with papillary structures projecting into dilated acini. H&E, ×160.

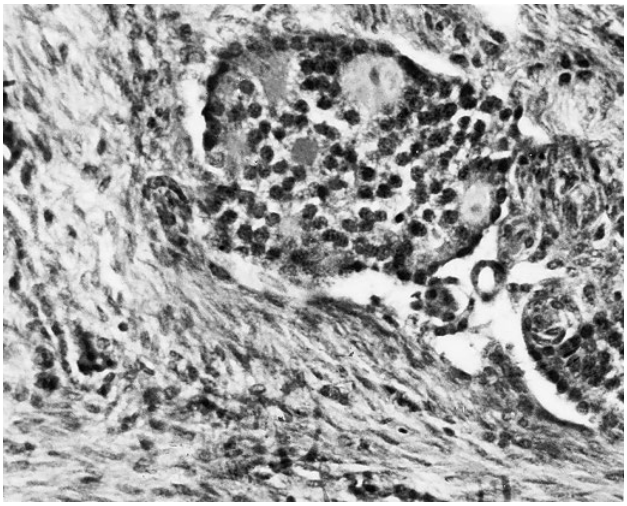


Figure 15.55 Another form of ovarian adenocarcinoma with dense bands of stromal cells enclosing clusters of neoplastic acinar cells with intensely basophilic nuclei. H&E, ×160.

membrane. Tumors occasionally exhibit peripheral thin-walled cysts filled with fluid; sectioned tumors are yellow and friable and some contain hemorrhages. These tumors enlarge while remaining attached by the thin ovarian pedicle. Tumors may become quite large without metastasizing, but metastases to visceral organs and serosal surfaces can occur (20, 37). Granulosa-theca cell tumors are by far the most common ovarian tumor of the young broiler chicken. In these birds, there is often marked precocious glandular hyperplasia of the oviduct (50). Histologically tumor cells are polyhedral to fusiform with

pale eosinophilic to vacuolated cytoplasm (Figure 15.56). Nuclei are generally irregularly round to ovoid. The arrangement of tumor cells varies widely from the formation of follicular structures to solid sheets composed of tightly packed thin cords (Figure 15.57) separated by a fine fibrovascular stroma, more elaborate gyriform arrangements (Figure 15.58), or rosettes arranged around small central spaces. The mitotic rate is variable but often low, and the stroma may be prominent.

Ultrastructurally, tumor cells are identified by transosomes, a structure specific to avian granulosa cells and those of some other lower vertebrates (37). Transosomes (lining bodies) are dense structures of the lateral and apical plasma membranes of granulosa cells that are taken up by the oocyte becoming associated with primordial yolk granules. These structures are involved in the transport of vitellogenin (a yolk precursor) into the oocyte (76). Although granulosa cells from normal avian follicles produce progesterone, theca interna cells produce testosterone, and theca externa cells estrogen (94): hens with large granulosa-theca cell tumors exhibit markedly elevated plasma levels of estrogen (37). Oviducts of affected hens are the same size as hens in lay, but eggs are not produced. A significant thecal cell component may be present in granulosa-theca cell tumors; the presence of numerous vacuolated theca-like cells may lead to characterization of a granulosa cell tumor as "luteinized" or even as a "luteoma" (20). Campbell (20) also includes a tumor composed primarily of thecal cells that he designated a thecoma. A metastatic granulosa-theca cell tumor has been described in a duck (16). A very low incidence of granulosa-theca cell tumors has been induced in meat-type chickens inoculated as embryos with ALV-J (92).

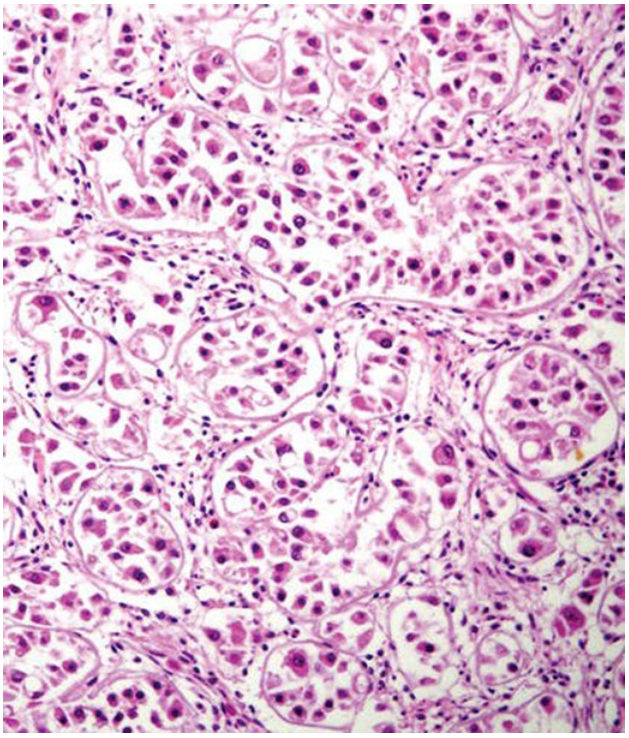


Figure 15.56 Lobules of vacuolated cells separated by moderate trabeculae in a granulosa-theca cell tumor. H&E, ×200.

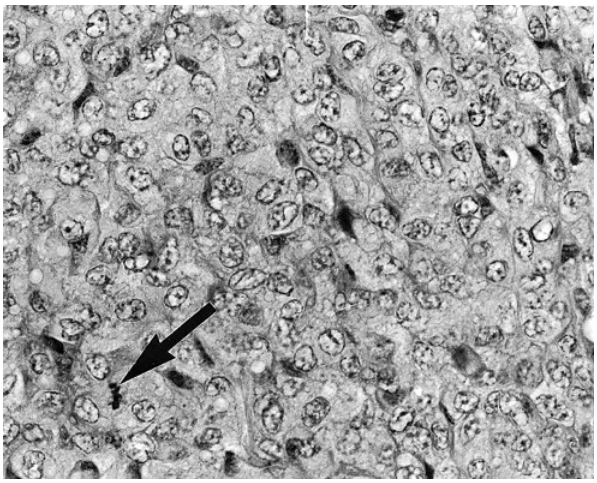


Figure 15.57 Granulosa-theca cell tumor composed of a uniform population of tightly packed tumor cells with plentiful, pale eosinophilic cytoplasm and uniform, round vesicular nuclei. Note the mitotic figure (arrow). H&E, ×600.

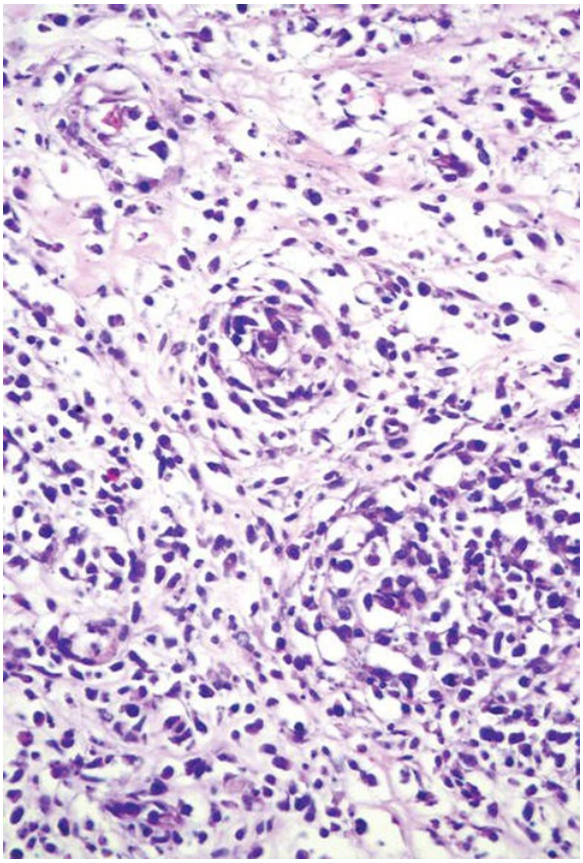


Figure 15.58 Gyriform arrangements of cells in one area of an ovary with a granulosa-theca cell tumor. H&E, ×400.

Arrhenoblastoma (Arrhenoma)

These tumors are associated with formation of seminiferous tubules within the ovarian stroma and are accompanied by evidence of sex reversal (virilism). Sex reversal in domestic fowl was well recognized in ancient times. Most cases of sex reversal in poultry are due to destruction of the functional left ovary and formation of an ovotestis in the remnants of the rudimentary right gonad rather than hormone production by ovarian tumors (20, 36). In birds, the male is the neutral sex and the young female is demasculinized by the production of her ovarian hormones (88). Well-differentiated arrhenoblastomas are histologically composed of branching cords of columnar epithelium that are often two cells thick; these resemble primitive seminiferous tubules. There is little, if any spermatogenesis. In less differentiated tumors, epithelial cells may be arranged in cords, nests, and rosettes that form incomplete tubules (Figure 15.59) separated by a prominent interstitium that may contain interstitial (Leydig) cells (98). Large tumors may be hemorrhagic. Arrhenoblastomas have been induced by the injection of radioactive isotopes into the left ovary (121).

Ovarian Sertoli Cell Tumors

Ovarian Sertoli cell tumors in the chicken seem to be limited to the five cases described by Fredrickson (38). These tumors were composed of compact masses of seminiferous tubules lying under the ovarian capsule. The tubules were lined by a single, radially arranged layer of tall columnar Sertoli cells (Figure 15.60); variable numbers of interstitial cells were present between tubules. There was no obvious sex reversal or alteration in circulating hormones.

Dysgerminoma

Dysgerminomas are the equivalent of mammalian ovarian seminomas, and have been detected in a few intersex (pseudohermaphroditic) pullets (123, 124). There was no overt sex reversal, but there was masculinization of the comb and plumage of the head. Tumor cells were round to polygonal and contained round to occasionally reniform nuclei. Some tumors had metastasized to the liver or peritoneum.

Mesosalpinx

Leiomyoma

Leiomyomas (at times termed fibroleiomyomas or even fibroids) are common in the ventral ligament of the oviduct and the oviductal wall in domestic fowl, including SPF hens (56). The prevalence of these tumors is variable, but may affect up to 60% of hens in the first egg laying cycle (6). Similar tumors were present in the oviduct ligaments in some rapidly growing Japanese quail (35). Leiomyomas of the ventral ligament of the oviduct are usually solitary. Unless tumors are quite large, there is little effect on oviductal function, but there may be increased egg yolk peritonitis (77). In hens affected by these tumors, there were elevated serum concentrations of estradiol, and oviductal leiomyomas were induced in hens treated with both diethylstilbestrol and

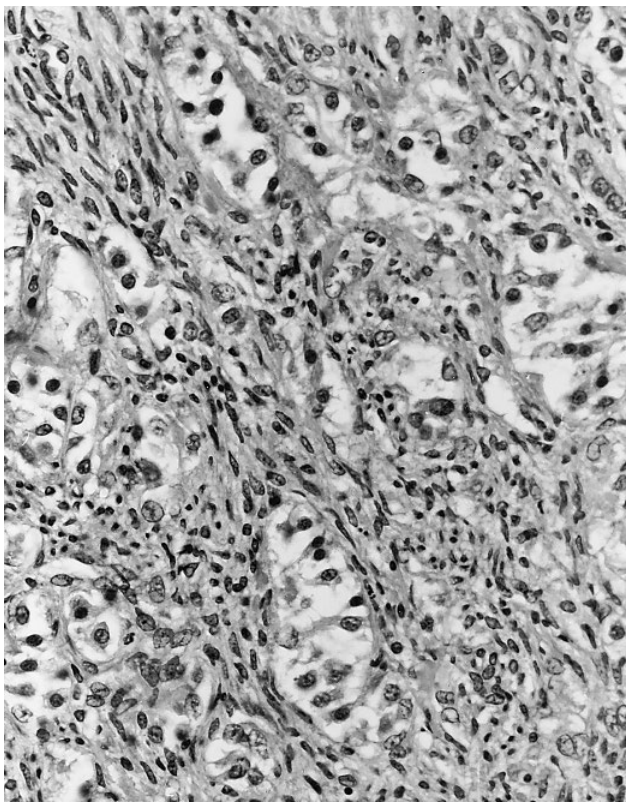


Figure 15.59 Arrhenoma from a hen that showed sex reversal. Network of epithelial cells arranged as ill-defined cords and tubules. H&E, ×350. (C.J Randall).

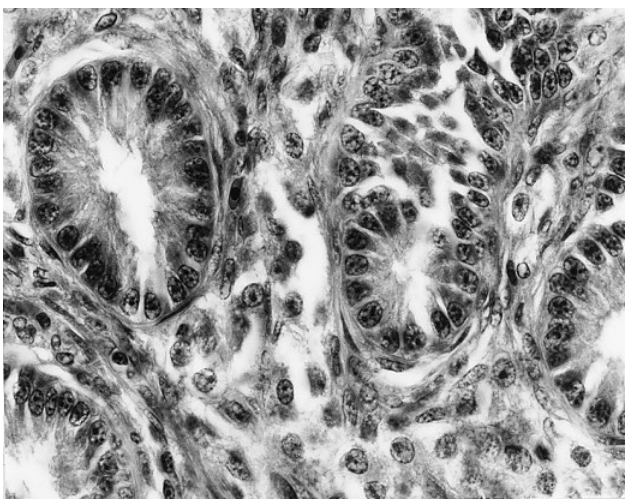


Figure 15.60 Ovarian Sertoli cell tumor composed of well-defined seminiferous-like tubules lined by Sertoli cells. Stroma contains interstitial cells. H&E, ×600.

progesterone (5, 6). Some reports (17) have also demonstrated that the tumor cells contain receptors for both estrogen and progesterone, suggesting steroid hormones are likely to be involved in the etiology of these tumors. Many tumors are discrete, pale, solid masses less than 1 cm in diameter, but some may be several centimeters in diameter and heavily vascularized (77, 98). Tumor cells are well-differentiated, interlaced, smooth muscle cells bundled by variable amounts of connective tissue (Figure 15.61). Mitotic figures are rare. These tumor cells are immunolabeled by α-smooth muscle actin, desmin, and vimentin (77).

Oviduct

Adenocarcinoma

Adenocarcinomas originating in the oviduct most commonly arise in the upper magnum, with occasional tumors seen in the uterus (shell gland) or infundibulum. These tumors, like ovarian carcinomas, are often metastatic to the mesentery and serous surfaces of abdominal organs, especially to the pancreas and duodenum, but also to the ovary and occasionally to the surface of the liver and spleen. Tumor cells may be spread transcoelomically in the ascitic fluid accumulating in the intestinal peritoneal cavity. In contrast, metastases to the lung may be hematogenous (2, 67). The prevalence of oviductal adenocarcinomas as determined by examining the oviductal mucosa for tumors varied widely (5 to 81%) in one survey (44) and in another survey only 4% were restricted to the oviduct (60). In some studies (4), a positive correlation existed between egg weight and body weight, and prevalence of tumors.

Oviductal adenocarcinomas in both chickens and turkeys may begin as small (2–10 mm) nodular areas of mucosal dysplasia, typically discovered during close examination of the mucosa of the oviduct (15). Histologically, these nodules are well-demarcated from adjacent glands and are composed of concentrically arranged columnar epithelial cells with acidophilic cytoplasmic secretory granules and pale enlarged nuclei (Figure 15.62). Adenocarcinomas are grossly evident as small pink to grey, firm masses that protrude into the lumen of the oviduct and may invade through the muscularis. There is often a distinct border between the normal magnal mucosa and tumor cells (Figure 15.63). Metastases are often well-encapsulated (Figure 15.64) and may be composed of well-differentiated cells. Implants on the enteric serosa are often anaplastic cells embedded in dense connective tissue (Figure 15.65) while implants on the oviduct serosa lack this intense scirrhous response (Figure 15.66). The metastases of oviductal adenocarcinomas may in some cases be histologically quite similar to those of ovarian adenocarcinomas and since the ovary is also a common site for metastasis, differentiating the tumors may be difficult. The ultrastructural characteristics of oviductal adenocarcinomas have been described (64). Immunohistochemical studies have demonstrated that the cells of oviductal adenocarcinomas contain ovalbumin (59), but more recent studies (42) have also identified ovalbumin in ovarian

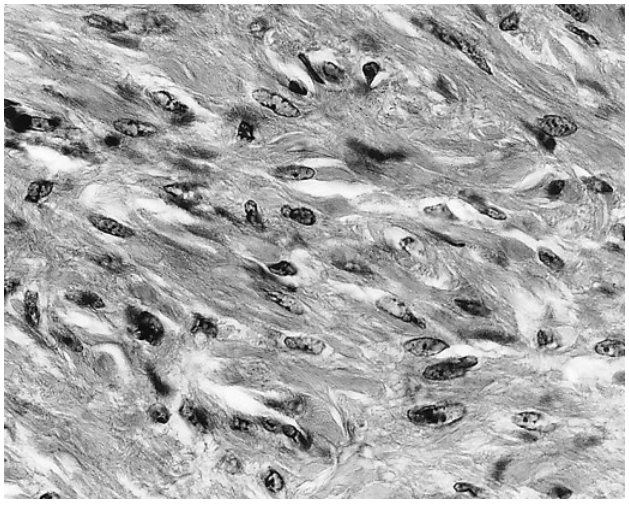


Figure 15.61 Leiomyoma of mesosalpinx composed of smooth muscle fibers arranged in compact whorls. Mitoses are absent from this field, and the nuclear/cytoplasmic ratio is low. H&E, ×600.

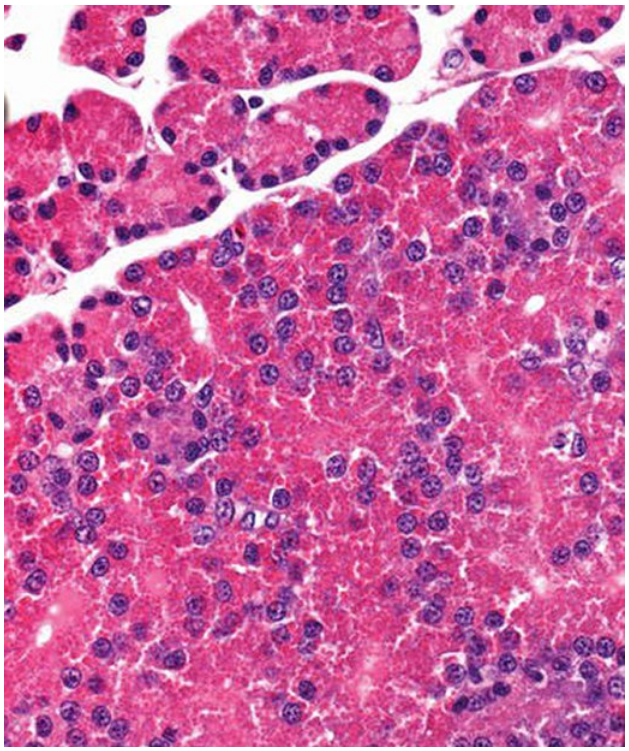


Figure 15.62 Dysplastic adenomatous focus in a fold in the magnum showing clear demarcation from surrounding normal glands. The columnar epithelial cells are densely packed and oriented concentrically. H&E, ×400.

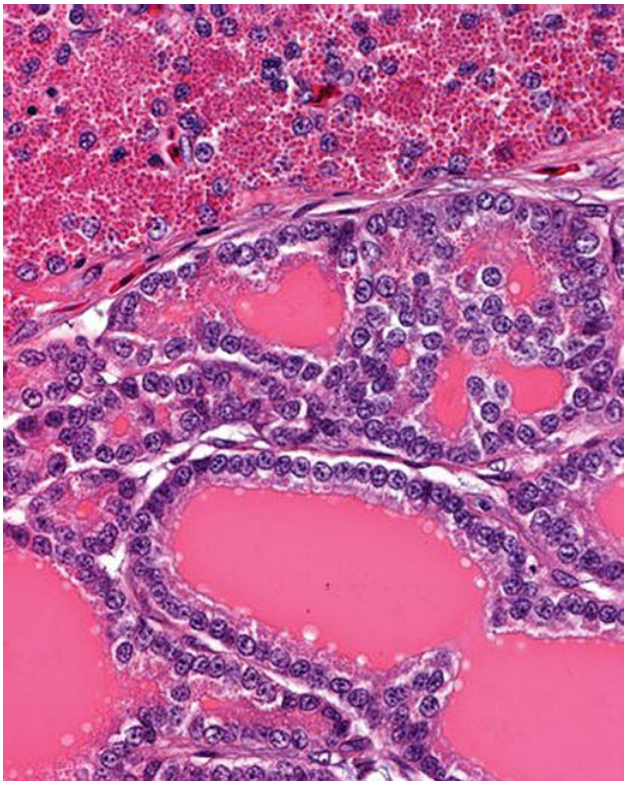


Figure 15.63 Magnal adenocarcinoma showing the well-defined margin between normal secretory tissue with cellular cytoplasm containing eosinophilic granules of ovalbumin (above) and very lightly granular tumor cells (below). H&E, ×400.

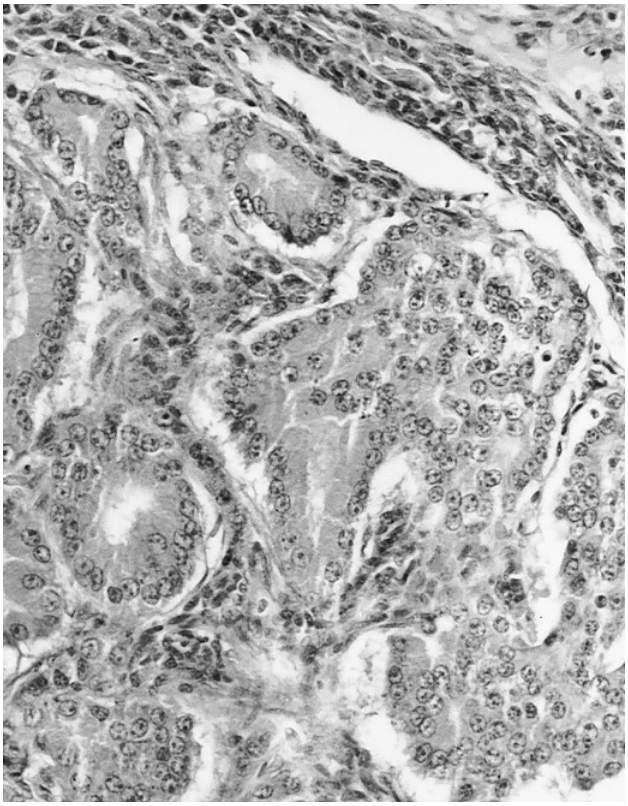


Figure 15.64 Implant of magnal adenocarcinoma deep in the ovary showing capsule around adenocarcinomatous cells. Despite the apparent aggressiveness of this tumor, mitotic figures are not prominent. H&E, ×200.

adenocarcinomas; the latter finding was attributed to cellular dedifferentiation or return of the cell to a less differentiated state. Cells of oviductal adenocarcinomas retain receptors for estrogen and progesterone (5) and the growth of these cells *in vitro* is stimulated by estrogen (4). In a recent study, few oviductal adenocarcinomas displayed immunostaining for v-erbB, the chicken oncogenic form of the epidermal growth factor homologous to human HER-2/neu, but it was present in many ovarian adenocarcinomas, especially those which were large (57). The organ or tissue of origin of many metastatic adenocarcinomas observed in the abdomens of poultry cannot be determined and these are best described as "of undetermined origin".

Testis

Sertoli Cell Tumor

Sertoli cell tumors are rare in chickens (20) and a few have been reported in Japanese quail (46), a duck (24), pigeon (96), and a goose (118). There is a single case report of a tumor arising in the testicular remnants of an incompletely surgically-caponized chicken (111). Elongated Sertoli cells are commonly intratubular (Figure 15.67) and arranged at right angles to tubular basement membranes, while in other cases there may be areas with solid sheets of cells. Nuclei are hyperchromatic and there often is a high mitotic rate.

Seminoma

Seminomas have been reported in chickens, ducks, quail, guinea fowl, and pigeons (56). These tumors are commonly unilateral and consist of intratubular or broad sheets of round to polyhedral cells (Figure 15.68) with eosinophilic cytoplasm and irregularly round, eccentrically placed nuclei. Multinucleated syncytia may be present. Some tumors have been metastatic.

Interstitial (Leydig) Cell Tumors

Interstitial cell proliferations may be present in seminomas of chickens (20), but interstitial cell tumors have not been described in poultry. Interstitial cells were a component of a mixed cell tumor affecting both testes of a duck, but only the Sertoli cell component was present in a metastatic site (73).

Renal

Renal nephroblastomas and adenocarcinomas occur in chickens. Some are associated with infections with avian leukosis/sarcoma viruses (see Leukosis/Sarcoma Group).

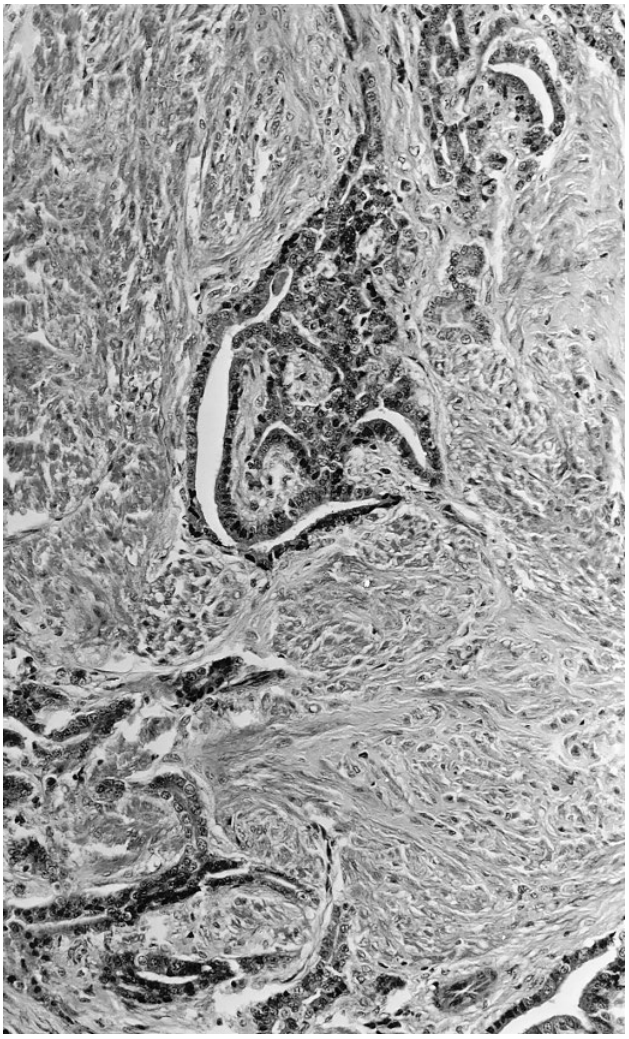


Figure 15.65 Compacted acini lined by cuboidal epithelium surrounded by dense drifts of fibrous tissue in this cirrhotic implant in duodenal serosa of a magnal adenocarcinoma. H&E, ×175.



Figure 15.66 Magnal adenocarcinoma implanted on the serosa of the isthmus is surrounded by little fibrous tissue. The dilated acinar lumina are lined by cuboidal epithelium. H&E, ×200.

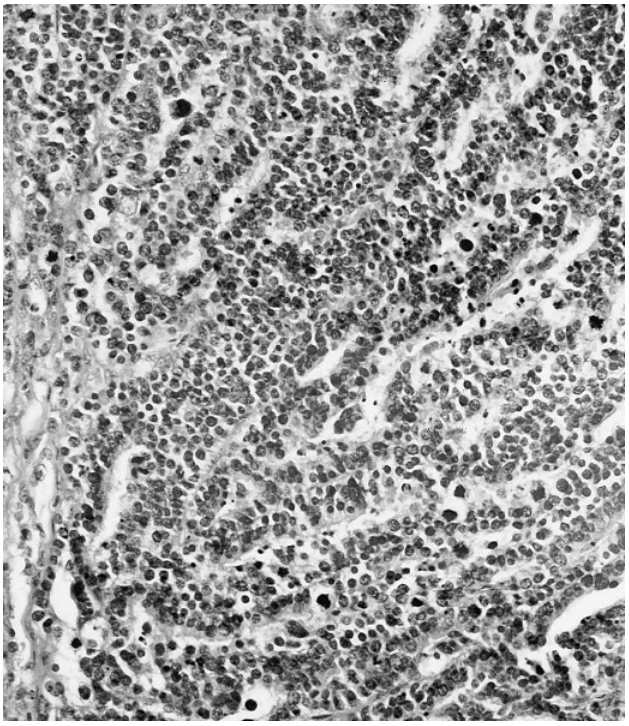


Figure 15.67 Sertoli cell tumor in a quail. The tubule-like structures are lined by cells two layers deep. H&E, ×360.

Digestive System

Alimentary Tract

Squamous cell carcinomas have been most commonly reported from the oropharynx, esophagus, and crop of chickens (56). Both humans and chickens from certain regions of northern China exhibit an increased prevalence of esophageal squamous cell carcinoma (108, 120), suggesting that common etiologic factors could play a role as chickens and humans may have common exposures to drinking water, food, and other environmental factors. However, detailed epidemiologic studies have not identified a specific cause (120, 129). Such tumors were often superficially ulcerated and composed of cords and islands of anaplastic squamous epithelial cells, some of which surrounded keratin (Figure 15.69).

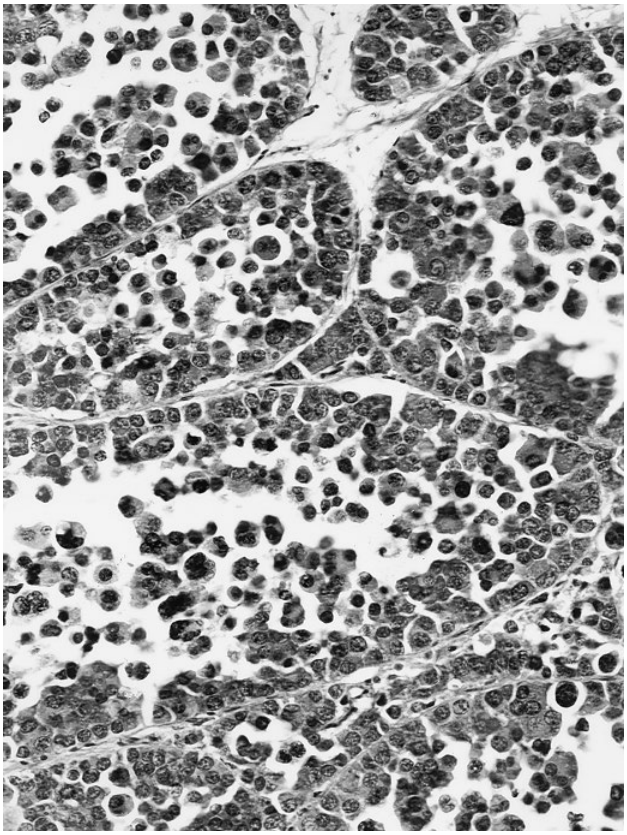


Figure 15.68 Seminoma in a duck. Lobules of pleomorphic polyhedral cells with finely granular cytoplasm; some multinucleated cells. Delicate stroma. H&E, ×180.

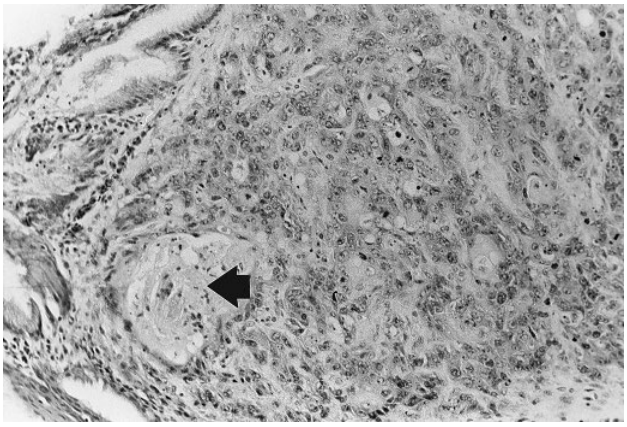


Figure 15.69 Oropharyngeal squamous cell carcinoma in an adult noncommercial chicken. Cords and islands of epithelial cells with some central keratin pearls (arrow). Mucosal gland is shown at top left. H&E, ×200.

Multiple papillomas have been described in the esophagus and crop of chickens, but that report lacks histological characterization (87).

In chickens, proventricular adenomas (21) and adenocarcinomas (20, 99) have been described, along with adenocarcinomas (20, 21, 98) of the gizzard (Figure 15.70). A proventricular adenoma was described in a duck (10).

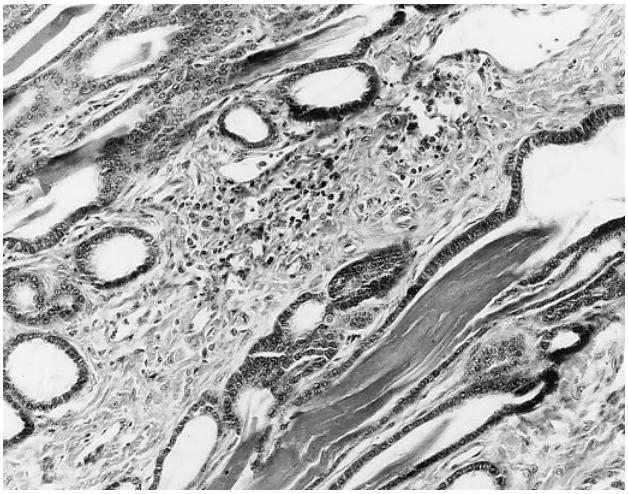


Figure 15.70 Adenocarcinoma of gizzard with growth of darkly staining cuboidal tumor cells downward into muscularis. The keratinous product of these cells is shown at lower right. H&E, ×200. (K. Langheinrich)

Adenocarcinomas of the small intestine have been described in chickens (56) and ducks (104); some of these involved the ileocecal junction. Primary adenocarcinomas affecting the intestinal tract must be differentiated from metastatic adenocarcinomas of the reproductive tract, which readily and rapidly metastasize to involve the intestine (20).

Enterogenous cysts that are lined by mucosa derived from the gastrointestinal tract have been described in young chickens. These usually present as fluid-filled cysts partially replacing the spleen and the lining mucosa often resembles that of the ventriculus (69).

Leiomyomas and leiomyosarcomas have been described affecting the gastrointestinal tract (see Musculoskeletal System). Pheasants are affected by a nodular typhlitis caused by infection with *Heterakis isolonche* with larval migration producing neoplastic submucosal nodules that are neurofibroblastic (63) or smooth muscle in origin (79) with possible metastasis to the liver.

Liver

Hepatocellular Tumors

Hepatocellular adenomas (hepatomas) and hepatocellular carcinomas are uncommonly reported in chickens (56). Adenomas consist of masses of large, polyclonal, well-differentiated hepatocytes that are compactly arranged in several cell thick cords separated by connective tissue septa (Figure 15.71); portal triads and central veins are, however, lacking in these masses and there is a low mitotic rate. Hepatocellular carcinomas often multifocally replace the liver parenchyma and there may be metastasis to the lung. In carcinomas, the tumor cells are less well differentiated, often exhibit mitoses, and may be multinucleated (20, 95).

Hepatocellular tumors in chickens have been induced with some oncogenic avian retroviruses (14) or by administration of diethyl-nitrosamine (68). These tumors appear to be not uncommon in ducks (15, 104) and some have been induced by feeding aflatoxin (23). Duck hepatitis B virus has been suggested to be involved in the development of hepatocellular carcinomas in Chinese ducks (31, 127), but aflatoxins were also suspected.

Cholangiocellular Tumors

Cholangiomas and cholangiocellular carcinomas are uncommon in poultry, with only a few cases being described in chickens, ducks, and pigeons (56). Tumors of bile ducts must be differentiated from the proliferation that accompanies chronic toxic damage to the liver due to the ingestion of hepatotoxins. Cholangiomas consist of clusters of dilated and often distorted ducts that are lined by well-differentiated epithelial cells; these ducts are separated by fibrous connective tissue (Figure 15.72). In cholangiocarcinomas, epithelial cells less commonly form defined ducts and are separated by fibroblasts (Figure 15.73).

Pancreas

Adenocarcinoma

Tumors of pancreatic epithelial cells are often difficult to distinguish from metastases of ovarian or oviductal tumors, as those tumors commonly implant upon the duodenal serosa and surface of the pancreas. Pancreatic tumors may arise from ducts (86, 97) or exocrine cells (38). Most probably arise from ductal epithelium and are composed of columnar epithelial cells with lightly basophilic cytoplasm (Figure 15.74). Tumors derived from exocrine cells morphologically resemble acini (Figure 15.75) and may or may not have cytoplasmic bodies resembling zymogen granules (38).

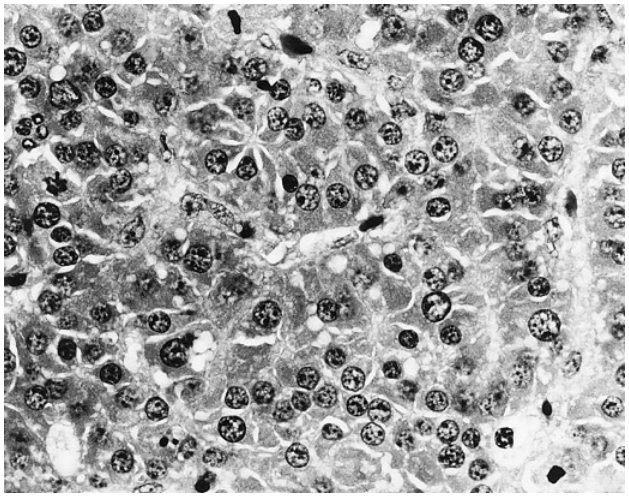


Figure 15.71 Hepatoma composed of large eosinophilic neoplastic cells, some in mitosis, forming irregular plates. H&E, ×600.

Peritoneum

Mesothelioma

Mesotheliomas have been reported in chickens (56) and ducks (74). Multiple papilliform abdominal nodules in which mesothelial cells and anaplastic fibrocytes were seen have been described in an aged female ostrich (87). There often is ascites, and tumors invest serosal surfaces of abdominal organs. Histologically, papillae are covered by cuboidal mesothelial cells supported on delicate stalks (Figure 15.76).

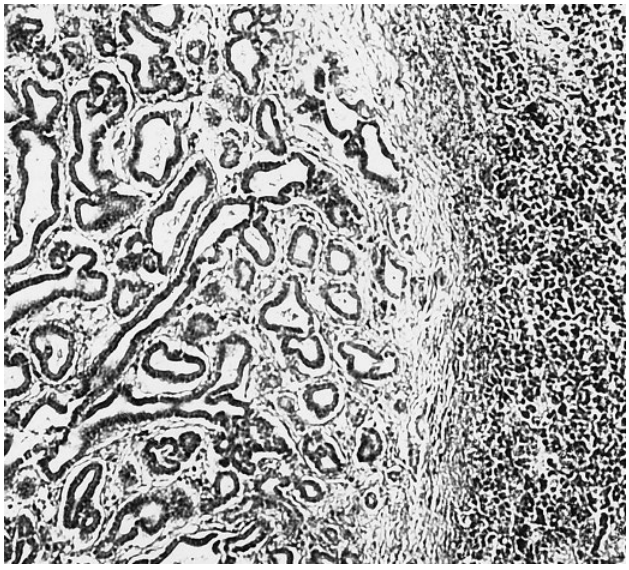


Figure 15.72 Cholangioma composed of dilated ducts in a loose fibrocytic stroma. H&E, ×75.

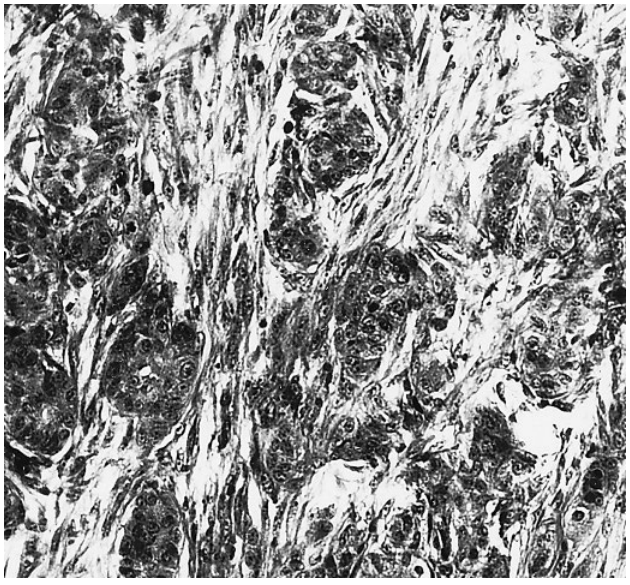


Figure 15.73 Cholangiocarcinoma composed of small clusters of epithelial cells in a fibroblastic stroma. H&E, ×190.



Figure 15.74 Pancreatic adenocarcinoma, probably of ductule cell origin, composed of columnar cells forming tubular structures among a few remnant acinar cells (arrow) ×160.

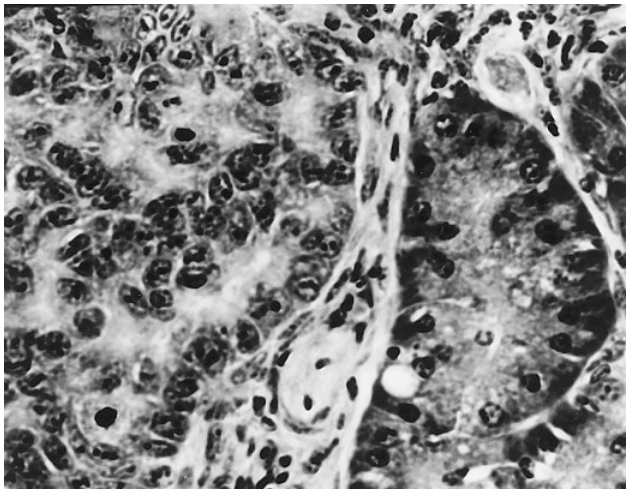


Figure 15.75 Pancreatic acinar cell adenocarcinoma with normal exocrine tissue (right) and agranular neoplastic cells (left). H&E, ×600.

Respiratory System

Infraorbital Sinus

Reece (98) reported two chickens with pea-sized cystic adenomas that extended into the infraorbital sinuses and were filled with mucin.

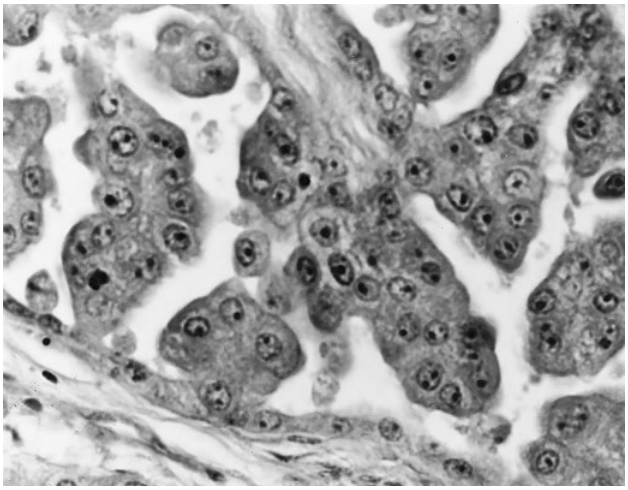


Figure 15.76 Mesothelioma with prominent neoplastic epithelial cells supported on a delicate stalk. H&E, ×600.

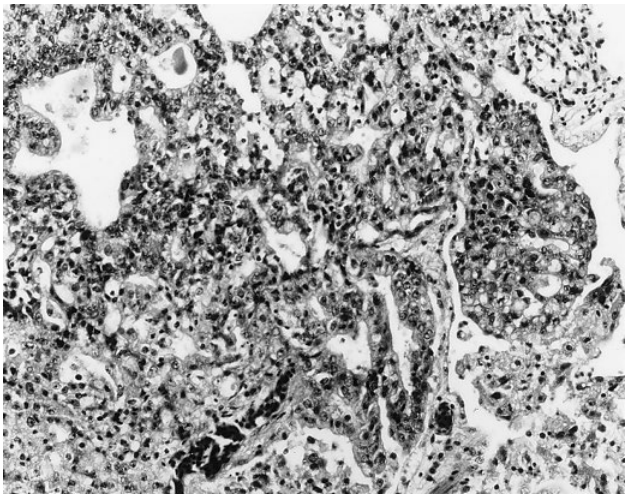


Figure 15.77 Adenocarcinoma of lung composed of a papillary growth of epithelial cells that have replaced most of the normal lung. H&E, ×200.

Lung

Adenocarcinoma

In chickens, primary adenocarcinomas of the lung appear to be extremely rare (8, 20). Campbell described three cases characterized by small nodules located near the primary bronchi and Fredrickson and Helmboldt described a papillary adenocarcinoma arising multifocally from parabronchi (38). In that case, cuboidal epithelial cells replaced most of the lung (Figure 15.77) and there were metastases in the thorax and abdomen. Primary tumors of the lung, especially in mature chickens, need to be differentiated from metastases of ovarian or oviductal adenocarcinomas, hepatocellular carcinomas, or metastases of other tumors (20, 99).

Reports of pulmonary adenocarcinomas are more common in ducks (75, 113, 128), particularly those maintained in zoos, than in other avian species. There are rare descriptions in pigeons (113).

Nervous System

Central Nervous System

Astrocytoma

Both sporadic and epizootic cases of astrocytoma (fowl glioma) have been described, commonly as multiple tumors in aged hens (56), and one case has been described in a domestic duck (110). These small masses are often near the thalamus or the base of the cerebellum (38). There frequently are perivascular accumulations of lymphocytes adjacent to the tumor, but no hemorrhage, giant cells, or areas of pressure necrosis (Figure 15.78). Masses are composed of astrocytes with extended cytoplasmic fibrillar processes (Figure 15.79). Similar masses in some breeds of chickens have been shown to be caused by infections with a specific subgroup A avian leukosis virus (61, 85, 116).

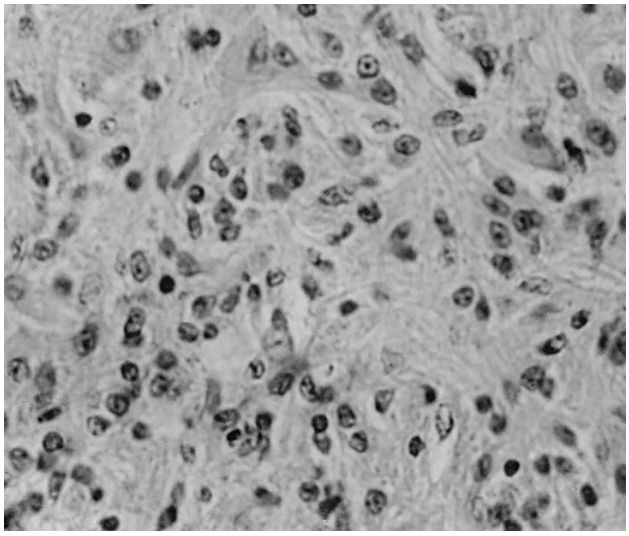


Figure 15.79 Astrocytoma composed of uniform astrocytes with extended cytoplasmic processes. H&E, ×190.

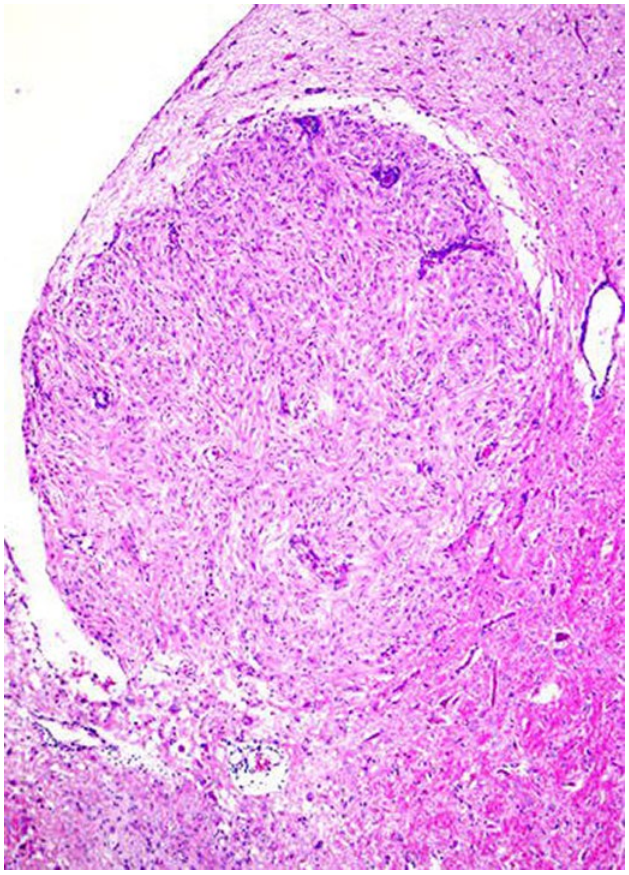


Figure 15.78 One of several clearly demarcated, but unencapsulated astrocytomas composed of fibrillar astrocytes, in anterior brain stem. There is a significant lymphocytic infiltrate around the blood vessels within the tumor and the adjacent tissue. H&E, ×100.

The small ependymoma described by Wight and Campbell (125) was discovered in the right cerebral hemisphere of a 60-day-old chicken affected by MD; the mass was within or adjacent to the lateral ventricle and consisted of vacuolated cells that occasionally formed palisades or rosettes. In the same report, two subdural angioblastic meningiomas were described in pullets displaying neurological signs: both were located near the cerebellum and consisted of multiple congested vascular sinuses that were lined by enlarged endothelial cells and separated by a prominent reticulin network.

Pineal Body Tumor

There are a few reports of pineal body tumors in chickens (20, 99, 115). In the young hen described by Swayne et al. (115), the mass was somewhat similar to a normal pineal gland, but was greatly enlarged (3×), displaced adjacent cerebellar tissue, and cells exhibited an increased mitotic rate. These characteristics, in addition to a relatively decreased number of follicular cells, identified this as a tumor rather than pineal gland hyperplasia. In the cases reported by Reece (99), there were clinical histories that included neurologic symptoms such as fine tremors and head pressing. In each bird, there was a large mass between the cerebrum and cerebellum that extended into or impinged upon the cerebellum. Tumors were composed of lobular masses of low columnar epithelial cells (Figure 15.80) surrounding a central lumen. These cells were surrounded by parafollicular cells.

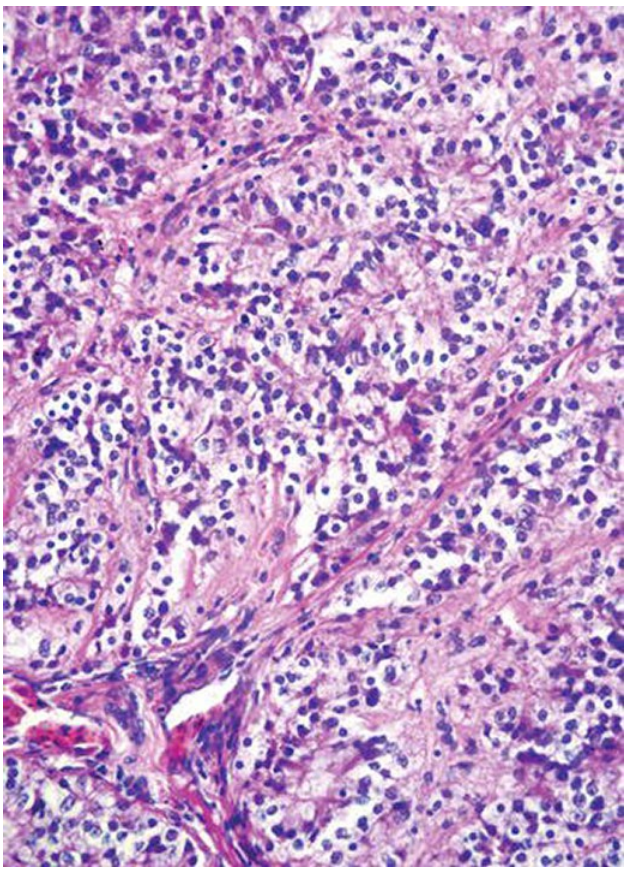


Figure 15.80 Lobules of a pineal body tumor separated by fine trabeculae. Palisaded low columnar epithelial cells with large vesicular nuclei are surrounded by smaller parafollicular cells. H&E, ×200.

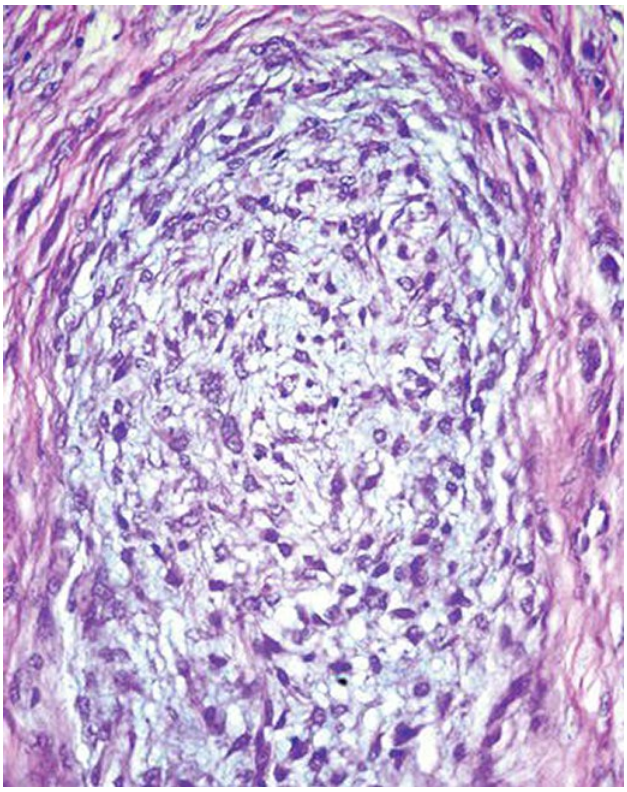


Figure 15.81 Schwannoma of the sciatic plexus showing a whorling pattern of spindle-shaped cells with central nuclei. H&E, ×400.

Peripheral Nervous System

Neurofibromas

The term neurofibroma is often used interchangeably with Schwannoma (neurilemmoma), however, Schwannomas are relatively homogenous proliferations of Schwann cells, while neurofibromas contain a mixture of nerve elements including Schwann cells. Separation of these tumors is not always possible in poultry and similar tumors have been referred to by some authors as neurogenic sarcomas (87). Tumors of the nerve sheath are well recognized in young broiler chickens (21) but also occur in older birds, often originating in major nerves or near dorsal root ganglia. Neurofibromas are often solitary masses, but multiple tumors have been described (3). Tumors are composed of fibroblast-like spindle cells that may form concentric whorls resembling nerve sheaths (Figure 15.81). In tumors with predominantly Schwann cell proliferation, there may be nuclear palisading or structures resembling sensory nerve endings (Wagner–Meissner corpuscles or Pacinian corpuscles) (20).

Neuromas

Post-traumatic neuromas commonly occur when afferent nerves attempting to regenerate become obstructed by, then entrapped within, densely collagenous scar tissue, resulting in tangled masses of poorly myelinated axons, Schwann cells, and perineurial cells separated by bands of connective tissue (Figure 15.82). These neuromas are not true neoplasms and are a well-recognized although relatively rare, sequela to beak-trimming (Figure 15.83) or toe amputation (39, 40). When young chicks or turkey poults are partially beak-trimmed, the resultant dermal scar tissue is less dense which may partially explain the relative absence of neuroma development in birds treated when they were young (28, 41).

Melanoma

Melanomas, including some described as originating in the ovary with metastasis to other organs have been described in the chicken (20). Multifocal melanomas with the appearance of malignancy have been seen affecting the skin, visceral organs, musculature, and other tissues in young broiler chickens and a 16-week-old pullet (99). Some authors described similar tumors as malignant melanomas and demonstrated that unbleached tumor cells and tumor cells bleached by potassium permanganate or hydrogen peroxide, were not immunoreactive with antibodies specific for S100 proteins, but did react with antibodies to neuron specific enolase, vimentin, and Melan-A (126). Melanocytes in avian melanocytic tumors often do not react with antibodies to S100 proteins even

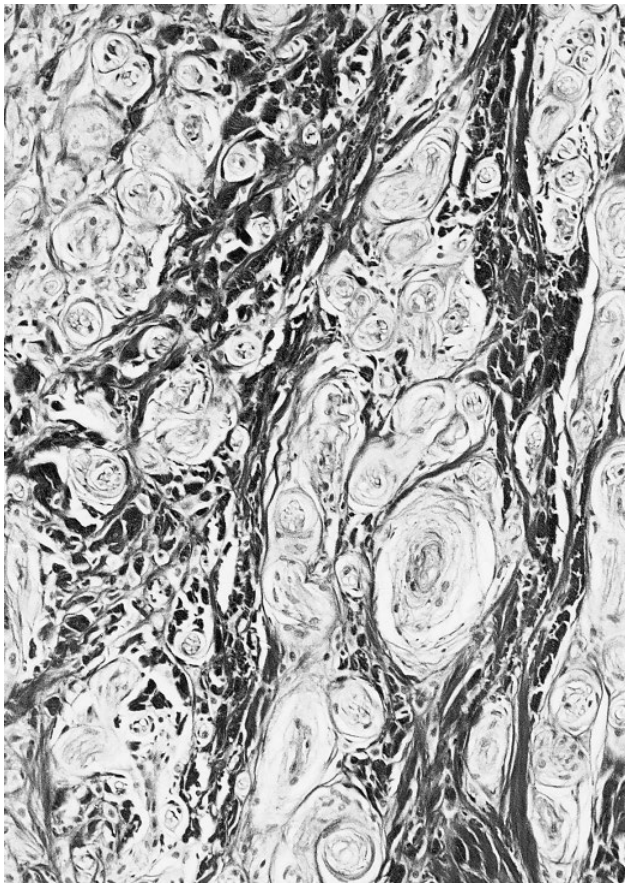


Figure 15.82 Post-traumatic neuroma in beak tip showing dense collagenous scar tissue and multiple whorls of nervous tissue composed of poorly myelinated axons, Schwann cells, and associated connective tissue. Martius scarlet blue, ×360. (C.J Randall)

though normal nerves are S100 immunopositive (55, 72). These cells may be spindloid or there may be closely packed masses of polyhedral melanocytes reminiscent of epithelial tissue (Figure 15.84). Malignant melanomas have also been described in ducks and a pheasant and melanomas, some of which were amelanotic, have been seen in the skin of pigeons (56). Benign melanomas have been seen within the eye of chickens (12, 32, 107). A small number of cases of multifocal melanomas were noted in young adult Japanese quail from one inbreed commercial strain (101).

Special Senses

Eye

Intraorbital rhabdomyosarcomas, thought to originate from the striated muscle of the iris, have been described in chickens (32). Retinoblastomas (20, 26) and melanomas involving the eye, have been described.



Figure 15.83 Lower beak neuroma due to beak trimming. The mass can range in size and may hinder food intake if large enough.

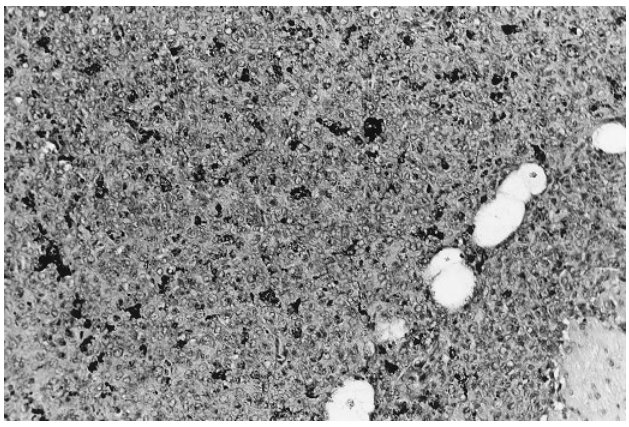


Figure 15.84 Subcutaneous melanoma of the wing of a pigeon. There is melanin pigment in many polyhedral cells. Mitotic figures are rare. The cells are densely packed, clearly demarcated from the surrounding tissue, and penetrate between muscle fibers and adipocytes (lower right). H&E, ×200.

Endocrine System

Adrenal Gland

The avian adrenal gland is composed of intermingled cords of cortical cells and enterochromaffin (medullary) cells rather than displaying a defined cortex and medulla. Two adrenal cortical adenomas have been described, one in a young chicken and one in an SPF hen (21, 99). The well-differentiated cells comprising these neoplasms contained abundant eosinophilic cytoplasm that was

occasionally vacuolated. The close anatomic relationship between the ovary and adrenal glands makes it imperative to eliminate markedly luteinized ovarian carcinomas when the ovary is also involved (20). A pheochromocytoma has been described in a 14-week-old pullet (20).

Pituitary Gland

Pituitary tumors of poultry appear to be limited to the two chickens reported by Campbell (20). One was described as a tumor of chromophobe cells that compressed the brain stem, and the second was an infiltrative acidophil adenoma.

Thymus

Thymomas have been most commonly described in chickens and one has been described in a duck (56). These have primarily been masses in the neck that are histologically characterized by sheets of epithelial cells lacking distinct cytoplasmic borders. Immunostaining of tumor cells with cytokeratin has been utilized to verify epithelial origin (11). There may be accompanying myoid cells (20).

Thyroid and Parathyroid Glands

Adenomas of the thyroid have been described in a 24-week-old pullet (87) and a 2-year-old hen (20). Both affected the left thyroid glands, contained multiple cysts and were described as containing relatively undifferentiated epithelium resembling embryonic thyroid. Microscopic cysts lined by ciliated epithelium are occasionally seen in the thyroid and are thought to be derived from thyroglossal duct remnants (20). Tumors of the parathyroid gland of chickens seem to be limited to an adenoma and a parathyroid carcinoma (48).

Integument

Subcutis

Of the various sarcomas, fibromas, and myxomas that are encountered in subcutaneous tissues of chickens, many may be induced by avian leukosis viruses (see Leukosis/Sarcoma Group), although some of these tumors are reported from SPF hens and other poultry species. Chickens systemically infected by some retroviruses may produce tumors in response to the inflammation associated with wounds, as postulated to be the cause of some mesenchymal tumors observed near the trimmed beak of hens (99). There is a report of a fibroma in a wild, hunter-killed, ring-necked duck with a large mass on the side of the head from the commissure of the beak to ventral of the ear (19).

Hemangiopericytoma

A few hemangiopericytomas have been described in chickens (38, 112), they were all benign and occurred as subcutaneous nodules in the cervical region. Histologically, they were arranged as concentric rings around blood vessels, and were composed of uniform spindle-shaped cells with a fusiform nucleus. The intervening reticulin fibers could be demonstrated by a silver stain (Figure 15.85).

Lipoma and Liposarcoma

Subcutaneous lipomas are not common in chickens (20, 97). They are generally benign, encapsulated and delicately trabeculated; and may exhibit necrosis and/or hemorrhage. These tumors are composed of large mature vacuolated adipocytes with a pale displaced nucleus: mitotic figures are rare. Liposarcomas of chickens are rare and may be locally invasive or metastasize (100); in some cases, the predominant cells are elongate and may be mistaken for fibrocytes except for the presence of small cytoplasmic fat vacuoles, others are composed of more typical immature adipocytes. Myelolipomas and erythrolipomas have not been reported thus far in chickens.

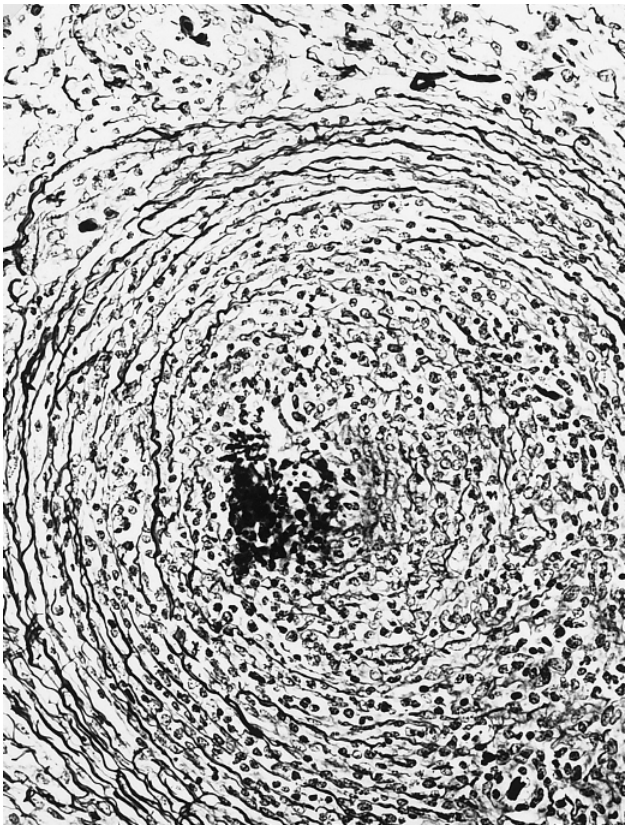


Figure 15.85 Hemangiopericytoma with concentric rings of pericytes clearly defined. Silver, ×90.

Cutis

Squamous Cell Carcinoma

The true squamous cell carcinoma of the skin of birds originates from the surface epidermis, forming infiltrating cords of anaplastic keratinocytes that invade downward into the underlying dermis. Epithelial cells are separated by intercellular bridges and resemble cells of the stratum spinosum. Since it is likely that some of these tumors, like those of other species, are induced by years of sun exposure, many have occurred on the feet and shanks of aged birds (20, 22). Although these tumors are locally invasive, only a few have been metastatic (1). The tumors originally described as dermal squamous cell carcinomas in young broiler chickens, have been designated avian keratoacanthomas, due in part due to origin from feather follicle epithelium rather than surface epidermis (109). Oropharyngeal, esophageal, and ingluvial squamous cell carcinomas also have been recognized, especially in chickens (see Digestive System).

Avian Keratoacanthoma

These tumors are most commonly found in the skin of carcasses of young chickens at slaughter (56), although some have been identified in live broiler chickens (52, 99) and a few have been seen in older chickens (66, 114). These tumors were formerly designated as "dermal squamous cell carcinoma", but avian keratoacanthoma was selected as a more descriptive term since tumors unaltered by the processing artifacts of de-feathering and scalding have the distinctive tissue architecture of keratoacanthomas, originate from feather follicle epithelium rather than the surface epidermis, are not metastatic, and, in live birds, regress (52). Carcasses with extensive lesions are condemned at slaughter, while less affected carcasses undergo trimming. The prevalence of broiler carcasses with multiple lesions commonly varies from 0.01% to 0.05%, but may be 0.09% or higher in individual flocks (53, 117, 122). In some studies, chickens slaughtered at less than 48 days of age exhibited more tumors than older birds and, tumor prevalence was cyclic, being lowest in summer months (53, 122). In other surveys, an increased number of condemnations due to these tumors were associated with dusty houses, birds placed in new houses, or certain farms (43). One report has commercial adult layer hens (55,000 hens) affected starting at 30 weeks of age and continuing for 28 weeks and regressing. Approximately 0.1% of the flock was affected in the region of the legs and toes (29).

The etiology of this condition remains unknown. Some authors have suggested an association with fowl pox because some studies by nested PCR detected DNA sequences specific for fowlpox virus in lesions (33), but other studies failed to detect evidence of fowlpox (81). In one study of these lesions, type C retroviruses were identified (109), but in other studies, viral particles were not seen ultrastructurally and evidence of avian leukosis viruses was not detected (29, 52, 81). Carcasses most commonly exhibit crater-shaped ulcers with raised margins within feather tracts (Figure 15.86), but both nodular and ulcerative lesions may be present. Smaller circular ulcers average 5 mm in diameter, but larger irregular, coalescing ulcers are present on some carcasses. In live chickens, these ulcers are filled with keratin and cell debris (Figure 15.87). Nodules are generally smaller (averaging 3 mm in one study) and appear grossly as

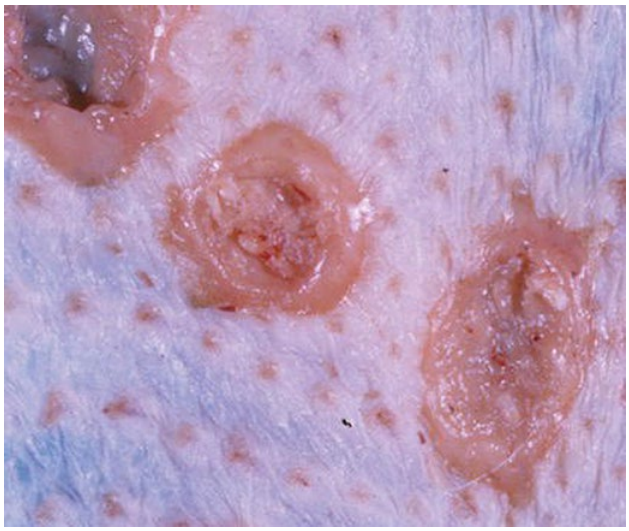


Figure 15.86 Typical carcass lesions of avian keratoacanthomas are craterous ulcers within feather tracts.



Figure 15.87 In live chickens with keratoacanthomas, ulcerative lesions contain central masses that are mixtures of keratin, cell debris, and bacteria.

enlarged feather follicles (Figure 15.88). Microscopically, nodular lesions appear as proliferative outgrowths of feather follicle epithelium (Figure 15.89), cysts originating from dysplastic feather follicles, or hyperplastic feather follicles that contain hyperkeratotic feathers.



Figure 15.88 Early keratoacanthoma lesions in the skin of live chickens are nodules in the base of feather follicles.

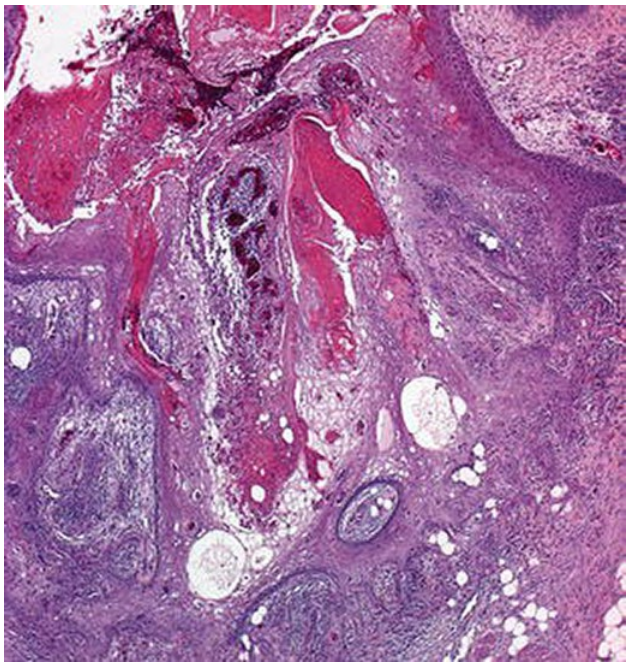


Figure 15.89 Microscopically, nodular lesions of avian keratoacanthoma are expansions of feather follicle epithelium. H&E, ×40.

Ulcers are composed of a central cup-shaped cavity lined by epithelium and filled with keratin, bacteria, and cell debris. Epithelial lips overhang this central mass of keratin and cell debris. The lining epithelium keratinizes toward the central cavity and extends thin strands of keratinocytes into the surrounding dermal fibroplasia (Figure 15.90). Carcass lesions are often extensively altered by post-slaughter de-feathering with loss of the central keratin core and much of the lining epithelium. In affected live chickens, nodules progressed to ulcers and all lesions eventually regressed (52).

Feather Folliculoma

Multiple cystic structures found on the medial surfaces of the wings of adult hens have been described (99). These cysts were filled with keratin and feather remnants, and were lined by squamous epithelial cells. In some areas, there was abrupt keratinization of the lining epithelium, while in other areas, there was disorganized feather follicle epithelium (Figure 15.91). There was an intense inflammatory reaction in the adjacent dermis. Similar cysts have been described in turkeys (27).

Intracutaneous Keratinizing Epithelioma

These benign masses were characterized by multiple nodules in the facial skin of adult hens, each marked by a central pore. These cysts were lined by stratified squamous epithelium with orderly maturation from a peripheral basal layer. The cyst lumen contained lamellated keratin (Figure 15.92) but no feather remnants. Little

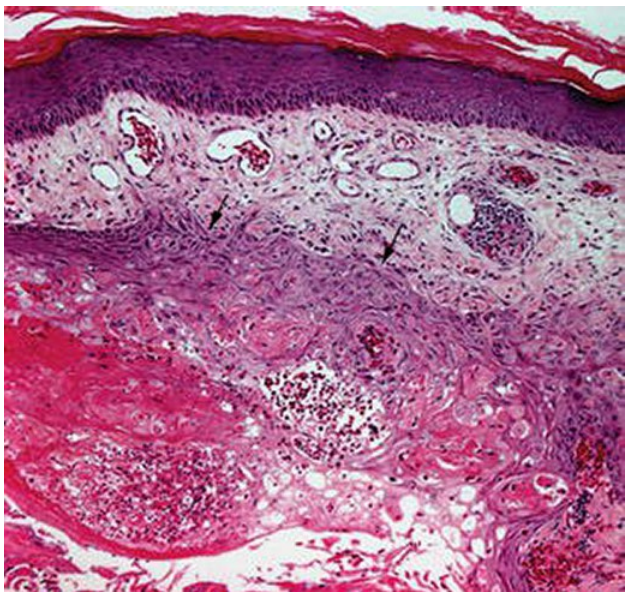


Figure 15.90 A section through the epithelial lip of an ulcerative keratoacanthoma from a live chicken shows the centrally keratinizing and peripherally invasive lining epithelium (arrows) adjacent to the central keratin and cell debris. H&E, ×100.

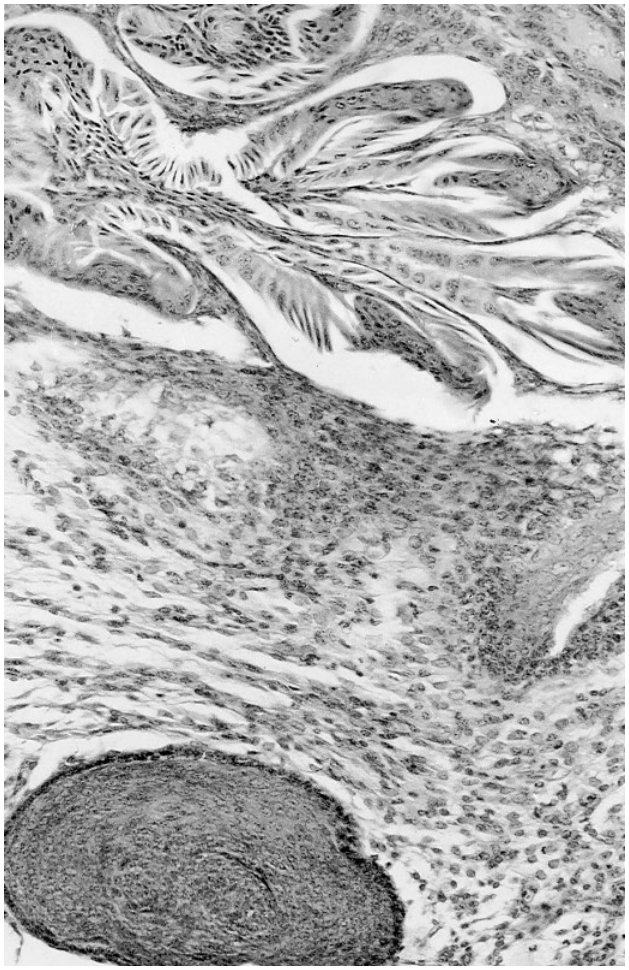


Figure 15.91 Edge of a feather folliculoma showing dysplastic specialized feather-forming epithelium and an adjacent cord of basal cells. The lumen was lined by stratified cuboidal to squamous epithelium with abrupt keratinization and contained keratin and feather remnants. H&E, ×180.

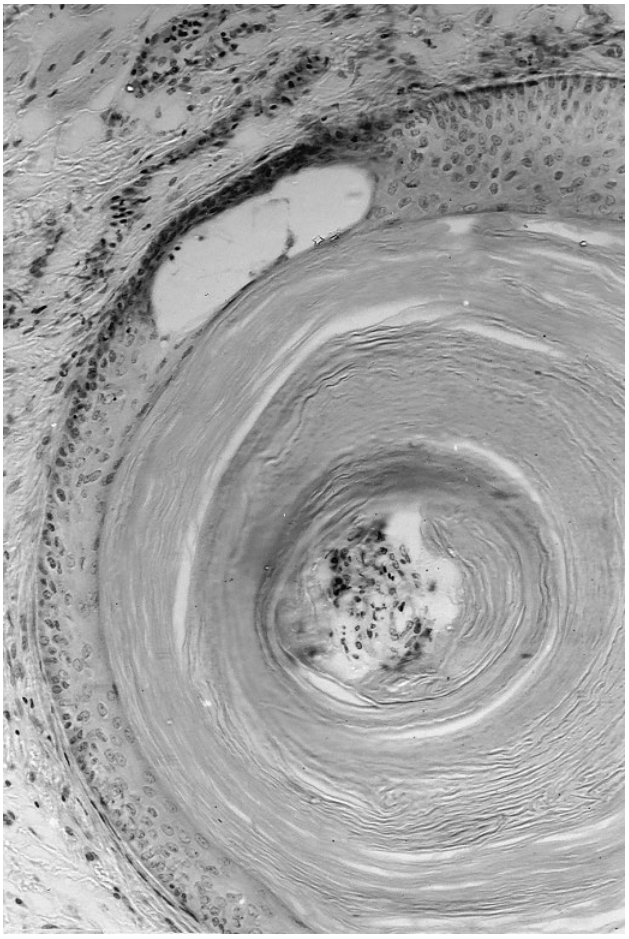


Figure 15.92 The lumen of this intracutaneous keratinizing epithelioma contains lamellated keratin. The epithelium shows basal cells aligned on a distinct basal lamina and progression to polyhedral cells with abrupt keratinization. Note the small intraepithelial bulla. H&E, ×190. (Reece (99). Courtesy of Taylor and Francis, Ltd, www.tandfonline.com, on behalf of Houghton Trust, Ltd.).

inflammation surrounded these cysts unless there was rupture of the cyst wall (99).

Other Tumors of the Cutis

Acanthomas are firm, often conical masses that protrude from the scaled epithelium on the plantar surface of the tarsometatarsus. Tumors are composed of keratin whorls on a base of fibrous connective tissue that contains islets of squamous epithelium with central basal epithelial cells (20).

Mast cell tumors are extremely rare in poultry, but have been described in a few adult chickens (54, 58, 91). In one, there was a solitary lesion of the eyelid, while in the other two birds, there were multiple dermal tumors composed of solid sheets of round to ovoid mast cells. In one hen, there was metastasis to the lung (54).

Xanthomas are not true neoplasms, but are masses of foamy macrophages and multinucleated giant cells that surround cholesterol clefts. The multiple xanthomas seen in chickens in the 1950s were attributed to chlorinated hydrocarbons contaminating the fat added to rations. The masses were considered to possibly be a reaction to metabolites of this material accumulating in dermal fat (106).

Musculoskeletal

Leiomyoma and Leiomyosarcoma

Leiomyomas of the ventral ligament of the oviduct are common in domestic fowl and Japanese quail (see Urogenital System), other smooth muscle tumors are more rarely reported. Leiomyomas have been described in the intestine of ducks (98), the trachea of broiler chickens (21, 98, 102) (Figure 15.93), attached to the pancreas (97) or in the liver of pigeons (25), and in the

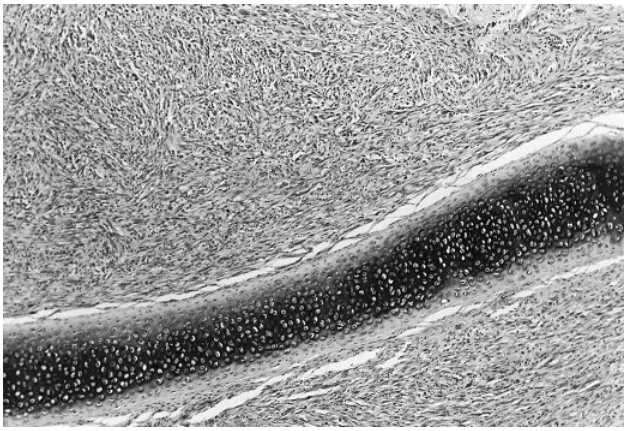


Figure 15.93 Leiomyoma of the trachea of a broiler chicken. Bundles and whorls of smooth muscle fibers in the lamina propria (upper portion) penetrate between the cartilaginous rings and extend into the adjacent adventitia (lower right). H&E, ×100.

muscularis of chicken proventriculus, ventriculus (gizzard), and duodenum (21, 99). Leiomyosarcomas have been described in chicken tracheal muscle (20), chicken ventriculus (gizzard) (105), hen ovary (65), chicken intestine (3), and thigh skeletal muscle of a hen with metastasis (70). Leiomyosarcomas have also been described in the lung and skin of pigeons (80, 82).

Rhabdomyoma and Rhabdomyosarcoma

Rhabdomyomas and rhabdomyosarcomas have been primarily seen affecting the skeletal muscle of the breast and legs and less commonly the musculature of the hearts of young chickens (20, 21, 87), and a rhabdomyoma has also been described in the eyelid of a racing pigeon (97). A rhabdomyosarcoma originating in cranial muscle and invading the brain occurred in a 7-month old hen (47), and rhabdomyosarcomas metastatic to the lung of chickens have also been described (71, 99). Two intraocular rhabdomyosarcomas have been described in chickens (32). These muscle cell tumors are generally histologically characterized by spindle, strap, flame, or racquet cells and multinucleated cells (Figure 15.94). Cross striations may be present only in rare cells; even examinations with polarized light or phosphotungstic acid-stained sections may not detect striations in the intensely eosinophilic cytoplasm of anaplastic myoblasts. Immunohistochemical examinations for vimentin, myoglobin, muscle specific actin, and desmin have been useful in some avian rhabdomyosarcomas (34).

Osteoma and Osteosarcoma

Osteomas have been described in the feet of ducks (97) and affecting various sites in a few chickens (20, 21, 99).

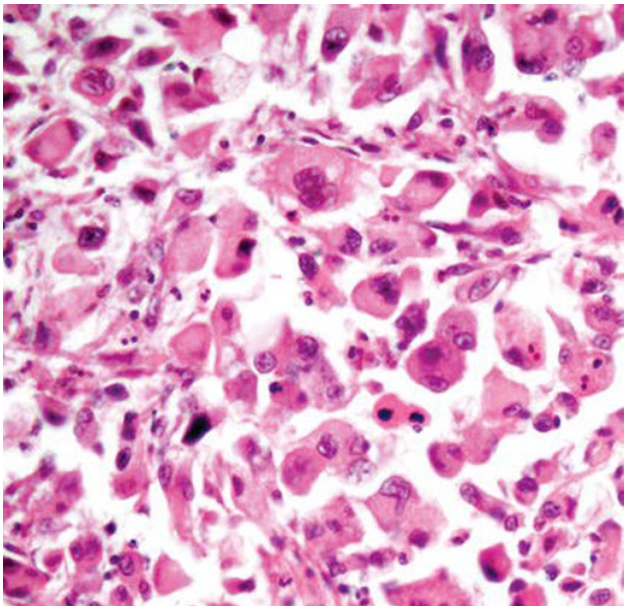


Figure 15.94 Rhabdomyosarcoma. Some cells are strap-like, whereas others are large and polyhedral. The cytoplasm is eosinophilic and some cells contain multiple nuclei. H&E, ×400.

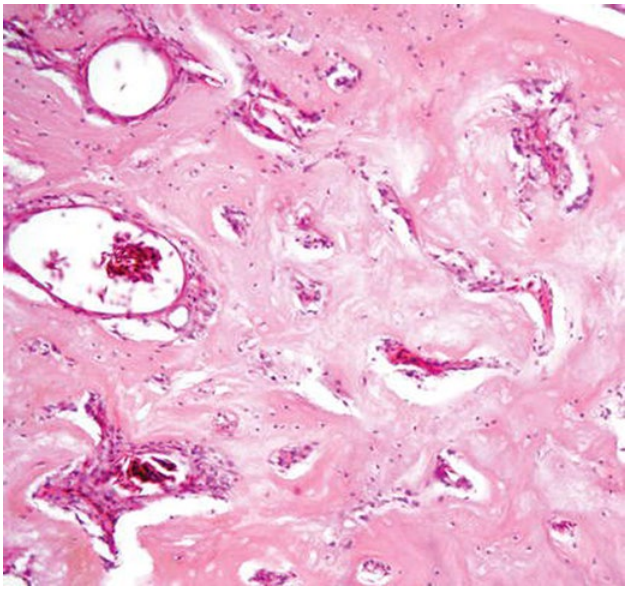


Figure 15.95 Osteoma showing thick irregular trabeculae. H&E, ×100.

These osteomas were well circumscribed and were composed of disorganized bone trabeculae (Figure 15.95). Several osteosarcomas, some of which were metastatic, were described affecting long bones, ribs, and vertebrae of young chickens (21). Another osteosarcoma affecting an aged hen was also metastatic (110). A report describing osteosarcoma in free range aged hen had involvement of the vertebrae with metastasis to the liver (30).

An osteosarcoma has also been described affecting the tibiotarsus of a Japanese quail (97) and the foot of a goose (78).

Chondroma and Chondrosarcomas

A single chondroma has been described as originating at a costochondral junction in a young chicken (20). Multifocal chondromas have been described in the footpads of geese and ducks (97). These tumors were characterized by lobules of chondrocytes separated by trabeculae (Figure 15.96). Dittmer et al. described a chondrosarcoma in an aged free range hen that involved the sternum and extended into muscle (30). Chondrosarcomas in poultry are exceedingly rare.

Other Tumors

Teratoma

Teratomas are composed of tissues arising from more than one germinative layer and commonly contain a disorganized mixture of variously differentiated epithelial cells, bone, cartilage, smooth muscle, fat, or other tissues including nervous tissue, melanocytes, or cardiac muscle. Often, ciliated columnar epithelial cells with goblet cell differentiation line tubules or cysts. Other epithelial islands may be composed of squamous epithelial cells that surround keratin (resembling primitive feather follicles). Some tumors are described simply as abdominal masses (62). In chickens, teratomas are reported to arise more commonly in the testis than ovary (20, 21, 62). Teratomas may also arise at other sites (20, 21, 48, 93). Teratomas have also been induced in chickens by the injection of various metallic ions into the testis (20, 49). Teratomas have been observed in several ducks and a goose (18, 89, 103).

In contrast to teratomas, hamartomas are a focal overgrowth of mature tissue indigenous to the organ or location in which it is found; these have not been reported in poultry.

Multicentric Histiocytosis

Multicentric histiocytosis (histiocytic sarcomatosis, systemic spindle-cell proliferative disease) primarily affects young broiler chickens, producing hepatomegaly and splenomegaly. Numerous small (0.5 to 2 mm) white masses are grossly evident in the spleen, liver, and kidneys. Some diseased birds are pale (anemic) and smaller than flock mates. Microscopically, nodules of spindle-shaped cells replace the spleen (Figure 15.97), liver, kidneys, and other organs most commonly including bone marrow, pancreas, intestine, proventriculus, and lungs (9, 45, 51, 90, 91). Germinal centers may be present within these nodules, especially in the spleen; liver nodules may contain a more heterogenous population of cells, including plasma cells and some typically bulge into portal vessels. Lesions are not accompanied by myeloid leukosis (myelocytomatosis). Spindle cells contain abundant eosinophilic cytoplasm and elongated ovoid to more pleomorphic nuclei (Figure 15.98). Mitoses are



Figure 15.96 Multifocal chondroma of the footpad of a goose showing lobules of cartilage that are separated by fibrovascular trabeculae. H&E ×100.

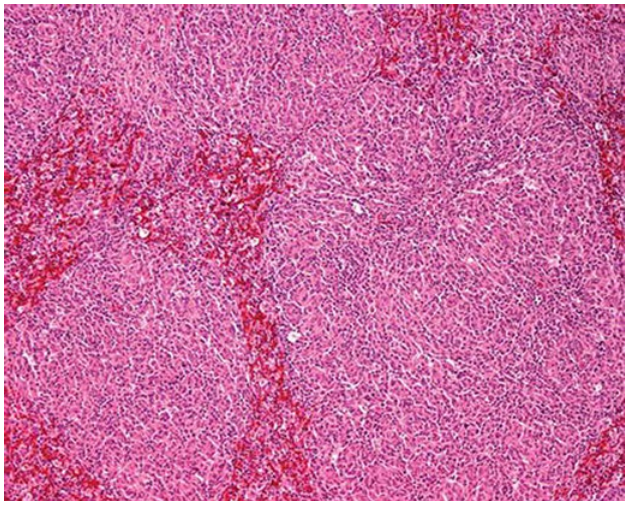


Figure 15.97 In multicentric histiocytosis, nodular masses of spindle-shaped cells multifocally replace the spleen. H&E, ×100.

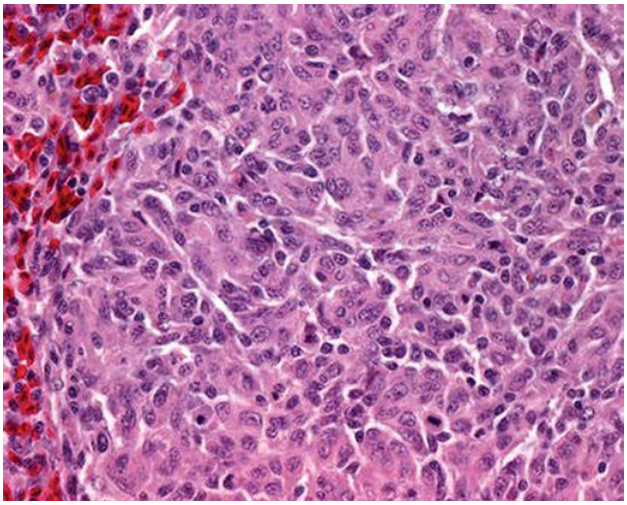


Figure 15.98 Histiocytic spindle-shaped cells in the spleen of a chicken with multicentric histiocytosis contain elongated and pleomorphic nuclei. H&E, ×400.

common, but there are no multinucleated cells. Spindle cells are immunohistochemically positive for markers characteristic of antigen-presenting tissue macrophages or dendritic cells (9, 90). Infection of meat-type chickens at day of hatch with strains of subgroup J avian leukosis virus have reproduced these lesions, but only in birds that are persistently viremic with an ineffective antibody response (90).

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Section III

Bacterial Diseases

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16

Salmonella Infections

Richard K. Gast and Robert E. Porter, Jr.

Summary

Agent, Infection, and Disease. Two nonmotile organisms, *Salmonella* Pullorum and *S.* Gallinarum, are host-specific for avian species, causing pullorum disease (an acute systemic disease of chicks or poults), and fowl typhoid (an acute or chronic septicemic disease that most often affects mature birds), respectively. Both have been responsible for serious economic losses since the inception of commercial poultry production and remain widely prevalent in some regions. Most other *Salmonella* serovars (i.e. paratyphoid salmonellae) are motile and can infect a wide variety of hosts, including invertebrate and vertebrate wildlife, domestic animals, and humans, and are principally vehicles of foodborne illness. Although paratyphoid infections of poultry are common, they seldom cause acute clinical disease except in highly susceptible young birds exposed to stressful conditions. More often, paratyphoid infections of poultry are characterized by asymptomatic (although sometimes persistent) colonization of the intestinal tract and internal organs, potentially leading to contamination of finished carcasses. Some serovars, especially *S.* Enteritidis, are deposited inside the contents of eggs laid by systemically infected hens.

Diagnosis. Clinical signs and gross lesions associated with *Salmonella* are not completely distinctive from other bacterial infections, so diagnosis generally requires the isolation or identification of causative organisms. Both conventional culturing and probes for specific DNA target sequences are applied to samples from tissues, eggs, voided feces, and poultry house environments.

Intervention. Because there are many potential sources for the introduction of salmonellae into poultry flocks, effective strategies for controlling these zoonotic pathogens require the sustained implementation of comprehensive risk reduction practices throughout the production continuum. Many *Salmonella* serovars, especially *S.* Pullorum and *S.* Gallinarium, are vertically transmitted so control efforts must include testing and eradication programs in breeding flocks.

Introduction

Salmonella infections in poultry flocks can cause acute and chronic clinical diseases but have received greater international attention in recent years because of their role in foodborne outbreaks of human illness. Contaminated poultry meat and eggs are among the most frequently implicated food vehicles of salmonellae. As a consequence of both public health and flock health concerns, *Salmonella* infections cause economically significant losses for poultry producers in many nations and absorb large investments of government and private resources for testing and control efforts. Accordingly, the present edition of this chapter focuses on these food-transmissible "paratyphoid" salmonellae.

Information about pullorum disease (*S.* Pullorum) and fowl typhoid (*S.* Gallinarum) has been reduced in this chapter from previous editions of *Diseases of Poultry*, and the general salient features of these microorganisms are included with other salmonellae. However, the importance of these diseases in early commercial poultry production in the United States should not be discounted, and they remain significant problems in countries that are developing intensive poultry production or practice high volume poultry breeding. Both pullorum disease and fowl typhoid were largely eliminated from commercial poultry flocks in the United States by the middle of the twentieth century after implementation of the National Poultry Improvement Plan (NPIP), a pullorum-typhoid control program, although outbreaks occasionally occur in small or backyard poultry flocks. The NPIP, a cooperative program of state agencies, the U.S. Department of Agriculture, and the poultry industry (312), was originally established in 1935 to control pullorum disease in chickens. The reader is referred to

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earlier editions of *Diseases of Poultry* for detailed information (history, transmission, microbiology, gross and microscopic lesions) regarding *S.* Pullorum and *S.* Gallinarum (286).

Salmonella enterica subspecies *arizonae* (*S.* Arizonae) is a paratyphoid *Salmonella* which is long recognized as economically significant in turkey production. This organism causes egg-transmitted disease involving septicemia and neurological signs in turkey poults and decreased egg production in turkey breeders (288). Chickens can be infected but generally without any significant productivity effects. This bacterium has no host specificity and has been isolated from a variety of avian, mammalian, and reptilian species (111). The reader is referred to previous editions of *Diseases of Poultry* for more details about this pathogen and its associated disease (285).

Economic Significance

Human illnesses resulting from the consumption of poultry products contaminated by *Salmonella* can be expensive for the poultry industry, governments, and affected individuals. The total combined costs of medical care, lost productivity, and premature deaths resulting from foodborne *Salmonella* infections of humans in the United States have been estimated as $4–11 billion per year (276). Widely circulated media reports regarding *Salmonella* contamination of particular foods can reduce consumer demand for those items. International markets for poultry products are increasingly subject to restrictions based on food safety considerations.

Poultry producers face many direct expenses from *Salmonella* infections in their flocks. Infections acquired vertically from parents or horizontally in the hatchery can cause growth depression or even mortality in young chicks or poults. Other diseases or stressful conditions can predispose mature poultry to severe infections. Likewise, infection with *Salmonella* can increase susceptibility to other pathogens. Preventing the transmission of salmonellae to progeny or to humans can be expensive for producers. For example, risk reduction practices for controlling *S.* Enteritidis infections in laying flocks (including biosecurity, facility cleaning and disinfection, rodent control, vaccination, and testing) were estimated to add nearly 1 cent per dozen to the costs of egg production (235). Proposed regulations for controlling this pathogen in the United States were projected to cost the egg industry $81 million annually, but save $1.4 billion in annual human health expenses (313). In most economically developed nations, the only costs regularly attributed to the poultry-specific pathogens, *S.* Pullorum and *S.* Gallinarum, are associated with the administration of widespread testing programs for commercial breeding flocks. However, in many developing countries, fowl typhoid remains a cause of economically significant disease losses.

Public Health Significance

Salmonellae are consistently reported to be among the leading international sources of foodborne human disease. The incidence of laboratory-confirmed human *Salmonella* infections in the United States in 2010 (17.6/100,000 population) was higher than any other foodborne pathogen (51). Poultry products are often identified as prominent sources of salmonellae which cause human illness. More than 70% of human *Salmonella* infections in the United States have been linked to the consumption of contaminated chicken, turkey, or eggs (151). An estimated 208,400 annual human *Salmonella* infections in the United States can be attributed to poultry meat, more than any other meat source (178). Throughout much of the world, eggs and egg-containing foods have been implicated as the principal vehicles for the transmission of *S.* Enteritidis infections (146). The incidence of human salmonellosis has been correlated with *Salmonella* prevalence in commercial poultry flocks. A multinational European study reported that laying hens were the leading reservoir for transmission of salmonellae to humans, associated with 42% of all cases (87). Humans can also be infected with *Salmonella* via handling live poultry. Many of the serovars that are most prevalent in humans (especially *S.* Enteritidis and *S.* Typhimurium) are similarly common in poultry.

Etiology

Classification and Nomenclature

The genus *Salmonella* is a member of the family Enterobacteriaceae and consists of 2 genetically distinct species, 1 of which *(S. enterica)* includes 6 subspecies determined by patterns of biochemical reactions. Only 1 subspecies *(S. enterica* subspecies *enterica*) is associated with disease in warm-blooded animals, and includes more than 2,500 motile and nonhost-adapted serovars such as *S. enterica* subspecies *enterica* serovar Enteritidis and *S. enterica* subspecies *enterica* serovar Typhimurium. The traditional serovar designations (*S.* Enteritidis, *S.* Typhimurium) are routinely employed for concise diagnostic classification and epidemiologic analysis. Although the causative agents of pullorum disease and fowl typhoid have been taxonomically assigned to a single serovar, *S.* Pullorum-Gallinarum, these 2 organisms are genetically and biochemically distinguishable and the 2 biovars are accordingly more commonly designated separately as *S.* Pullorum and *S.* Gallinarum.

Morphology and Staining

Salmonellae are straight, nonspore-forming rods, measuring about 0.7–1.5 × 2.0–5.0 μm. Salmonellae are Gram-negative, but cells can readily be stained with common dyes such as methylene blue or carbolfuchsin. Paratyphoid *Salmonella* are usually peritrichously flagellated and motile, although naturally occurring nonmotile mutants are occasionally encountered. Both *S.* Pullorum and *S.* Gallinarum are characteristically nonmotile.

Growth Requirements

Salmonellae are facultatively anaerobic and can grow well under both aerobic and anaerobic conditions. The optimum temperature to support *Salmonella* multiplication is 37 °C, but some growth is observed over a range from about 5 °C to 45 °C. Salmonellae can grow within a pH range of approximately 4.0–9.0, with an optimum around 7.0, although cellular components such as flagella and fimbria may not be expressed under extreme pH conditions. The nutritional requirements of salmonellae are relatively simple, and most culture media that supply sources of carbon and nitrogen can support their growth. The viability of *Salmonella* cultures can be maintained for many years in simple media, such as peptone agar or nutrient agar, which have been stab-inoculated, sealed, and held at room temperature.

Colony Morphology

Typical *Salmonella* colonies on agar media are about 2–4 mm in diameter, round with smooth edges, slightly raised, and glistening.

Biochemical Properties

Typical paratyphoid salmonellae ferment glucose (to produce both acid and gas), dulcitol, mannitol, maltose, and mucate, but do not ferment lactose, sucrose, malonate, or salicin. However, some *S.* Arizonae strains are able to ferment lactose slowly, but may not utilize dulcitol. Most *Salmonella* strains can produce hydrogen sulfide on many types of media, decarboxylate ornithine and lysine, utilize citrate as a sole source of carbon, and reduce nitrates to nitrites. Paratyphoid salmonellae do not hydrolyze urea or gelatin and do not produce indole.

Most paratyphoid salmonellae can be readily distinguished from the avian host-adapted biovars, *S.* Pullorum and *S.* Gallinarum, on the basis of the inability of *S.* Pullorum strains to ferment mucate or dulcitol and the inability of *S.* Gallinarum strains to decarboxylate ornithine or produce gas from glucose fermentation. In addition, paratyphoid salmonellae are usually motile but *S.* Pullorum and *S.* Gallinarum are typically nonmotile.

Susceptibility to Chemical and Physical Agents

Chemical Disinfectants

Diverse chemical treatments have shown efficacy for reducing the levels of salmonellae associated with hatching eggs, feed, and poultry facilities. However, recontamination after disinfection can diminish any potential benefits. Sublethal chemical treatment has been reported to induce bacterial thermotolerance and antibiotic resistance (6, 273), but exposure to disinfectants may also suppress the ability of salmonellae to infect chickens (263).

Significant reductions of salmonellae on hatching eggs have been achieved using a variety of disinfectants, including formaldehyde, hydrogen peroxide, ozone, lactic acid, peroxidase catalyzed compounds, quaternary ammonium compounds, and biguanides (43, 72). Disinfectants can be applied to hatching eggs by fumigating, spraying, or dipping. Significant *Salmonella* reductions in contaminated poultry feeds have been reported following the inclusion of ethyl alcohol or organic acids such as zinc acetate and zinc propionate (152, 250). However, a study of 12 potential antagonists of salmonellae in poultry feed (including organic acids) found that only formalin was consistently effective (293).

Chemical disinfectants (especially phenolic and quaternary ammonium compounds) are also widely used in poultry housing facilities. However, poultry houses sometimes remain contaminated with *Salmonella* after cleaning and disinfection (94). Heavily contaminated areas of poultry houses, including floors, dropping boards or belts, and nest boxes, can be especially difficult to disinfect completely (45). Disinfectants may not be effective against all bacterial strains, especially those which form biofilms (221). The presence of chick fluff, feces, feed, or wood shavings can interfere with disinfectants (30). Some disinfectants may be less potent when used with well, stream, or pond water (84). Improper performance of cleaning and disinfection protocols, recontamination of the environment by infected rodents, or bacterial tolerance to biocides can all compromise the efficacy of disinfection efforts (223, 237). Formalin disinfection or formaldehyde fumigation can be highly effective for decontaminating poultry facilities, but safety considerations have limited their use (45). Ozone fumigation has been considered as a safer (although less effective) alternative (333).

Physical Agents: Heat and Irradiation

Except for a few distinctively thermoresistant strains such as *S.* Senftenberg 775W, salmonellae are generally susceptible to destruction by heat. Thermal decontamination (during cooking or pasteurization) is critical for ensuring the microbial safety of poultry products, but heat disinfection has fewer applications

during live poultry production. Steam pelleting treatment of poultry feed under precisely defined conditions inactivates salmonellae in a manner dependent on temperature, time, and moisture (165). At high relative humidity, a 7-log reduction in *Salmonella* contamination was achieved by heating fresh chicken litter to 70 °C for 80–100 minutes (194). Irradiation has also been considered for eliminating salmonellae from poultry feeds and environmental surfaces. Gamma radiation was successfully applied to diminish *Salmonella* levels in poultry feeds (208). Ultraviolet radiation has reduced *Salmonella* contamination on eggs and egg belts (115).

Environmental Factors

Environmental persistence by paratyphoid salmonellae creates continuous opportunities for horizontal transmission of infection within and between flocks. *S.* Enteritidis has been observed to survive in litter and feed for 26 months after removal of an infected flock (81). *S.* Senftenberg persisted for more than 2 years in a poultry house despite depopulation, cleaning, disinfection, drying, and numerous intervening negative environmental tests (254). High moisture levels are particularly important risk factors for *Salmonella* persistence. The numbers of viable *Salmonella* in poultry litter are directly related to water activity and accordingly tend to increase in regions of houses with reduced airflow (240). Reducing water activity or manipulating pH to antibacterial extremes have reduced *Salmonella* populations in poultry litter (253). Strains of *S.* Pullorum and *S.* Gallinarum are also capable of extended survival under favorable environmental conditions, although they are generally less resistant to heat and chemicals than most paratyphoid salmonellae.

Antigenic Structure

The traditional Kauffmann–White classification of salmonellae into serovars is based on both somatic and flagellar antigens. Somatic "O" antigens are determined by polysaccharides associated with the cell body and are identified by arabic numerals. Serogroups (designated with uppercase letters) are defined by somatic antigens unique to members of that group. Most *Salmonella* isolates found in poultry belong to serogroups B, C, or D. The "H" antigens are determined by flagellar proteins and are usually identified by lowercase letters. Flagellar antigens sometimes occur in 2 different phases. The serovar of a particular *Salmonella* isolate is determined by the combination of its O and H antigens. Isolates of *S.* Pullorum fall into 3 distinct groups which express different variants of O antigen 12, thereby complicating the application of serologic screening tests for infection (117).

Strain Classification

Phage Typing

Differentiation of epidemiologically relevant strains within serovars is often based on their patterns of lysis with a defined set of bacteriophages. Phage typing has sometimes provided better strain discrimination than antimicrobial susceptibility testing, plasmid analysis, ribotyping, or pulsed-field gel electrophoresis (314). The various phage types of *S.* Enteritidis have provided the foundation for establishing relationships between isolates from different sources (167). However, the dependability of phage typing is limited by the potential for the conversion of isolates to different phage types by mutation or by the introduction of plasmids or temperate phages (265).

Molecular and Antibiotic Susceptibility Typing

Diverse genetic analyses have been evaluated for identifying and differentiating *Salmonella* isolates according to their source or epidemiological relevance. However, no single approach is demonstrably superior for all applications. The relative discriminatory abilities of methods sometimes differ between serovars (214). Moreover, genetic homogeneity within serovars such as *S.* Enteritidis has limited the meaningful differentiation of epidemiologically relevant strains (246). Pulsed-field gel electrophoresis of chromosomal DNA, ribotyping, random amplification of polymorphic DNA, plasmid profiling, repetitive extragenic pallindromic-polymerase chain reaction, and multilocus variable-number of tandem repeats analysis are among the most widely used molecular typing methods for *Salmonella* isolates (92, 213, 218). These techniques have distinguished between outbreak-related strains of salmonellae and unrelated strains (61) and between strains from different geographic locations (179). They have also been used to link isolates obtained from diverse sources within integrated commercial poultry enterprises (214) and to establish relationships between isolates from poultry flocks and human disease outbreaks (38). The combined use of 2 or more typing methods can provide the most detailed differentiation of *Salmonella* strains (211). The pattern of resistance to antimicrobial agents has often been used for strain differentiation in conjunction with molecular analyses (149).

Virulence Factors

Toxins

Two general categories of toxins play roles in the pathogenicity of salmonellae. Endotoxin is associated with the lipid A portion of *Salmonella* cell wall lipopolysaccharide (LPS). If released into the bloodstream of an infected animal upon lysis of bacterial cells, endotoxin can produce fever. Intravenously administered *S.* Enteritidis endotoxin

caused liver and spleen lesions in 2-week-old chickens (308). LPS also contributes to the resistance of the bacterial cell wall to attack and digestion by host phagocytes. Loss of the ability to synthesize complete LPS impairs the ability of *S.* Typhimurium to colonize the ceca and invade the spleen in broiler chicks (75).

Several proteinaceous toxins have also been identified in *Salmonella*. Enterotoxin activity induces a secretory response by epithelial cells that results in fluid accumulation in the intestinal lumen (201). The heat-labile cytotoxin causes structural damage to intestinal epithelial cells, perhaps by inhibiting protein synthesis (200).

Adherence and Invasion

Adherence to intestinal epithelial cells is the pivotal first step in the sequence of events by which salmonellae cause disease. Inefficient colonization of the intestinal tracts of chicks by *Salmonella* strains correlates with severely attenuated virulence. Variations between serovars in their persistence in chicken ceca have been attributed to differential regulation of core motility and adherence genes (59). Several characterized *Salmonella* virulence genes are upregulated in the high-osmolarity intestinal environment (95). Both flagella and fimbria have been identified as mediators of intestinal attachment, although neither are absolutely essential (96). Mutants of *S.* Enteritidis lacking flagella exhibited reduced adherence to cultured avian intestinal cells and competed poorly with wild-type strains to colonize the ceca of chicks (5). Strains of *S.* Enteritidis lacking fimbria were isolated less often than fimbriated strains from the ceca of inoculated chicks (301). LPS may also play a role in gastrointestinal attachment by salmonellae (46).

Salmonella virulence also requires mucosal invasion following adherence. Adherence and invasion appear to be separately regulated activities. The expression of important virulence genes in *Salmonella* pathogenicity island 1 strongly influences invasion of internal tissues such as livers and spleens, but has much less effect on initial cecal colonization (89). Mutations affecting intestinal colonization and lethality after oral inoculation of chicks did not have similar effects after intraperitoneal administration (257). Flagella and some types of fimbria may play roles in *S.* Enteritidis invasion and dissemination to internal organs of chicks. Flagella-deficient (but not fimbria-deficient) mutants were less able to invade to the livers and spleens of chicks (4). Type 1 fimbria appear to mediate the colonization of tubular gland cells in the upper oviduct (85). Both *S.* Pullorum and *S.* Gallinarum have lost the functionality of flagella and other genes essential to efficient colonization of the gut, so their virulence is accordingly more highly dependent on survival and multiplication in internal tissues (303).

Plasmids

Plasmids are transmissible extrachromosomal DNA elements which are sometimes associated with bacterial pathogenicity. Serovar-specific plasmids of characteristic molecular weights have been directly linked with virulence in salmonellae, although considerable homology has been demonstrated between virulence-associated plasmids of different serovars. Genes that promote survival within macrophages are found on many *Salmonella* virulence plasmids (272). A large plasmid is essential to the pathogenicity of both *S.* Pullorum and *S.* Gallinarum (25). The acquisition of a plasmid by a widely prevalent clone of *S.* Kentucky was hypothesized to confer increased ability to colonize chicken ceca and cause extraintestinal disease (184). Plasmid-mediated virulence among *S.* Typhimurium and *S.* Enteritidis isolates has been associated with survival and multiplication in serum (56). *Salmonella* strains cured of their virulence-associated plasmids were less persistent in the ceca of chicks (326). However, elimination of a serovar-specific plasmid of *S.* Enteritidis did not affect colonization and invasion of internal tissues in chickens (153). Plasmids carrying genes related to virulence have been found to also carry genes for antimicrobial resistance and conjugative transfer (154).

Pathogenicity Differences of Strains, Serovars, and Phage Types

Salmonella strains can differ greatly in their characteristic pathological effects in poultry. Differences between serovars have been reported in the associated frequencies of mortality in chicks and systemic infection in mature hens. Distinctively high levels of systemic infection and mortality are often attributed to strains of *S.* Pullorum and *S.* Gallinarum (53, 270). Significantly higher frequencies of invasion of cecal lamina propria and reproductive organs have been attributed to *S.* Enteritidis isolates than to many other serovars, as well as significantly more frequent deposition inside eggs (114, 126). Conversely, widely prevalent but avirulent *S.* Sofia isolates were impaired in adherence, invasion, and intracellular survival in comparison with other serovars (113). Despite their high degree of invasiveness, *S.* Pullorum and *S.* Gallinarum are generally less efficient colonizers of the ceca than most paratyphoid serovars (280). Diverse aspects of *Salmonella* infections, including colonization of the intestinal tract and invasion to internal tissues, can also vary tremendously within individual serovars. Differences between strains of *S.* Enteritidis, sometimes crossing phage-type boundaries, have been noted in their propensity to cause egg contamination by infected hens (132). Even within the same clonal genomic lineage, *S.* Enteritidis strains may not have entirely identical virulence properties (245), and considerable dissimilarity in metabolic properties has been found within phage types (234). Pathogenicity differences between *S.* Enteritidis

phage types have been extensively investigated, with phage type 4 sometimes associated with higher invasiveness and lethality for newly hatched chicks (121). However, in mature hens, infection with phage type 4 strains has caused intestinal colonization, systemic invasion, horizontal transmission, and egg contamination at frequencies similar to strains of other phage types (122, 132).

Although some virulence-associated genes are distributed widely in *Salmonella* isolates from diverse sources, genetic regions associated with specific attributes have accumulated differentially in individual strains (64). Particular genes, notably those of the type III protein secretion systems of *Salmonella* pathogenicity islands 1 and 2, are demonstrably important for invasion and survival in macrophages and internal organs (186, 337). Downregulation of some of these genes at the body temperature of poultry may result in lower virulence than is typically observed in mammals (306). The *in vivo* persistence of *S.* Enteritidis in the reproductive tract of chickens has also been associated with genes involved in amino acid and nucleic acid metabolism, cell wall integrity, and lipopolysaccharide structure (266). Genes related to invasion, cell division, metabolism, and bacterial defense contributed to *in vivo* survival of *S.* Pullorum (141). Conversely, *S.* Enteritidis colonization of the oviduct was more efficient when functional flagella were absent, perhaps caused by a reduced inflammatory response (192).

The changing environmental conditions to which enteric pathogens are exposed before and during the course of infection may induce corresponding changes in the expression of virulence-related genes. Many *Salmonella* genes are differentially expressed in response to environmental stress. Resistance to extreme osmotic, oxidative, and iron-limiting conditions has been directly linked to pathogenicity (281). Stress-induced responses have been postulated to mediate persistent oviduct contamination and survival in egg white by *S.* Enteritidis (320). Egg contamination by *S.* Enteritidis has been linked to the aggregate occurrence of a large number of single-nucleotide polymorphisms (233). The complex series of events from initial intestinal colonization to eventual systemic consequences may be orchestrated by sequential expression of complementary phenotypic properties, relevant to conditions experienced by the bacteria at different stages of infection (147).

Pathobiology and Epizootiology

Incidence of Salmonella in Poultry

Numerous and varied estimates have been made of the incidence of salmonellae in poultry and poultry house environments around the world. Recent surveys of the incidence of *Salmonella* infection among turkey flocks have ranged from 16% to 54%, with 20% of breeding flocks also reported as positive (10, 290). Likewise, recent surveys of the incidence of *Salmonella* infection among broiler chicken flocks have ranged from 9% to 57%, with up to 47% of breeding flocks also identified as positive (206, 290). Incidences of infection can differ substantially across national borders, even within the same geographic region, and national *Salmonella* isolation rates can sometimes change considerably from year to year. The actual prevalence of infection or contamination within *Salmonella*-positive flocks can also vary widely. For example, within turkey flocks, salmonellae were isolated from 13% of litter samples and 11% of cecal samples in 1 study and 79% of litter samples and 70% of fecal samples in another (244, 275). Moreover, the incidence of infection or contamination among flocks does not necessarily correlate with either the prevalence of infection within flocks or the quantitative level of contamination within poultry facilities (70, 327).

Recent surveys for the incidence of *Salmonella* in egg-type poultry in various nations have generated similarly diverse results, ranging from 12% to 65% of laying flocks and up to 26% of breeding flocks (256, 290). The frequency of *Salmonella*-positive samples within egg-laying flocks is often far less than the overall incidence among flocks. The within-flock *Salmonella* prevalence for laying hens in 5 European nations was reported to range from 0% to 27.5% (319). The distribution of salmonellae within contaminated laying houses is not necessarily uniform, as illustrated by a study which reported that 10.5% of laying houses, but only 1% of individual cage rows within these houses, were positive for *S.* Enteritidis (50). The number of *Salmonella* found in individual environmental samples from laying houses is typically relatively low, although somewhat higher levels can be present at the beginning of egg production and induced molting (268).

Distribution of Salmonella Serovars

Only about 10% of the known paratyphoid *Salmonella* serovars have ever been found in poultry, and an even smaller subset of these are consistently common. The distribution of serovars from poultry sources varies geographically and changes over time, although several serovars are consistently found at a high incidence. Among clinical and environmental isolates submitted in the United States in 2014, the most frequently identified serovars were *S.* Senftenberg, *S.* Kentucky, *S.* Mbandaka, *S.* Enteritidis, *S.* Typhimurium, and *S.* Infantis in chickens; and *S.* Senftenberg, *S.* Anatum, *S.* Hadar, *S.* Muenster, *S.* Agona, and *S.* Heidelberg in turkeys (236). The important epidemiological connection between poultry and human reservoirs of salmonellae is sometimes evident in

similar serovar distributions. For example, *S.* Enteritidis and *S.* Typhimurium, which have both been highly prevalent in poultry for many years, were the most frequently isolated serovars from humans in the United States in 2012 (52). Similar associations between the distributions of *Salmonella* serovars from poultry and humans have been reported in many countries. The implementation of new food safety regulations in Australia changed the relative incidences of *Salmonella* serovars, but not the linkage between the serovars found in poultry and those causing human illness (295).

Reports of the frequencies at which paratyphoid salmonellae are isolated from poultry sources around the world vary widely, although several serovars are of continuing international significance. The unique epidemiologic association of *S.* Enteritidis with transmission of disease to consumers via contaminated eggs has made the prevalence of this serovar a topic of particular interest. *S.* Enteritidis has been the most common serovar found in surveys of egg-producing chickens in many nations (299) and has been reported as the principal serovar present in eggs even when other serovars are predominant in associated laying flocks (247). *S.* Enteritidis has also become a prominent isolate from broiler chickens in some countries (220). Several other serovars are highly prevalent in particular types of poultry or certain geographic regions. Serovar Typhimurium has been reportedly associated with the widest variety of commercial poultry species (145). An increased prevalence of *S.* Kentucky has been recently observed in both egg-type and meat-type poultry in the United States (110). *S.* Infantis has been common in both layers and broilers in Japan (182). *S.* Sofia has predominated for a number of years in Australian poultry (228). Other widely prevalent serovars in poultry include *S.* Heidelberg, *S.* Hadar, *S.* Mbandaka, and *S.* Anatum (110, 290).

The host-adapted pathogens *S.* Pullorum and *S.* Gallinarum have rarely been reported in recent decades from economically developed regions such as the United States, Canada, Western Europe, and Australia, except in sporadic instances from backyard poultry flocks (331, 341). However, in Asia and Latin America, these organisms (and the diseases they cause) remain highly prevalent in both subsistence and commercial flocks (207, 297).

Natural and Experimental Hosts

Salmonella Infections in Chicks and Poults

Salmonella infections often have far different consequences for newly hatched poultry than for older birds. In very susceptible young chicks and poults, *Salmonella* infection can sometimes lead to illness and death at high frequencies. For example, oral doses of 10^9 *S.* Typhimurium cells were lethal for 50% of broiler chicks at 1 day of age and 20% at 3 days, but for 0% at 7 days (104). Mortality associated with naturally occurring infections in poultry often reaches peak levels at 3–7 days of age. Older birds are much less susceptible to the lethal effects of salmonellae and may experience intestinal colonization and even systemic dissemination without significant morbidity or mortality. Mortality associated with *S.* Pullorum infection is generally confined to the first 2–3 weeks of life, although birds that survive early infection may become inapparent carriers of the pathogen in splenic macrophages and the reproductive tract (334). *S.* Arizonae infections are sometimes associated with high morbidity and mortality in young turkey poults (287). The development of resistance to salmonellae in young birds is mostly attributable to the acquisition of protective microflora from their feed and environment. These organisms compete with salmonellae for intestinal receptor sites or produce inhibitory factors. Significantly more orally administered *S.* Typhimurium cells adhered in the ceca of chicks at 2 days of age than at 3–7 days (118). Naturally exposed birds can be heavily infected on their first day of life (221), and the frequency of fecal shedding of salmonellae in broiler flocks has been reported to peak at 14 days of age (222). Only 4% of cecal samples collected from naturally infected turkey poults were *Salmonella*-positive at 1 day of age, but 55% were positive at 9 days (332).

Salmonella infection has been experimentally established by oral, intracloacal, intratracheal, intraocular, navel, and aerosol administration to chicks (68). The usual outcomes of paratyphoid infections in chicks and poults involve 3 stages. Orally introduced salmonellae first establish intestinal colonization, often resulting in persistent fecal shedding. *Salmonella* levels in the intestinal contents of colonized chicks may greatly exceed intracellular levels in the intestinal epithelium (291). Second, invasion beyond the gastrointestinal tract can lead to *Salmonella* multiplication in the macrophage–phagocyte system of the liver and spleen, and eventual dissemination to colonize a variety of internal tissue sites. Third, extensive bacteremia sometimes occurs, resulting in high mortality. The bacterial incidence at all individual stages of infection correlates strongly with the dose of orally administered salmonellae. Infectious doses of salmonellae may vary between different avian species (344). Unlike paratyphoid strains which typically cause rapid intestinal inflammation in the early stages of infection, *S.* Pullorum has been shown to preferentially target sites such as the bursa of Fabricius (160).

Salmonella intestinal colonization, invasion to internal organs, and persistence in colonized tissues are all higher in newly hatched chicks than in older birds. Intestinal persistence was far greater following oral inoculation of chicks with *S.* Typhimurium or *S.* Enteritidis at 1 day of age than at 7 days (32, 118). After oral inoculation of

1-day-old chicks, *S.* Enteritidis was shed in the feces of nearly half of these birds at 6 months of age (131). *Salmonella* infection has been reported to persist for as long as 64 weeks after the exposure of young chicks (255). Administering 10^2 *S.* Enteritidis cells to 1-day-old chicks led to more persistent intestinal infection than administering 10^9 cells to 1-week-old birds (321). Some internal tissues reportedly remained *Salmonella*-positive for as long as 1 year after inoculation of 1-day-old chicks (292).

Salmonella Infections in Older Poultry

Morbidity or mortality are not consistently associated with paratyphoid *Salmonella* infections in older poultry, although fowl typhoid can sometimes cause significant disease losses in adult flocks. Experimental infections of adult chickens with large oral doses of most serovars may result in extensive intestinal colonization, bacteremia, and systemic dissemination to diverse internal organs, but they rarely cause any evident signs of clinical illness. During the first 2–4 weeks following experimental oral infection of adult chickens or turkeys, salmonellae are typically isolated from intestinal tracts and voided feces at high frequencies (129). Although the incidence of gut colonization and fecal shedding steadily decline thereafter, some *S.* Enteritidis strains can persist in the intestines of chickens for several months after oral inoculation (119). *Salmonella* was isolated from 42% of commercial broilers at 6–8 weeks of age (73). The frequency of fecal shedding of salmonellae by commercial layers peaked at 18 weeks of age and then declined steadily (210).

Gut colonization by salmonellae is usually followed by invasion through the intestinal epithelium and dissemination to colonize internal tissues. In commercial broilers, 35% of sampled spleens, livers, and gall bladders were *Salmonella*-positive at 6–8 weeks of age (73). Various serovars (including *S.* Typhimurium and *S.* Heidelberg) are known to be invasive for poultry, but the patterns and consequences of systemic invasion have been documented most extensively for *S.* Enteritidis. After experimental oral inoculation of laying hens, *S.* Enteritidis has been isolated from numerous internal tissues, including the liver, spleen, ovary, oviduct, heart blood, and peritoneum (21, 119). Dissemination of *S.* Enteritidis to diverse internal organs, including the ovary and oviduct, has also been recorded following intravenous, intratracheal, conjunctival, intravaginal, or intracloacal inoculation, exposure to contaminated aerosols, or insemination with contaminated semen (124, 188). The magnitude of internal organ colonization is directly related to the infecting dose (125). *S.* Enteritidis has also been isolated from a wide range of internal organs in naturally infected poultry.

The production of contaminated eggs is an aspect of *Salmonella* infections in mature chickens which has unique public health ramifications. Among egg-transmitted human salmonellosis outbreaks, 74% have been caused by *S.* Enteritidis (146). Investigations of egg-laying flocks implicated in human disease outbreaks have sometimes found *S.* Enteritidis isolates of the same phage types, plasmid profiles, or chromosomal DNA profiles found in affected people. Internal contamination of eggs with *S.* Enteritidis is a consequence of invasion to the ovary and oviduct (189). *S.* Enteritidis can colonize diverse regions of the reproductive tract of chickens, although the relationship between the colonization site and the frequency or location of egg contamination is uncertain (127). Both *S.* Heidelberg and *S.* Typhimurium have also been found inside eggs laid by experimentally infected hens (239, 336). However, some strains of these serovars can colonize reproductive organs but are rarely deposited in eggs (126, 248). The reported incidence of *S.* Enteritidis contamination of eggs from infected commercial flocks has usually been extremely low, even when laying house environments are contaminated (100), but transiently higher incidences may sometimes lead to human disease outbreaks (37). After oral inoculation of hens, *S.* Enteritidis can be deposited in both yolk and albumen, although usually at very low initial concentrations (132). Naturally contaminated eggs also typically contain very small numbers of *S.* Enteritidis cells, but these populations can expand to more dangerous levels if eggs are held at growth-promoting temperatures.

Predisposing Factors

A number of factors can increase the likelihood or severity of *Salmonella* infections in poultry. Several other infectious agents are known to influence the course of *Salmonella* infections. Prior infection with coccidia such as *Eimeria tenella* can increase *Salmonella* colonization of the intestinal tracts of chickens and *E. tenella* exposure may cause recrudescence of an earlier *Salmonella* infection (260). Coccidial infection decreases intestinal levels of *Salmonella*-inhibiting volatile fatty acids (11). However, *E. tenella* infection also diminished the frequency at which subsequently administered *S. enteritidis* invaded to the internal organs of chicks, possibly by thickening the intestinal lamina propria (298). Infections with immunosuppressive viruses or bacteria can also affect the outcome of *Salmonella* infections in poultry. Exposure to reticuloendotheliosis virus or bursal disease virus at 1 day of age increased mortality among chicks inoculated subsequently with salmonellae (238, 342). Suppression of cell-mediated immunity by *Corynebacterium parvum* led to increased morbidity in chicks after *Salmonella* infection (66). Perhaps via protection against compromising infections, both coccidial and viral vaccines have been associated with reduced *Salmonella* detection in broiler flocks (328).

Environmental and management factors also influence the susceptibility of poultry to salmonellae. Stressful

conditions facilitate or exacerbate infections. Lowering the brooding temperature of chicks by 5–8 °C increased mortality among newly hatched chicks inoculated with *S.* Worthington (300). Stress from environmental heat or cold, water deprivation, and the onset of egg laying have all been associated with higher frequencies or duration of *Salmonella* isolation from experimentally infected chickens (39, 261). The incidence of *Salmonella* in broiler chickens sometimes increases after they are transported for processing (222). Both preslaughter feed withdrawal and exposure to heat alter intestinal morphology and microbial communities, and increase *Salmonella* attachment to intestinal cells (44). Inducing molting by feed restriction has been identified as a highly significant risk factor for *S.* Enteritidis infection in laying flocks (116). Feed deprivation lowers crop levels of protective lactobacilli and volatile fatty acids while increasing pH (99). In experimental infection studies, feed restriction of hens increased intestinal colonization and fecal shedding, intestinal lesions, invasion to livers and spleens, recurrence of prior infections, and horizontal or airborne transmission of *S.* Enteritidis infection (169, 175). Molting can also be induced via alternatives such as fiber-based diets without affecting *Salmonella* susceptibility (267).

A diverse assortment of poultry facility characteristics and management practices, including larger flock size, greater flock age, housing in older facilities, access to outdoor areas, and multiple-age stocking, have been linked to high *Salmonella* prevalence in commercial egg production operations (88, 256). Housing systems for laying hens (including conventional cages, cage-free options such as aviaries, and intermediate alternatives such as enriched colony cages) have been intensively investigated as potential *Salmonella* risk factors, but no definitive overall consensus has yet emerged from these studies. Some investigations have reported greater frequencies of *Salmonella* infection or environmental contamination in conventional cage-based housing (319), but others have observed greater prevalence or transmission of *Salmonella* infection in cage-free housing systems (91). In a large field survey, different housing systems did not significantly affect the environmental prevalence of salmonellae, but distinctive *Salmonella* reservoirs and risk factors were identified for each system (185). Experimental infection studies have associated higher hen stocking density with increased susceptibility to both intestinal colonization and invasive infection by *S.* Enteritidis (128, 129). Stress from overcrowding has been implicated in the suppression of immune responsiveness to *Salmonella* infection (144).

Sources, Vectors, and Transmission

Salmonellae can be introduced into poultry flocks from many sources. Feeds containing contaminated animal proteins, vegetable proteins, or cereals, or contaminated by vermin or wildlife, are potential sources of *Salmonella* in both chickens and turkeys. Chicks can be infected by very low levels of salmonellae in feed (166). *Salmonella* contamination was found in 28% of finished feed samples from commercial broiler farms (3), although much lower frequencies of contamination are more common (284). Meal or mash feeds more often harbor salmonellae than pelleted feeds (77). Salmonellae can survive for 2 years in inoculated feeds (82). *Salmonella* isolates from feedstuffs given to commercial poultry have been found to have genetic profiles which matched those of isolates from poultry meat and eggs produced by those flocks (283). Contaminated sources of drinking water can also transmit infection (196).

The wide paratyphoid *Salmonella* host range creates numerous reservoirs of infection for poultry. Biologic vectors introduce, disseminate, and amplify salmonellae in flocks. In Belgian egg-laying farms, molecular fingerprinting linked *S.* Enteritidis isolates from farm environments, rats, mice, mites, flies, hens, and eggs (93). Insects and other invertebrates, including flies, darkling (litter) beetles, ground beetles, cockroaches, red mites, and centipedes can carry *Salmonella* organisms externally or internally (81, 329). *Salmonella* infection has been transmitted by feeding contaminated flies or darkling beetles to chickens (174, 269). Mice and rats are important vectors for *S.* Enteritidis in laying flocks. A study of commercial laying flocks in the United States reported an *S.* Enteritidis prevalence in mice from environmentally positive poultry houses which was 4 times that of negative houses (116). High rodent densities have been correlated with a greater likelihood of *Salmonella* infection in laying hens (205). A single rodent fecal pellet can contain 10^8 *Salmonella* cells (309). Wild birds and other wildlife are sometimes identified as *Salmonella* risk factors for poultry (177, 212). Humans can also transmit *Salmonella* infection into poultry flocks (196).

Molecular typing of isolates has demonstrated *Salmonella* dissemination from a breeding flock to multiple broiler flocks, and ultimately into the abattoir (217). Vertical transmission of salmonellae to the progeny of infected breeding flocks can result from internal or external contamination of eggs. Egg shells can be contaminated with feces during oviposition. *Salmonella* penetration into or through shells and shell membranes can either directly transmit infection to developing embryos or allow exposure of chicks to infection when the shell structure is disrupted during hatching. *S.* Enteritidis can be deposited in the contents of eggs before oviposition (127, 189). The resulting transovarian transmission of infection to progeny is an important aspect of *S.* Enteritidis epidemiology in chickens. The same *Salmonella* serovars responsible for mortality in naturally infected chicks or poults are often also found in

their parent flocks. Vertical transmission from breeders has been identified as the principal route for dissemination of infection with host-adapted *S.* Pullorum in chickens (183) and has also been implicated in the introduction of *S.* Arizonae infection into turkey flocks (76). The *Salmonella* status of newly hatched chicks is a leading risk factor for infection in mature commercial layer and broiler flocks (242). Any salmonellae carried in or on eggs can spread extensively in hatcheries. As chicks or poults pip through egg shells, salmonellae are released into the air and circulated around hatching cabinets on contaminated fluff and other hatching debris (327). Samples of egg fragments, belting materials, and paper pads from broiler hatcheries have been *Salmonella*-contaminated (69). Newly hatched birds, lacking protective intestinal microflora, are highly susceptible to intestinal colonization by salmonellae. Chicks from uncontaminated eggs became infected with *S.* Typhimurium when hatched along with surface-contaminated eggs (49). Horizontal contact between newly hatched chicks played an important role in an outbreak of *S.* Pullorum infection in commercial broilers (183). *Salmonella* prevalence in broiler hatcheries is reportedly greater than at later production stages, and the serovars isolated from hatcheries sometimes correlate with those found on finished carcasses (16). *Salmonella* serovars carried from hatcheries by chicks often predominate in broiler flocks.

Poultry environments are often implicated as principal sources of salmonellae. Serovars present in broiler houses during the rearing period are likely to appear on processed carcasses. Litter contamination during production was reported as highly predictive of eventual carcass contamination (34). Studies in commercial laying flocks suggested that *S.* Enteritidis infection was more often acquired from farm environments than from breeders (316). Dust levels are also important risk factors for salmonellae in poultry (33). *S.* Enteritidis persisted for at least 1 year in dust in an empty poultry house, even after cleaning and disinfection (82). In commercial laying houses, voided feces, manure belts, egg belts, and floors are recurrent areas of high *Salmonella* contamination (94). Horizontal transmission of *Salmonella* occurs both within and between flocks. Infection in previous flocks is a significant risk factor for *Salmonella* in both broilers and layers (242). *Salmonella* infection spreads via direct bird-to-bird contact or ingestion of contaminated feed, water, feces, or litter. *S.* Enteritidis was found in the internal organs of uninoculated laying hens housed in cages adjacent to orally inoculated birds (119). Circulation of personnel, equipment, aerosols, and dust throughout houses facilitates bacterial dissemination. Air samples collected both inside and outside of commercial laying houses were *Salmonella*-positive (83). Airborne transmission of *Salmonella* infection has been reported, apparently mediated by contaminated dust (135). Levels of airborne salmonellae in broiler houses have been linked to levels in litter (60). Infection can spread rapidly among chicks reared on litter.

Clinical Signs

In general, paratyphoid *Salmonella* infections of poultry typically cause clinical signs only in very young chicks or poults. *Salmonella* contamination within eggs may lead to embryo mortality or rapid death among newly hatched birds before clinical signs are observed. Typical signs of severe *Salmonella* infection in chicks and poults include progressive lassitude and somnolence with closed eyes, drooping wings, ruffled feathers, shivering and huddling near heat sources, anorexia, emaciation, and profuse watery diarrhea (often resulting in dehydration and pasting of the vent area). Morbidity and mortality can be high during the first 2–3 weeks of life, with significant body weight loss or growth retardation, but signs of disease are infrequent in older birds. The course of illness is normally relatively brief in individual birds. Signs of severe *Salmonella* infection in young poultry are generally similar to those observed with other bacteria which cause acute septicemia. Although clinical disease is not characteristic of paratyphoid infections in mature poultry, some *S.* Enteritidis strains have caused anorexia, diarrhea, and reduced egg production in experimentally infected laying hens (289). Among other paratyphoid salmonellae of note, *S.* Arizonae mostly affects turkey poults up to 4 weeks of age, causing relatively nonspecific clinical signs of depression, weakness, and diarrhea, with mortality approaching 50% in some instances. Other signs often associated with *S.* Arizonae infection in turkey poults include paralysis with torticollis and terminal opisthotonus (287). Infection of turkey breeding hens with *S.* Arizonae can decrease the production and hatchability of eggs.

Salmonella Pullorum infection causes egg-transmitted disease in young chicks and turkey poults, often associated with white diarrhea and high mortality, but signs can be minimal with mortality peaking at 2–3 weeks after hatch. Blindness, labored breathing, and lameness caused by swelling of the hock joint are occasionally observed (287). Dead chicks may be found in the hatcher, but mortality is usually greatest after 10 days of age. Clinical signs, if any, include diarrhea with urate staining of the vent, decreased feed consumption, and huddling near heat sources. Adult birds are generally more resistant to infection and infected individuals are often asymptomatic, but they can transmit infection through eggs to hatchlings. *S.* Gallinarum infections of mature birds are sometimes responsible for substantial levels of morbidity and even mortality.

Pathology

Severe outbreaks of *Salmonella* infection in young chicks or poults can involve rapidly developing septicemia with high mortality and few apparent lesions. Omphalitis and yolk sac infection are commonly associated with *Salmonella* infection of newly hatched poultry. The navel skin is often red or covered with a scab, and the yolk sac is typically filled with either blood or watery yellow fluid with flocculent material (Figure 16.1). When the course of disease is longer, enteritis with mucosal necrosis is observed (possibly with tan pseudomembrane; Figure 16.2), along with white to yellow caseous exudate in the lumen of the ceca ("cecal cores"; Figure 16.3). The liver is enlarged and often has pinpoint, pale to hemorrhagic foci of necrosis (Figure 16.4). Splenic enlargement is also common. Kidneys may be enlarged and congested. Both the heart and liver are frequently covered with fibrin. Other occasionally observed lesions of paratyphoid *Salmonella* include accumulation of fibrin or fibrinopurulent exudate on the surfaces of the heart, liver, coelomic viscera, lung, and also within the eye (hypopyon) and wing or leg joints (arthritis).

Gross lesions associated with *S.* Arizonae infection in turkeys include omphalitis, retained yolk sacs, hepatosplenomegaly and white, caseous exudate in the ceca. Poults with neurological signs may have white (fibrinopurulent) exudate within the eye (hypopyon opthalmitis; Figure 16.5) and also on the meninges of the brain (meningoencephalitis).

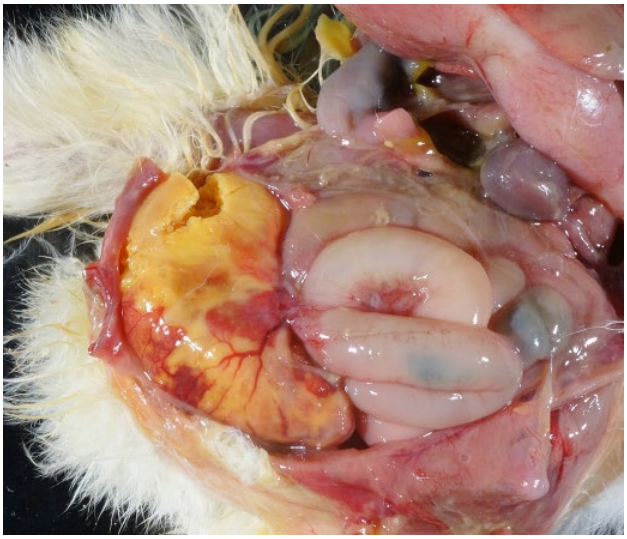


Figure 16.1 Paratyphoid *Salmonella* infection. Retained, hemorrhagic yolk sac containing caseous exudate in a 5-day-old broiler chick.

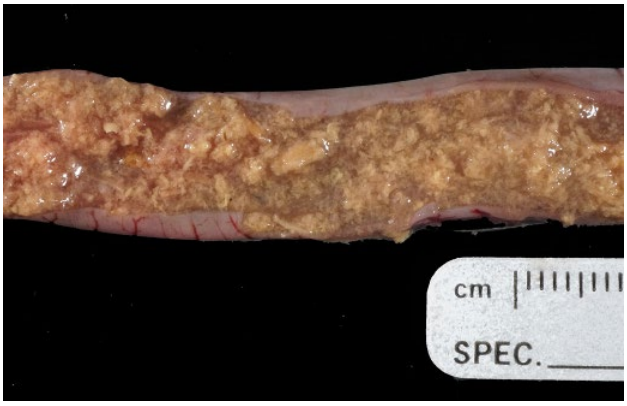


Figure 16.2 Paratyphoid *Salmonella* infection. Mucosal necrosis and hemorrhage (fibrinonecrotic enteritis) in small intestine of a white leghorn pullet.



Figure 16.3 Paratyphoid *Salmonella* infection. Caseous cecal exudate (fibrinonecrotic typhlitis) in a 10-day-old pheasant.

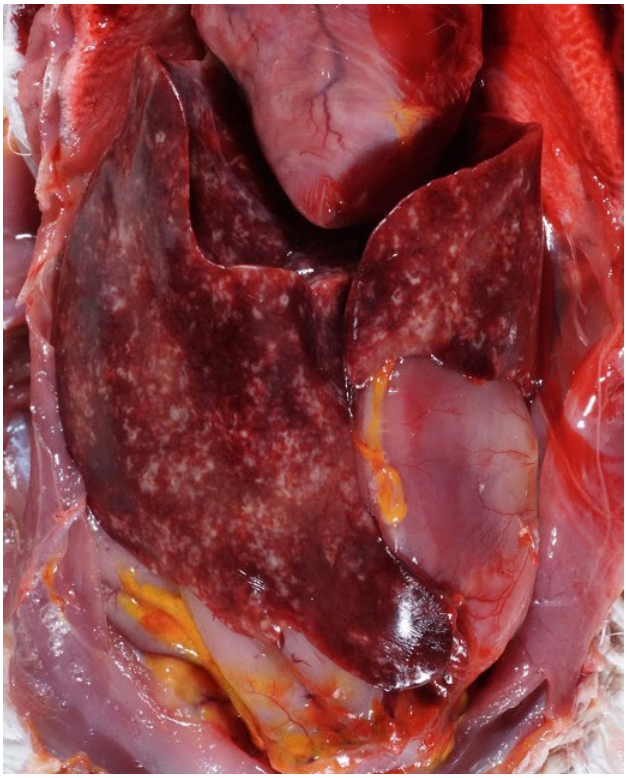


Figure 16.4 Paratyphoid *Salmonella* infection. Enlarged liver with pale, pinpoint, coalescing foci of necrosis in a pigeon.



Figure 16.5 *Salmonella* Arizonae infection. Eye of 3-week-old turkey poult with severe opthalmitis.



Figure 16.6 *Salmonella* Pullorum infection. Nodular lesions in heart caused by chronic infection; note thickened pericardium (arrows).

Young birds infected with *S.* Pullorum sometimes die with no gross lesions, but they can also show hepatosplenomegaly with white to grey nodules (granulomas) in the heart (Figure 16.6), liver, and gizzard; caseous, white to yellow exudate in the cecal lumen; and white to yellow, caseous exudate within the joints of the legs and wings. Adult birds often show no gross lesions, but can have fibrin-covered, misshapen or atretic ovaries (oophoritis), and fibrin on both the heart and coelomic viscera.

Pathogenesis of the Infectious Process

Although salmonellae invade epithelial cells throughout the intestinal tract, the ceca and ileocecal junction are sites of particular affinity (308). After oral inoculation of chicks, *S.* Enteritidis adhered to epithelial cells at the tips of villi (90). *Salmonella* invasion changes the density and morphology of intestinal epithelial cells, affecting intestinal fluid and electrolyte regulation, and ultimately causing cell death and diarrhea (105). Oral inoculation of laying hens with *S.* Enteritidis produces inflammation of the epithelium and lamina propria of the ceca and colon caused by heterophilic infiltration (160). Epithelial invasion may also allow removal of salmonellae through the basement membrane into the lamina propria by macrophages (41). The ability of salmonellae to survive and multiply in internal organs, particularly within mononculear phagocytes of the liver and spleen, is a key element of their virulence. *S.* Pullorum is unable to multiply inside these cells in ducks, perhaps accounting for their resistance to pullorum disease (24). *S.* Enteritidis was recovered from several internal organs of laying hens within 1 hour after oral infection (181). *S.* Enteritidis numbers peaked at 24–36 hours after oral inoculation of chicks (158). The spleen may provide a protected site for intracellular *Salmonella* multiplication without exposure to host defense mechanisms (98). Many *S.* Enteritidis strains, as well as some strains of serovars such as *S.* Heidelberg and *S.* Typhimurium, are invasive for cells of the reproductive tract (13).

Immunity and Resistance

The immune response of poultry to salmonellae minimizes the duration and severity of infection and protects against reinfection. This response also serves as the basis for protecting birds against infection by vaccination and permits serologic detection of infected flocks. Administering immunosuppressive agents to chicks increased mortality associated with *S.* Typhimurium infection (101). *Salmonella* infection of chickens can cause lymphocyte depletion, atrophy of lymphoid organs, alteration of host immune signaling pathways, and immunosuppression, thereby facilitating the establishment of a persistent carrier state (155, 199). The invasive behavior of *S.* Pullorum and *S.* Gallinarum may be facilitated by their lack of immune-stimulating flagella, perhaps enabling them to avoid provoking strong inflammatory responses in infected birds (112).

Salmonellae can elicit strong antibody responses in infected poultry. In a naturally infected broiler breeder flock, 70% of the birds were found to be positive for antibodies to *S.* Enteritidis (65). Experimental infection of chicks with *S.* Typhimurium induced strong IgG, IgA, and IgM responses (156). When laying hens were orally infected with *S.* Enteritidis, serum antibodies were produced by most birds by 1 week postinoculation, reached peak values at 2 weeks, and remained at high levels for more than 6 months (133). Serologic positivity to *Salmonella* persisted among infected chickens throughout

a 1-year period (292). However, a highly virulent strain of *S.* Pullorum was found to elicit a lower serum antibody titer in broiler chickens than did a less virulent strain (22). *S.* Enteritidis infection of laying hens also induces antibody production by gastrointestinal lymphoid cells (324). Specific antibodies to *S.* Enteritidis have been detected in the yolks of eggs laid by infected hens, reaching peak levels several weeks after the serum antibody response (139). The progeny of immune breeding hens may acquire partially protective immunity via transfer of maternal antibodies in egg yolks (32).

Cell-mediated immunity to salmonellae is also important in poultry. Avian heterophils are phagocytic and bactericidal, and may play a vital role in restricting organ invasion during early phases of *Salmonella* infection. A temporary decrease in T-cell responsiveness to S. *Pullorum* at the onset of egg laying was observed to coincide with invasion of the pathogen to reproductive tracts of infected hens (335). Stimulating the immune responses of heterophils increased the resistance of chicks to *S.* Enteritidis colonization (159). Cytokines produced by sensitized T lymphocytes expand the pool of circulating phagocytic heterophils and recruit them to the site of infection (198). Various intestinal cytokines and antimicrobial peptides are sequentially expressed during the course of *Salmonella* infection in chickens. *S.* Enteritidis infection in chicks induced the expression of chemokines which recruit macrophages and monocytes (58). Cytokine and chemokine expression profiles have been observed to vary for different *Salmonella* serovars (13).

Both humoral and cell-mediated immune responses appear to play important roles in protecting poultry against *Salmonella* infection. The timing of clearance of *S.* Typhimurium infection in chickens correlated with the emergence of strong antigen-specific cellular and humoral immune responses (28). Likewise, a decline in *S.* Enteritidis isolation from reproductive tissues of laying hens during the second week of infection was associated with the proliferation of both T and B lymphocytes (338). Both the opsonic activity of specific antibodies and the phagocytic and lytic activity of cellular effectors may be necessary for the full expression of immunity. In addition to antigen-specific adaptive immune responses, innate host phagocytic capabilities also contribute significantly to resistance during the early stages of infection by salmonellae. Chicken macrophages can internalize higher numbers of *S.* Enteritidis cells and clear intracellular salmonellae more rapidly than lymphocytes (202). Innate responses are especially critical in newly hatched poultry which are immunologically immature and unable to mount fully protective adaptive immune responses to infection (173).

Selection for genetically based differences in the innate resistance or immunity of lines of chickens to *Salmonella* infection has been considered as a potential protective strategy for flocks. Chicks from distinct lines can vary significantly in *Salmonella*-associated mortality (150). Different incidences of fecal shedding, organ invasion, and egg contamination have been observed for lines of mature chickens infected with *S.* Enteritidis (97, 143). Lines of chickens differing in *Salmonella* resistance have differed in T cell responses and the expression of cytokines, antimicrobial peptides, and pathogen-specific cellular receptors (27, 62). However, effective selection for *Salmonella*-resistant lines of chickens is complicated by negative genetic correlations between resistance in chicks and adults and between resistance traits and production traits (29, 296).

Diagnosis

Isolation and Identification of Causative Agent

Because clinical signs and gross lesions associated with *Salmonella* infection can resemble those caused by a variety of other bacteria, final diagnosis depends on the isolation and identification of causative organisms. Using conventional culture methods, this requires 48–96 hours (and even longer for some protocols). A concise summary of traditional methods for isolating *Salmonella* from poultry was provided previously (330). Numerous faster alternative strategies for detecting and identifying salmonellae have been proposed and studied. Serologic detection of specific antibodies is sometimes employed as a rapid preliminary screening test to identify flocks that have been exposed to salmonellae.

Sample Selection

Samples from a variety of sources, including tissues, eggs, voided feces, and poultry house environments, are collected and tested to identify *Salmonella* infection in flocks. The number of samples which must be processed to achieve a predetermined level of confidence of accurate detection is directly related to flock size and inversely related to the prevalence of infection. In very large flocks estimated to have low *Salmonella* prevalence, multiple samples are sometimes pooled together before culturing to improve the likelihood of detection within the limitations of existing laboratory resources.

Many *Salmonella* serovars are highly invasive and can spread systemically to numerous internal tissues, so a diversity of sites (including liver, spleen, ovary, oviduct, testes, yolk sac, heart, heart blood, kidney, gallbladder, pancreas, synovia, and eye) can provide samples for diagnostic culturing. Because lesions do not reliably indicate infected tissues, several different organs should be cultured from each bird (separately or together). Livers and spleens, as filtering organs, are most likely to

be contaminated and thereby are the most effective culture targets for identifying infected birds. Egg culturing is often applied to assess the potential threat to public health posed by laying flocks infected with highly invasive serovars, particularly *S.* Enteritidis, which are deposited in the contents of eggs before oviposition.

Because paratyphoid *Salmonella* infections in poultry almost invariably involve intestinal tract colonization, samples of intestinal tissues and contents are frequently a focus for testing. In a diagnostic survey, salmonellae were found exclusively in intestinal samples in 78% of chickens and 70% of turkeys (103). In experimentally infected laying hens, *S.* Enteritidis was recovered more often from the intestinal tract than from any other sampled tissue (119). The caudal ileum, ceca, cecal tonsils, and cecal contents are often recommended for *Salmonella* recovery, although no specific sample ensures detection of all infected birds. Cloacal swabs or fecal samples can provide sensitive indicators of persistent intestinal colonization in individual birds, but their diagnostic reliability is diminished by the intermittent shedding of salmonellae in the feces of infected birds (318).

Fecal shedding of *Salmonella* into poultry house environments by infected birds makes culturing environmental samples a valuable diagnostic tool. Environmental samples also provide opportunities to monitor the introduction of salmonellae into poultry houses by feed, vectors, personnel, and equipment. Sampling floor litter has sometimes provided a level of detection comparable with fecal samples (271). Dragging moistened gauze pads across floors in poultry houses has detected salmonellae with high sensitivity (195). Swabs dragged through wet areas of manure are more productive than dry areas. Foot covers worn in poultry houses can also provide effective samples for detecting environmental salmonellae (34). Nest boxes, egg belts, dropping belts or scrapers, fan blades, and dust are particularly productive sources for *Salmonella* sampling in laying houses (78). Dust samples may provide more consistent *Salmonella* detection than fecal samples, but combined testing of both optimizes detection sensitivity (7). Hatcher fluff and hatched eggshell membranes are frequently contaminated with salmonellae, offering opportunities for early detection of infection (55). Air sampling detected *Salmonella* in both hatching cabinets and rooms housing infected chickens (36, 136).

Standard Culture Methods for *Salmonella* Detection

Although diverse culture conditions are applied to isolate and identify salmonellae, most standard methods follow a general scheme involving 4 principal stages. First, nonselective pre-enrichment encourages the growth of very small numbers of salmonellae or resuscitates injured cells. Pre-enrichment is sometimes omitted when testing samples (such as intestinal contents or feces) with large numbers of competing organisms that might overgrow salmonellae in nonselective media. Second, selective enrichment allows additional expansion of the *Salmonella* population while suppressing the growth of other organisms. Third, plating on selective agar media yields isolated colonies, each derived from a single cell. Nonselective agar plating media are also sometimes used with swabs from internal organs. This can be especially useful for detecting strains of *S.* Pullorum, which may grow slowly or produce very small colonies in the presence of some selective media ingredients (259). Fourth, colonies with appearances characteristic of salmonellae are subjected to biochemical and serologic tests to confirm their genus and serovar identity. Virtually all proposed methods require the last 2 of these steps, but enrichment requirements vary according to the nature of the sample.

Internal organs (except for intestinal samples) from infected birds ordinarily contain relatively few competing organisms. Swab or loop samples taken from internal tissues are often transferred directly to plates of both selective and nonselective agar media, without broth enrichment. Excised tissue samples, and any samples derived from the intestinal tract, are generally transferred initially into selective enrichment broth.

Because fecal contamination may result in the presence of diverse flora, eggshells are usually sampled for *Salmonella* without pre-enrichment (unless detecting the presence of other bacterial contaminants is also of interest). The surface of eggshells can be sampled by immersion or rinsing in broth media or the entire shell (including interior structures and shell membranes) can be sampled by aseptic breaking to release the contents followed by manual crushing and the addition of enrichment broth. Before culturing egg contents for contamination by salmonellae, the exterior surface of shells must be disinfected to prevent the transfer of fecal contaminants to the contents during breaking.

Salmonella contaminants inside eggs are typically present at both very low prevalences and in very small numbers, so the entire liquid contents of 10–20 eggs are often pooled together for sampling to minimize demands on laboratory resources. Egg contents pools are usually incubated before further culturing to allow *Salmonella* populations to expand to consistently detectable levels. Supplementation of whole egg pools with iron can increase *Salmonella* multiplication during incubation (130). Pre-enrichment of egg contents leads to more sensitive *Salmonella* detection than direct selective enrichment, perhaps by allowing expansion of small initial levels of bacteria before they are exposed to harsh selective enrichment conditions (345). Direct plating of incubated egg pools onto selective agar media reduces time, media, and labor requirements, but provides significantly poorer detection sensitivity (130).

Environmental samples are typically collected in sterile plastic bags and subsequently cultured by transfer into selective enrichment broth. Moistened gauze pads can be used to sample environmental surfaces or can be dragged across floor litter or dropping pits. Transporting environmental swab samples in double-strength skim milk or buffered peptone water may improve *Salmonella* detection. Poultry feed should be tested by collecting several representative samples from each lot and transferring into selective enrichment broth. Pre-enrichment of environmental and feed samples has not consistently improved *Salmonella* recovery (277).

Culture Media

A broad assortment of media has been developed and evaluated for isolating and identifying salmonellae. Although some evidence suggests that proper selection of culture media is contingent on the type of sample being tested, several commercially available formulations have been consistently effective for a variety of applications. Suggested broth media to pre-enrich samples for salmonellae include trypticase soy broth and buffered peptone water. The selective broth media most often used for *Salmonella* detection in recent years are tetrathionate broth and Rappaport–Vassiliadis broth. Modified semi-solid Rappaport–Vassiliadis (MSRV) medium has sometimes performed as well as traditional broth media for selectively enriching *Salmonella* from poultry samples.

Numerous agar media are available for isolating salmonellae. Among the most commonly used plating media are brilliant green, xylose-lysine-deoxycholate (XLD), xylose-lactose-tergitol 4 (XLT4), Rambach, bismuth sulfite, and Hektoen enteric agars. Brilliant green agar has been widely and successfully applied for *Salmonella* isolation from diverse poultry sources, including tissue, environmental, egg, feed, and air samples. XLT4 agar has also been effectively employed for detecting salmonellae in poultry house environmental samples. Novobiocin addition to media improves *Salmonella* recovery by suppressing the growth of competitors such as *Proteus*. Samples should always be plated on 2 different agar media, preferably with dissimilar indicator systems for differentiating salmonellae from other organisms.

Most culture media are incubated for 24 hours at 37 °C. Shorter selective enrichment is usually inadequate to suppress competing microflora in heavily contaminated samples. Incubation of some selective enrichment media at elevated temperatures (41 °C –43 °C) has been recommended to restrict the growth of competing organisms, especially in intestinal samples or samples containing fecal material.

Confirmation of Genus and Serovar

Characteristic *Salmonella* colonies on selective agar plates must be tested further to confirm their genus identity and determine their serovar. Combined use of triple sugar iron agar and lysine iron agar provides an effective presumptive test for salmonellae. The observed pattern of fermentation with a battery of 6 carbohydrates can provide further differentiation of *Salmonella* isolates from other organisms (74). The serogroup of isolates can be determined by agglutination tests with polyvalent antisera for somatic O antigens, and the serovar can then be determined by slide agglutination tests with monovalent antisera to specific O antigens and tube agglutination tests with antisera to flagellar H antigens. Molecular genetic *Salmonella* serotyping methods, such as intergenic sequence ribotyping (148), have become more widely utilized in recent years.

Rapid Detection Technologies

Obtaining *Salmonella*-negative results using conventional culturing methods requires several days for most types of samples, and confirming positive results adds even more time. Considerably faster techniques are available and are increasingly becoming accepted as standard practices, although rapid methods have not altogether supplanted traditional culturing for most applications. Rapid methods typically reduce time requirements for testing by 1 or more days, and many are adaptable to some degree of automation. The principal limitations of rapid methods are related to cost and detection sensitivity. Rapid methods nearly always require at least 1 enrichment step to achieve detectable cell densities. Particularly high detection thresholds have been reported for nonmotile, slow-growing strains of *S*. Pullorum (294). Most efforts to develop rapid *Salmonella*-detection methods have centered around the use of specific antibodies or DNA probes.

Specific antibodies have been utilized in a variety of enzyme immunoassay (EIA) methods to detect *Salmonella* antigens. Polyclonal antibodies to LPS or flagella have detected salmonellae in eggs, tissues, cloacal swabs, environmental drag swabs, litter, and feed (157). Likewise, assays using monoclonal antibodies to LPS, outer membrane proteins, or flagella have been applied to detect *Salmonella* (or specific serovars) in eggs, tissues, and environmental samples (42). Recent innovations in antibody-based methods have attained sensitivities comparable with standard culture methods, although 1 or more initial enrichment steps are typically required to support expansion of the *Salmonella* population to detection threshold values of at least 10^5 cells/mL (42). Other antibody-based assay formats have also been effectively applied for detecting salmonellae in poultry samples (134). An important limitation on the usefulness of antibody-based tests is their propensity to yield false-positive results from competing flora which are able to grow in enrichment media.

Another application of antibodies for *Salmonella* detection is immunomagnetic separation (IMS), which

employs small magnetic beads coated with specific antibodies to bind *Salmonella* target antigens in samples and remove them when a magnetic field is applied. IMS offers a faster alternative to broth enrichment for concentrating salmonellae without adversely affecting sublethally injured cells. IMS concentration supported detection of small numbers of *S.* Enteritidis in pooled egg samples by both culturing and EIA (171). IMS concentration plus an EIA detected *Salmonella* from poultry environmental swabs with 98% of the sensitivity of traditional culturing, but required only 48 hours for testing (209).

An increasingly prominent approach to rapid testing for *Salmonella* is based on detecting genus-specific or even serovar-specific genetic sequences by hybridization of specific probes with target DNA extracted from samples. DNA hybridization typically detects salmonellae at sensitivity thresholds similar to EIA, and thus usually requires 1 or more preliminary enrichment culturing steps (216). Moreover, DNA hybridization assays are procedurally complex and are often more expensive than other available methods. The development of polymerase chain reaction (PCR) technology has allowed amplification of specific target segments of DNA, thereby enabling hybridization reactions with probes to detect salmonellae in tissues, environmental swabs, feces, and eggs with a high level of sensitivity (1, 279). After enrichment culturing, PCR methods have detected initial contamination loads of fewer than 10 *Salmonella* cells in eggs and poultry environmental samples (215). Carefully chosen DNA probes can be used with PCR to detect salmonellae with particular characteristics, such as genes for virulence factors, biochemical properties, or surface structures such as fimbria (204, 339). A duplex PCR assay (specific to 2 different gene targets) was used to effectively differentiate *S.* Pullorum and *S.* Gallinarum isolates from other salmonellae and also from each other (26). Multiplex PCR assays can simultaneously detect the presence of several serovars (176). Some PCR-based tests are reportedly able to distinguish attenuated *Salmonella* vaccine strains from wild-type field isolates (225).

Serologic Diagnosis of Infection

Specific antibodies to salmonellae can be found in infected poultry with high sensitivity using diverse agglutination and EIA methods. Serologic testing has played an especially critical role in programs to control *S.* Pullorum-Gallinarum infections in poultry breeding flocks of economically developed nations, such as the NPIP in the United States (312). These programs apply assays for serum antibodies to screen flocks for the presence of serologic reactors. Subsequent testing for the pathogen in internal organs of sampled birds provides confirmation of active infection and serves as the basis for removal of positive individuals or the depopulation of infected flocks.

Detectable serum antibody titers are often present long after clearance of all salmonellae from tissues and cessation of fecal shedding. Because antibody tests only document prior *Salmonella* exposure, and do not provide unequivocal evidence of ongoing infection in flocks, positive serologic results must be followed by bacteriologic culturing for confirmation. Serology also yields positive results much later after infection than bacteriologic culturing (187). Other serologic testing limitations include subclinical infections which lead to fecal shedding without eliciting detectable antibody responses, immunologic unresponsiveness in very young birds, cross-reactions between antibodies to antigenically related *Salmonella* serovars, and vaccine-induced antibody responses which confound serologic differentiation of vaccinated and infected birds (137, 232).

Agglutination tests have detected both natural and experimental infections of chickens with salmonellae (120). Agglutination assays are performed on both whole blood and serum in plate, tube, and microwell formats. All of these tests rely on the ability of specific antibodies to visibly agglutinate killed whole *Salmonella* cells, which are stained (except in tube tests) to improve visualization of agglutination reactions.

Salmonella infections in poultry can also be detected using diverse EIA approaches. An international survey reported a high degree of correspondence in the performance of a wide assortment of EIA formats and antigens for detecting *S.* Enteritidis infections (23). EIA using LPS, flagella, or outer membrane protein antigens has identified chickens infected naturally or experimentally with *S.* Typhimurium, *S.* Enteritidis, or *S.* Pullorum (31, 37, 193). Screening for serum antibodies using a flagella-based EIA was applied successfully for controlling *S.* Enteritidis in Dutch breeder flocks (322). By using very precisely defined antigens, EIA often achieves a high degree of specificity and thus produces fewer false-positive cross-reactions between serovars than agglutination reactions (193). Assays employing fimbrial antigens have shown especially high specificity for identifying *S.* Enteritidis infections in chickens (262).

Antibodies deposited in egg yolks by infected hens offer uniquely convenient samples for *Salmonella* testing. A variety of assay formats have been applied to find antibodies to salmonellae in eggs from naturally and experimentally infected chickens, correlating directly with the incidence of fecal shedding and organ invasion (302, 316). Antibodies were detected by flagella-based EIA in egg yolks from hens inoculated orally with as few as 10^3 *S.* Enteritidis cells (139). In both experimental infection studies and epidemiological field investigations, the detection of egg yolk antibodies to *S.* Enteritidis and the detection of the pathogen in feces or environmental swabs were similarly effective for predicting the production of contaminated eggs by infected hens (138, 197).

Intervention Strategies

Risk Reduction

The diversity of sources for *Salmonella* introduction into flocks or houses complicates the identification of critical control points for preventing infection of poultry. Because intervention options are not always individually effective, successful prevention and control programs must involve coordinated and sustained implementation of diverse risk reduction practices throughout the production continuum (305). Financial analysis has indicated that the costs of intensive *Salmonella* control efforts in poultry are justified by even greater savings in public health costs (313). Eggs and chicks (or poults) should be obtained only from demonstrably *Salmonella*-free breeding flocks. Hatching eggs should be properly disinfected and hatched under stringent sanitation standards. Poultry houses should be thoroughly cleaned and disinfected by recommended procedures between flocks. Rodent and insect control measures should be incorporated into house design and management and verified by periodic testing. Rigidly enforced biosecurity practices should be implemented to restrict movement of personnel and equipment onto poultry housing premises and between houses. Only pelleted feed or feed containing no animal protein should be used. Water provided to poultry should come only from sources treated to ensure cleanliness. Treatments such as gastrointestinal colonization control or vaccination can be applied to reduce the susceptibility of birds to infection. Finally, the *Salmonella* status of poultry and their environment should be monitored by periodic testing to verify the effectiveness of risk reduction practices. Multifaceted risk reduction programs in breeding and laying flocks have been internationally associated with significant reductions in both the prevalence of *S*. Enteritidis infection in poultry and egg-association human illness (9, 340).

Gastrointestinal Colonization Control

Newly hatched chicks and poults are highly susceptible to *Salmonella* infection, but quickly become more resistant as they acquire protective intestinal microflora from their environment. The ability of the normal bacterial flora of the gastrointestinal tract to inhibit colonization by pathogens is the basis for a diverse group of treatments often referred to collectively as competitive exclusion (CE). Defined or undefined CE cultures are administered to poultry to diminish *Salmonella* colonization. Various nonmicrobial manipulations of gastrointestinal biochemistry have also been explored as colonization control options. CE treatment reduces the incidence and magnitude of *Salmonella* colonization in poultry, but rarely prevents it altogether. Accordingly, CE treatment can make a valuable contribution toward controlling salmonellae, but it cannot entirely supplant comprehensive risk reduction programs.

Competitive exclusion treatment with intestinal or fecal material from mature birds or undefined anaerobic cultures derived from such material has diminished both intestinal colonization and subsequent invasion to internal tissues by salmonellae in both chickens and turkeys (323). In commercial broiler flocks, CE treatment significantly reduced the incidence of salmonellae both in live birds and on carcasses (20). CE administration to egg-type pullets before transfer into a contaminated laying house reduced subsequent *Salmonella* isolation from fecal and environmental samples (80). Treatment with CE cultures has sometimes enhanced the clearance of concurrent or preexisting *Salmonella* infections (161). Only live CE preparations exert protective effects, but their efficacy can be maintained by continuous flow culturing (163, 168). CE cultures are effective when administered via oral gavage, spraying, or addition to drinking water. The protective efficacy of CE can be overcome by severe *Salmonella* challenges. Disruption of the normal intestinal microflora by feed or water deprivation, or by antibiotic administration, can interfere with the activities of CE cultures (14). Because CE cultures are most effective when administered before exposure to pathogens, infection during hatching can compromise the value of subsequent treatment (15). Individual CE preparations may not be equally protective in different avian species (230).

Efforts to identify the microflora constituents responsible for CE effects have reported that cultures of diverse bacterial genera, including *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Escherichia*, *Pediococcus*, and *Streptococcus*, and the yeast *Saccharomyces*, exhibit protective (probiotic) activity against salmonellae in chickens (310, 315). Defined mixtures of microorganisms potentially perform with greater consistency and more assurance of safety than undefined cultures of unknown organisms. The protective efficacy of defined CE preparations is often higher for mixtures of greater diversity or complexity (162).

Protection by CE cultures has been attributed to direct steric interference with *Salmonella* attachment to the intestinal epithelium, altered intestinal permeability to pathogens, or inhibition of intestinal *Salmonella* growth via lowered pH (47, 258). Some probiotic cultures may also influence gene expression by salmonellae or modulate the host immune response to infection (107, 164). Diverse "prebiotic" additives either directly inhibit pathogen colonization or support the growth of protective microflora. Feed or water supplementation with various complex carbohydrates (including chitosan, mannanoligosaccharide, and fructooligosaccharide) has reduced crop or cecal colonization of chickens by salmonellae (54, 229). Supplementing feed with medium-chain fatty acids (formic, propionic, butyric, caprylic, or caproic acids),

or providing feedstuffs which are easily fermented to yield these acids, has been associated with lower frequencies of *Salmonella* isolation (231, 325). Plant-derived antimicrobial compounds such as *trans*-cinnamaldehyde have also demonstrated protective efficacy as feed additives (311).

Vaccination

Administration of either killed or live vaccine preparations can significantly reduce the susceptibility of poultry to *Salmonella* infection. Vaccination of laying flocks with several different combinations of killed and live vaccines all reduced the frequency of egg contamination with *S.* Enteritidis, although a lesser effect was observed on fecal shedding and poultry house environmental contamination (8). Decreased incidences of human *S.* Enteritidis infections have followed the widespread implementation of vaccination programs for egg-laying hens (63). Neither type of vaccine has consistently provided an impenetrable barrier against infection, especially when high *Salmonella* challenge doses are involved (140). Feed or water deprivation and environmental stresses such as heat can compromise vaccine efficacy. Vaccination is often unable to confer protection against heterologous serovars (343). Like competitive exclusion, vaccination is most effectively used as a component within comprehensive risk reduction programs.

The emergence of *S.* Enteritidis as an egg-transmitted source of human illness generated renewed interest in killed vaccines (bacterins) for poultry. Killed vaccines have been associated with decreased incidences of *S.* Enteritidis infection in Dutch broiler breeder flocks (106) and *S.* Enteritidis contamination in eggs from Japanese laying flocks (304). Subcutaneous or intramuscular vaccination of laying hens with adjuvanted bacterins induces long-lasting antibody responses (306) and has significantly reduced *S.* Enteritidis isolation from feces, internal tissues, and eggs following subsequent oral challenge (140). Consistently negative *S.* Enteritidis testing results were obtained from a vaccinated laying flock transferred into previously contaminated facilities (79). Bacterin administration to laying hens moderated the effects of induced molting by feed restriction on fecal shedding of *S.* Enteritidis (241). Progeny of breeding flocks vaccinated with *Salmonella* bacterins may display some degree of maternally acquired protective immunity to infection (35). Multivalent bacterins, prepared from a mixture of serovars or strains, can provide an expanded spectrum of protection (86). Immunization of chickens with subunit vaccines composed of outer-membrane, fimbrial, or flagellar proteins has conferred significant protection against *S.* Enteritidis colonization (190, 249). Bacterins which highly express these immunogenic surface components have been shown to have enhanced protective capabilities (274).

Live attenuated vaccines must persist in tissues long enough to induce protective immune responses, but should be avirulent and cleared from vaccinated birds within a few weeks of administration. A wide variety of *Salmonella* vaccine strains have been evaluated for protective efficacy in poultry, often incorporating multiple deletion mutations to ensure irreversible attenuation. Mutant strains unable to synthesize or utilize essential metabolites, produce flagella or fimbria, express virulence-related proteins or lipopolysaccharides, or multiply at poultry body temperatures have all reduced *Salmonella* colonization after administration to chicks or poults by spraying or addition to drinking water (191, 224). Recombinant vaccine strains can express molecules with adjuvant properties to enhance immunogenicity (243). Nonflagellated mutants may elicit protective immunity without preventing serologic detection of paratyphoid-infected flocks (2). Live vaccine strains have been reported to enhance the innate immune response and to induce both humoral and cellular adaptive immune responses (40, 48). Attenuated vaccines have been useful for protecting hens against increased susceptibility to *S.* Enteritidis infection following induced molting by feed deprivation (172) and for enhancing protection by maternal antibodies in chicks from bacterin-vaccinated hens (18). Evidence for cross-protection by live vaccine strains against other *Salmonella* serovars has been inconsistent (251). Protection against challenge with antigenically unrelated *Salmonella* strains has been attributed to a combination of immunological and competitive exclusion mechanisms (170). The commercially available "rough" 9R strain of *S.* Gallinarum has been widely and successfully used for many years as a vaccine to control the incidence of fowl typhoid in regions where this disease is endemic (203). More recently, *S.* Gallinarum attenuated deletion mutants and "ghosts" (cell envelopes after expulsion of their cytoplasmic contents) have also been developed and demonstrated to have protective efficacy against infection (57, 108).

Live *Salmonella* vaccines have sometimes been associated with more complete protection of poultry, perhaps because of more persistent presentation of relevant antigens to the host immune system or because these antigens are adversely affected during the preparation of killed vaccines (12). Bacterins may also fail to fully elicit the cell-mediated portion of the protective immune response (239). However, killed vaccines frequently stimulate protection of greater duration (19). Protection against infection following the combined application of both live and killed vaccines has often exceeded the performance of either product administered separately (109). Prophylactic administration of cellular lymphokines from immunized chickens can protect chicks against organ invasion following subsequent *Salmonella* challenge (226). This effect is associated with increased phagocytosis and

killing of *Salmonella* by avian heterophils (90), but it may be of relatively transient duration (142).

Treatment

The efficacy and wisdom of medicating with antibiotics to prevent or treat *Salmonella* infections in poultry have been debated for many years. Antibiotics have a long history of widespread utilization in poultry at both therapeutic and subtherapeutic (growth-promoting) levels. Their usefulness for these purposes has been extensively documented in both experimental and commercial settings. A variety of antibiotics have demonstrated either prophylactic or therapeutic activity against salmonellae in poultry, in some instances leading to decreased fecal shedding when used as feed additives (67, 71). Antibiotics were employed effectively for *S.* Enteritidis control in broiler and broiler breeder flocks in Northern Ireland (227). Treatment with a fluoroquinolone antibiotic, followed by provision of a competitive exclusion culture to restore protective normal microflora, has reduced fecal shedding of *S.* Enteritidis in broiler breeders, egg-type pullets, and molted laying hens (278). *In ovo* administration of gentamicin controlled *Salmonella* infection without affecting the viability of competitive exclusion cultures given to the hatched chicks (17).

However, current control practices for poultry salmonellosis in many nations no longer regularly rely on antibiotics because of both inconsistent performance of these drugs in eliminating *Salmonella* colonization and concerns that indiscriminate veterinary and agricultural use could promote microbial resistance (67). Limited efficacy of antibiotics for controlling *Salmonella* infections in poultry has been documented on numerous occasions (180). In some instances, antibiotic administration has actually increased susceptibility to *Salmonella* infection, perhaps by suppressing the growth of other competitive or inhibitory microflora (219). Discontinuing antimicrobial use for growth promotion in Denmark was followed by decreased *Salmonella* prevalence in broilers (102). Both therapeutic and subtherapeutic antibiotic administration can select for drug-resistant strains of salmonellae (264), thereby imperiling the medicinal usefulness of those (and related) agents for animals and humans. Drug resistance determinants can accumulate over time in salmonellae present in poultry flocks. Very high incidences of antibiotic resistance have been reported among *Salmonella* isolates from both poultry production facilities and poultry products (182, 252), with a large proportion of these strains often displaying resistance to multiple antimicrobials (282).

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17

Campylobacteriosis

Qijing Zhang and Orhan Sahin

Summary

Agent, Infection, and Disease. Thermophilic *Campylobacter* species, primarily *C. jejuni* and *C. coli*, are frequent colonizers of the intestinal tract of domestic poultry species including chickens and turkeys. Despite the extensive colonization, *Campylobacter* is generally regarded as a commensal in birds, and its infection rarely results in clinical disease or significant pathological lesions. However, fecal colonization leads to consequent carcass contamination in processing plants and foodborne transmission of *Campylobacter* to humans, a significant burden for public health worldwide. Additionally, vibrionic hepatitis associated with *C. jejuni* and *C. coli*, once thought to be a rare condition, has recently become an important problem in free-range laying hens because it causes mortalities and reduced egg production in some parts of the world. This "new" condition, termed spotty liver disease, is shown to be induced by a novel *Campylobacter* species, *C. hepaticus*, and is characterized by multifocal, small white-grey lesions in the liver. In addition, *C. jejuni* is reported to induce intestinal inflammation and diarrhea in certain breeds of broilers under laboratory conditions.

Diagnosis. The most commonly used and accurate diagnostic methods are culture-based isolation of the organism and molecular detection of its DNA from affected sites, along with characteristic lesions in the case of spotty liver disease.

Intervention. At present no single measure (e.g., biosecurity, vaccination, competitive exclusion, etc.) is completely effective in controlling *Campylobacter* infections on poultry farms. The emerging importance of *Campylobacter* in layer chicken health and the significance of *Campylobacter* in food safety necessitate enhanced efforts to develop practical and effective interventions to control *Campylobacter* in poultry.

Introduction

Domestic poultry including chickens, turkeys, ducks, and geese are frequently infected with the members of thermophilic *Campylobacter*, primarily *C. jejuni* and *C. coli* (21, 116, 121, 124). As enteric organisms, *C. jejuni* and *C. coli* are well adapted to the avian host and reside in the intestinal tract of birds. Despite extensive colonization, *Campylobacter* infections produce little or no clinical diseases in poultry (21, 54, 79, 100, 121). However, vibrionic hepatitis associated with *C. coli* and *C. jejuni* has been reported in laying hens and ostriches, causing high morbidity and mortality (11, 131). An experimental study suggested that *Campylobacter* alone is not sufficient to cause vibrionic hepatitis and a predisposing factor in chickens might be required for the development of the disease (65). Interestingly, there have been several reports in recent years describing a condition named spotty liver disease (with pathology and epidemiology similar to vibrionic hepatitis) affecting predominantly free-range layer chickens in Australia and the United Kingdom (24, 144, 145). As a novel *Campylobacter* species, *C. hepaticus* was isolated from the livers of affected chickens and was shown to induce the disease in experimentally infected layers (144, 145). In addition, intestinal inflammation and diarrhea were produced in fast growing breeds of broiler chickens following experimental infection with *C. jejuni* in a recent study (59), suggesting that the organism may be associated with disease in poultry under some circumstances.

Although thermophilic campylobacters are not significant pathogens for poultry, they are of importance to food safety and public health, with *C. jejuni* being responsible for the majority of human campylobacteriosis, followed by *C. coli*, and rarely by *C. lari*. *Campylobacter* has now emerged as a leading bacterial cause of foodborne gastroenteritis in humans around the world (37). The infections are characterized by self-limiting watery and/or bloody diarrhea, abdominal cramp, and possible fever; however, severe conditions

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may occur in immunocompromised patients, requiring antibiotic treatment (92). In addition, *Campylobacter* infection is associated with Guillain–Barré syndrome, a postinfectious autoimmune disease characterized by acute and progressive neuromuscular paralysis (97). The majority of *Campylobacter* infections in humans are sporadic and predominantly associated with poor handling of raw chicken or consumption of undercooked chicken (33, 149). In the United States, a recent study ranked *Campylobacter* in poultry as the highest pathogen–food combination with the largest burden on public health considering the number of cases, hospitalization, death, economic cost, and health-related quality of life (7).

Etiology

Classification

At present, the genus *Campylobacter* contains at least 30 valid species and subspecies, with *C. fetus* being the type species (78, 103). The family *Campylobacteraceae* represents a diverse but phylogenetically distinct group within the group of Gram-negative bacteria and placed in the epsilon division of the *Proteobacteria* (78). In addition to *Campylobacter*, this family comprises the genera *Arcobacter* and *Sulfurospirillum*. Members of this family are characterized by their low chromosomal guanine and cytosine (G + C) content, inability to ferment carbohydrates, and microaerobic growth requirements.

The members of the genus *Campylobacter* are associated with a wide variety of diseases in humans and animals although they are generally commensals in poultry (78). Within the genus, 3 species (*C. jejuni*, *C. coli*, and *C. lari*) known as thermophilic *Campylobacter* are of clinical significance as they are the dominant causative agents of human campylobacteriosis (54, 78, 79).

Growth Requirements

Thermophilic *Campylobacter* spp. grow *optimally* at 37°C–42°C on artificial media with no growth observed at temperatures below 31°C (49, 78). They are slowly growing fastidious organisms and require a microaerobic atmosphere for optimal growth (78, 117). In general, *Campylobacter* is sensitive to oxygen, desiccation, osmotic stress, low pH, and high temperatures (10, 78). *Campylobacter* cells are S-shaped spirally curved rods in size of 0.2–0.8 μm wide and 0.5–6.0 μm long, although cells may transform to spherical or coccoid forms in response to stress or deleterious conditions (78, 124). The members of the genus are Gram-negative, nonspore-forming, and possess a single bipolar flagellum, mediating a characteristic corkscrew-like or darting motility (12, 78). *Campylobacter* spp. are typically unable to ferment or oxidize carbohydrates, and thus energy is mainly derived from the degradation of amino acids or tricarboxylic acid cycle intermediates (73). However, recent studies indicate that some *C. jejuni* strains are able to utilize L-fucose as a substrate for growth (94).

Pathobiology and Epizootiology

Incidence and Distribution

Campylobacter jejuni and C. coli are widespread in avian hosts, especially in commercial chickens and turkeys (21, 100, 116, 121). Generally, the carriage rate of *Campylobacter* in domestic poultry is found to be much higher than that in wild birds (124). This is probably because of the high bird density in commercial poultry houses, which facilitates the spread of *Campylobacter* between birds. The numbers of *Campylobacter*-positive poultry flocks are generally high, but vary by region, seasons, and the production stages and types (conventional, free range, or organic). It appears that the prevalence of *Campylobacter* is lower in Scandinavian countries (e.g., Norway, Sweden, Finland, and Iceland) than in other European countries, North America, and other regions (100). Many prevalence studies have been conducted in Europe and the United States, which reported *Campylobacter*-positive flocks ranging from 0% to 100% (37, 87, 100, 121). Similarly, our recent longitudinal study in commercial broilers indicated high levels of *Campylobacter* prevalence at the flock level (45%) and farm level (93%) in the United States (120). This study also indicated substantial variation in *Campylobacter* prevalence, with some farms consistently rearing *Campylobacter*-free flocks whereas others produced *Campylobacter*-positive flocks over multiple production cycles. Even though the majority of on-farm surveys were conducted with broiler chickens, breeder flocks and laying hens are also commonly infected by *Campylobacter* (61, 118, 124).

Seasonal variations were observed in the prevalence of *Campylobacter* flocks with a peak in warm months (54, 121, 134, 152). The exact reason(s) for this seasonal variation is unknown, but it is proposed that the peaking prevalence of *Campylobacter* in warm months is because of an increased fly population and fly-mediated transmission (41). On commercial poultry farms, *Campylobacter* is rarely detected in birds younger than 2–3 weeks of age regardless of production type or species of poultry (54, 121, 155). Typically, the prevalence of *Campylobacter* increases as the birds grow and reaches to the highest point at the slaughter age for broiler chickens. There is a general trend that *Campylobacter* is more prevalent in organic and free-range flocks than in

conventional production birds (55, 87, 143). For organic and free-range operations, birds have free access to the outside environments and are slaughtered at an older age, both of which may contribute to the increased prevalence rates of *Campylobacter*. Once a broiler flock is infected with *Campylobacter*, the majority of the birds within that flock can become colonized in a short time (36, 142).

With respect to species distribution of campylobacters isolated from chickens, *C. jejuni* accounts for the majority of isolates, followed by *C. coli*, and rarely by *C. lari* (10, 55, 87, 113, 124). However, a higher or even sometimes predominant proportion of *Campylobacter* isolates from turkeys or organic and free-range birds are *C. coli* (55, 87, 113, 154). In China, it was observed that the dominant species in chickens shifted from *C. jejuni* to *C. coli* (151). The exact reason for the shift is unknown, but it is possible that antibiotic selection pressure contributed, at least partly, to the species shift because *C. coli* tends to be more resistant to antibiotics than *C. jejuni*. The isolation of other *Campylobacter* spp. including *C. upsaliensis* and *C. hyointestinalis* in poultry is at a low rate (124, 152). Poultry *Campylobacter* isolates are of multiple genotypes and great genetic diversities. Chicken flocks can be colonized by a single or multiple genotypes of *Campylobacter* (16, 30, 121). Even during a single rearing cycle, a broiler flock can be infected by different species or genotypes of *Campylobacter* at different time points, reflecting the dynamic changes of *Campylobacter* populations on poultry farms.

Transmission, Carriers, and Vectors

Horizontal Transmission

Many farm-based studies have indicated that horizontal transmission from the environment to poultry houses is the most common source of infection of *Campylobacter* on poultry farms (1, 54, 100, 121). Potential sources of infection include old litter, untreated drinking water, other farm animals, domestic pets, wildlife species, house flies (and lack of fly screens), insects, equipment and transport vehicles, farm workers, and thinning (the practice of partial depopulation). Because *Campylobacter* growth is very sensitive to oxygen and temperature, the organism is usually unable to grow in feed, litter or water under normal ambient conditions (100, 116). The organism is usually absent in fresh litter or feed before broilers are infected. Used litter may become contaminated by *Campylobacter* and may play a role in maintaining *Campylobacter* in the farm environment (72, 85). Because of its low moisture content, feed is unlikely to be an original source for the introduction of *C. jejuni* into the poultry houses (62, 116). However, feed can be contaminated by feces in chicken houses (39), which may facilitate the spread of *Campylobacter* within production facilities.

Unchlorinated water supplies have been implicated as a source of *Campylobacter* infection in broiler chickens (107, 157). Because of its requirement for microaerobic conditions and its inability to grow below 31°C–32°C (49), *C. jejuni* is unlikely to propagate in environmental water. The presence of this organism in water systems is probably a sign of a recent contamination by feces of livestock or wild birds (68). Therefore, it is likely that contaminated water serves as a passive carrier of *Campylobacter* rather than a niche for growth of *Campylobacter*. Also, drinking water on poultry farms usually becomes positive with *C. jejuni* only after chickens are colonized (157), questioning the role of drinking water in transmitting *Campylobacter* on poultry farms. Water supplies of intensively reared broilers are often inhabited by protozoa. It was shown that *Campylobacter* could enter into protozoan cells and was able to survive for prolonged periods inside the protozoan cells (4). Protozoa in the aquatic environments could serve as a potential reservoir for *Campylobacter* and may facilitate the survival and transmission of *Campylobacter* in animal reservoirs.

Insects (house flies, darkling beetles, cockroaches, mealworms, etc.) can act as mechanical vectors, and may transmit *Campylobacter* to poultry houses (62, 111, 121). Several studies reported that identical serotypes and genotypes of *Campylobacter* were isolated from both broilers and insects within broiler houses (8, 62, 71). There were also reports indicating that insects in poultry houses were not positive for *C. jejuni* until the organism was isolated from broiler chickens in the same houses (8, 99). A number of studies in Denmark consistently implicated flies as an important risk factor for the introduction of *Campylobacter* into broiler houses (6, 42, 71). These studies indicated a significant reduction in the number of *Campylobacter*-positive flocks, as well as the disappearance of the normal summer peak in prevalence, in broiler houses with fly screens present on ventilation openings. Thus, flies can serve as a vector for transmitting *Campylobacter* on farms, especially during the warmer summer months.

Several studies have shown that rodents and other small wild animals such as raccoons also harbor *Campylobacter* in their intestine, and thus these wild animals can potentially introduce *Campylobacter* into grow-out houses (100, 121). The persistence of some *C. jejuni* clones during successive broiler flock rotations was suggested to be a result of survival of the organism in rodents and insects that were able to evacuate the house during cleaning and disinfection and then return (108). Improper rodent control was inconsistently found to be a risk factor for introduction of *Campylobacter* into broiler houses (25, 43, 121). Because most commercial

poultry production facilities have effective vermin control measures in place, rodents and other small wild animals are unlikely to be a common source of *Campylobacter* infection for commercial broiler flocks.

Campylobacter has a wide distribution in wild birds (44, 82, 150). Wild birds in the vicinity of poultry production facilities are often found to be infected with *C. jejuni*; however, the *Campylobacter* isolates from wild birds are usually different from those of chicken origin (39, 40, 44, 99). Because wild birds often carry *Campylobacter* in their intestines, and owing to their great mobility, wild birds may spread *Campylobacter* to domestic poultry through fecal contamination of pastures, forage, surface water, or feed. Thus, wild birds likely contribute to the overall epidemiology of *Campylobacter* on poultry farms.

Presence of other farm animals on broiler farms including pigs, cattle, sheep, and fowl other than chickens has been found to be associated with an increased risk of *Campylobacter* infection in broiler chickens (100, 121). Even though the direction of transmission (from or into the poultry flocks) was uncertain in many cases, farm animals, particularly cattle, are a likely source of flock infection because livestock is a well-known amplification reservoir for *Campylobacter* (29). Similar genotypes of *Campylobacter* were frequently, but not always, isolated from broilers and cattle in the vicinity (106, 133, 139). In a longitudinal study by Ridley et al. (119a) identical genotypes of *Campylobacter* were detected from a broiler farm and a nearby dairy cattle farm prior to their detection from commercial broiler flocks, suggesting transmission of *Campylobacter* from cattle to poultry. Pigs are also frequently colonized by *Campylobacter* (99). Tending pigs before entering broiler houses was indicated as a risk factor for *Campylobacter* colonization of chickens (70). However, pigs are usually infected with *C. coli* instead of *C. jejuni* (137), whereas poultry (especially chickens) are frequently colonized by *C. jejuni* (121).

Farm workers and equipment may carry *Campylobacter* between broiler flocks or farms and into poultry houses, and were shown to be potential risk factors in some studies (3, 47, 121). Contamination of transport crates by *Campylobacter* occurs frequently and it is proven to be difficult to disinfect them effectively. As such, transport crates were shown to carry identical genotypes of *Campylobacter* that were also recovered from broiler flocks and abattoirs (3, 7, 106), suggesting that such equipment could contaminate birds during transport or could even introduce *Campylobacter* into the broiler houses. Supporting evidence was that there was a noticeable decrease in the proportion of *Campylobacter*-positive flocks when hygiene by farm workers (such as hand washing, use of separate boots for each house, clean anterooms, and proper use of footbath disinfectants) was strictly adhered to (48, 91, 139).

In summary, it appears that poultry houses can be invaded by *Campylobacter* in many different ways from various sources. The complexity of *Campylobacter* transmission and the widespread presence of *Campylobacter* in the production system greatly undermine the success in controlling *Campylobacter* on poultry farms by using management-based strategies.

Vertical Transmission

There was a major debate regarding whether vertical transmission plays a role in introducing *Campylobacter* into poultry flocks, but the current understanding is that vertical transmission of *Campylobacter* does not occur or occurs very rarely on poultry farms. However, some controversy still exists (23, 54, 100, 121). This argument is supported by the following evidence. First, young broiler chickens usually lack *Campylobacter* before 2 or 3 weeks of age even though they are hatched from eggs originated from breeder flocks infected by *Campylobacter*. Second, progeny broiler flocks are frequently infected with strains different from those of their breeder flocks (10, 18, 108, 109, 139). Finally, isolation of *Campylobacter* from eggs and hatchlings has been scarce, and to date there has been only 1 report on isolation of live *Campylobacter* cells from hatcheries or young hatchlings (12). Evidence suggesting vertical transmission of *Campylobacter* was reviewed in previous publications (23, 54, 100, 121). Some earlier studies showed that *C. jejuni* could be isolated from both the outer and inner surface of egg shells laid by naturally infected commercial layers or broiler breeders (123). Furthermore, *C. jejuni* was isolated from the reproductive tract of healthy hens (13, 57) and from semen of commercial broiler breeder roosters (22). However, these findings do not necessarily indicate vertical transmission because *Campylobacter* needs to survive in eggs and the hatching process for transmission to young chicks. Several studies using molecular detection methods demonstrated the presence of *Campylobacter* DNA in embryos, newly hatched chicks, and hatcheries (19, 56), but these methods do not allow differentiation of dead and live *Campylobacter*.

Incubation Period

Experimental studies demonstrated that *Campylobacter* colonization could occur as early as 1 day after inoculation (74, 119, 156). In a few cases where diarrhea, mucosal damage, and inflammation as well as liver lesions (in the case of spotty liver disease) were observed, the incubation time ranged between 2 and 5 days (59, 145, 156). The minimal infective dose to establish colonization in day-old chicks was shown to be as low as 2 cfu (74), although other studies indicated higher infectious doses (125, 132). Once *Campylobacter* colonization

is established, it can persist in the intestinal tract for multiple weeks (79, 116, 132), but a gradual decrease in the level of colonization usually occurs after a prolonged plateau period (88, 156).

On poultry farms, *Campylobacter* is rarely detected in birds younger than 2–3 weeks of age. The reason for this lack of infection in young birds is unclear and may be related to multiple factors including the presence of maternally derived antibodies (15, 115, 119) or differences in environmental or host-related factors. Once a flock is infected, *Campylobacter* spreads rapidly within the flock, leading to colonization of the majority of the birds within a few days (100, 121). Despite the fact that *Campylobacter* infection rarely occurs in young flocks on poultry farms, newly hatched chickens can be readily infected experimentally with *Campylobacter* (54, 156).

Clinical Signs and Pathological Lesions

Campylobacter infections in poultry usually produce no clinical signs of disease under natural conditions. However, it has been reported that in ostriches natural *Campylobacter* infection can cause clinical illness and pathological lesions in the liver and intestines (131). Vibrionic hepatitis was prevalent during the 1950s and 1960s in commercial laying hens in North America but is only occasionally reported nowadays (156). It was suspected that *Campylobacter* might be the cause of the disease, but the etiologic agent(s) for vibrionic hepatitis was not formally identified (124, 156). However, more recently there has been a significant increase in reports describing the occurrence of a condition named "spotty liver disease/syndrome" predominantly in free-range layer chickens in the United Kingdom and Australia, associated with significant mortality (up to 11%), reduced egg production (10%–25%), and loose feces (24, 145, 146). The disease has also been observed in caged layers and broilers, albeit to a much lesser extent. It is now known that the condition is also present in US poultry flocks (39a). Pathologically, spotty liver disease is very similar to vibrionic hepatitis and characterized by the presence of multifocal, 1–2 mm grey-white lesions in the liver (24, 145). Less often, birds may also have fibrinous perihepatitis and excessive clear fluid in the abdomen and/or pericardium. Both epidemiological observations and experimental studies indicated that this condition was caused by a novel *Campylobacter* species, *C. hepaticus* (24, 144, 145). Unlike *C. jejuni* and *C. coli*, the organism grows slowly and may take up to 7 days for first isolation, and requires blood in the cultivation media (24). *C. hepaticus* was detected in the liver and bile as well as in the large intestine of chickens with clinical and pathological signs of the disease, and its transmission among birds is thought to be via the fecal–oral route (146). The organism was shown to be highly invasive in a cell culture model using the chicken hepatoma cell line and produced the typical liver lesions within 5 days following experimental oral gavage (145). The clinical significance of *C. hepaticus* is further corroborated by anecdotal evidence observed by clinical poultry veterinarians in Australia, who indicate spotty liver disease as a major problem in the free-range egg layer industry and to a lesser extent in barn layers (cage free) and chicken meat breeders.

Some studies reported that experimental challenge of young chickens with *Campylobacter* can induce clinical diseases including watery/mucoid/bloody diarrhea, mucosal damage and inflammation, weight loss, or even mortality (59, 156). In an early report, 3-day-old chickens inoculated with a high dose of *C. jejuni* developed diarrhea within 72 h, which lasted for 10 days and resulted in considerable weight loss as well as a mortality of 32% (114). Welkos (153) reported that almost one-third of the day-old chicks and nearly all of the newly hatched chicks, but none of the 3-day old chickens, developed signs of gastroenteritis when orally challenged with *C. jejuni*. Similarly, Sanyal et al. (122) observed watery/mucoid diarrhea in 81% of 36- to 72-hour-old birds 5 days after inoculation with *C. jejuni*, and also found that the Starbro strain of chickens was more likely to develop diarrhea than the white leghorn strain. *Campylobacter* infection in commercial broilers of younger than 2 weeks of age (a rare event) was found to be associated with diarrhea, decreased weight gain, and excess mortality (98). Another study using newly hatched or 4-day old turkey poults also observed reduced weight gain and transient watery diarrhea in the birds after inoculation with *Campylobacter* (77). Oral inoculation of 3-week-old Japanese quails with *C. jejuni* resulted in diarrhea that lasted for 2 weeks (90). In a more recent study (59), production of intestinal inflammation and diarrhea were observed in fast-growing broiler breeds following oral inoculation with *C. jejuni*. Despite these isolated reports, many other studies did not observe any clinical diseases associated with *Campylobacter* infections in poultry (54, 100, 121).

Gross pathologic lesions associated with *Campylobacter* infection in experimentally infected chicks are minimal and primarily confined to the gastrointestinal tract (156). Because of accumulation of fluid, gas, or excess mucus, distention of intestines including ceca with watery/foamy material may be a common finding (122). Blood and mucus in the lumen of the small intestine and petechial hemorrhages in the gizzard mucosa of chicks can be seen occasionally. There is a report that *Campylobacter* was isolated more frequently (21% of 223 livers) from broiler chicken livers with necrotic lesions than from normal livers (12% of 50 livers) obtained from slaughter plants in Canada (9); however, there was no evidence that *Campylobacter* directly

contributed to the lesions. In contrast, *C. hepaticus* was shown to be the direct cause of the liver lesions (1–2 mm multifocal white-grey miliary spots) and the organism was readily detected from such livers (24, 145).

Microscopic lesions following experimental infection of chicks are mostly inapparent or minimal, but exceptions occur in birds with severe clinical and gross pathological signs. Usually examination of gastrointestinal tissue reveals no necrosis or invasion of the epithelium or any other pathological changes; however, a mild edema of the lamina propria and submucosa of the intestines, mostly in ceca, was reported with *Campylobacter* infections in chickens (125, 132, 156). In some cases, *Campylobacter* cells can be seen attaching to the brush borders on enterocytes, within intestinal epithelial cells, and inside or outside of the cells of lamina propria with minimal tissue or cell damage (122). Mononuclear infiltration in the submucosa and villous atrophy resulting in accumulation of red blood cells and leucocytes in small and large intestinal lumen may occur in more severe cases (74). In a recent experimental study, *C. jejuni* was shown to be associated with thickening and shortening of villi in the ileum because of infiltration of inflammatory cells (59). In spotty liver disease, the liver of laying hens manifests mild to severe multifocal necrotic hepatitis, fibrin deposition, hemorrhage, and infiltration of inflammatory cells (24, 145).

Pathogenesis of the Infectious Process

Birds become infected with campylobacters via the fecal–oral route. The universally observed lag phase (i.e., lack of detection in birds younger than 2–3 weeks of age) in the colonization of poultry by *Campylobacter*, even in the presence of likely exposure to positive birds and other sources (69), implies that a biological mechanism of colonization resistance may be present in young birds. As an enteric organism, *Campylobacter* is able to survive the harsh conditions in the stomach (gizzard) and small intestine and eventually reach the lower intestine, where the organism establishes colonization in the cecal and cloacal crypts (156). To a lesser extent, the organism can also be recovered from the small intestines and the gizzard, and infrequently from liver, spleen, blood, and gall bladder. Several distinct features are associated with colonization of *Campylobacter* in chickens (156). First, it appears that *Campylobacter* does not adhere directly to intestinal epithelial cells, but primarily locates in the mucous layer of the crypts. Second, usually no gross or microscopic lesions are induced in the chickens. Third, invasion of the intestinal epithelium occasionally occurs with *Campylobacter.* Even when the invasion of internal organs occurs in some cases, no clinical signs of illness are observed. It was suggested that *C. jejuni* establishes colonization by utilizing a strategy that involves transient invasion of intestinal epithelium to avoid mucosal clearance combined with rapid replication in the intestinal mucus (141). Once a broiler chicken becomes infected, large numbers of the organism (up to 10^9 cfu/g feces) can be detected in ceca and excreted in feces for a prolonged period (e.g., at least until the slaughter age), which has been observed with both experimental and natural infections (100, 121). In a broader context, colonization of chickens by *Campylobacter* can be affected by such factors as the age of the bird, strain of the bacterium, and the diet (16, 45, 54). In a very recent laboratory study, the gut microbiota composition was shown to affect significantly the colonization (and pathology) of broilers both locally within the ceca as well as systemically in the spleen and liver (46). Genotype of the broiler chicken (i.e., growth rate and breed) does not appear to have any significant influence on colonization of birds by *Campylobacter* in field conditions (38). With respect to spotty liver disease, the fecal–oral route is also the likely source for initial infection; however, *C. hepaticus* must be able to invade the intestinal tract to be able to reach the liver and induce specific lesions (145).

Many bacterial factors contribute to the colonization of *Campylobacter* in poultry. These include flagella and motility, DnaJ (heat shock protein), CiaB (*Campylobacter* invasin antigen B), PldA (phospholipase A), CadF (*Campylobacter* adhesin to fibronectin), CmeABC (multidrug efflux pump), CmeR (a pleiotropic regulator), MCP (a methyl-accepting chemotaxis protein), RpoN (sigma factor), the CPS locus (capsule biosynthesis proteins), the Pgl locus (protein glycosylation system), SOD (superoxide dismutase), Fur (ferric uptake regulator), a lipoprotein encoding gene, FucP (a fucose permease), CbrR (a bile resistance response regulator), and poly P (inorganic polyphosphate) (50, 75, 76, 83, 96, 156, 158). A comprehensive review of the colonization factors in chickens was published previously (52) and the list of genes contributing to the infection likely will continue to grow with the advance of genomics and functional genomics.

Immunity

Despite the commensal relationship between *Campylobacter* and the avian host, the infection indeed elicits a mild pro- or anti-inflammatory innate response in the intestinal mucosa (51, 110) as well as both systemic and mucosal humoral responses (14, 112). Following experimental infection of day-old chickens via oral gavage, production of *Campylobacter*-specific IgM and IgA antibodies in serum reached significant levels within 1–2 weeks of infection and peaked at weeks 4–6 postinfection, followed by gradual decreases as birds aged (14). In contrast, detectable levels of IgG responses developed later than IgM and IgA responses, peaked at 8–9 weeks

of infection, and persisted for a longer period (14). Naturally occurring *Campylobacter* colonization in chickens also elicits overt immune responses, and anti-*Campylobacter* antibodies readily transfer from hens to their progenies as maternally derived (115). Maternal antibody plays a partial role in protecting young chickens from infection by *Campylobacter* (15, 119). Additionally, a wide variety of *Campylobacter* antigens are recognized by sera of naturally or experimentally infected chickens (14, 115, 126). There is a trend that with the development of specific anti-*Campylobacter* antibodies, the level of *Campylobacter* colonization diminishes, and some infected chickens eventually clear the infection (156). However, the nature of protective immunity has not been elucidated, and it is unknown if humoral immunity or cellular immunity (or both) contributes to the clearance of *Campylobacter* from the host.

Diagnosis

Culture-based Isolation and Detection Methods

Thermophilic campylobacters are fastidious and slow-growing, requiring microaerobic atmosphere (containing 5% O_2, 10% CO_2, 85% N_2) and elevated temperature (42°C) for optimal growth under laboratory conditions, with no growth observed at temperatures below 31°C (49, 117). Thus, culturing *Campylobacter* spp. from fecal or environmental materials with a high level of background flora requires the use of selective culture media and special culture conditions. The first selective medium for culturing *C. jejuni* and *C. coli* was developed in 1977 by Skirrow (128). Since then, more than 40 solid and liquid selective media for culturing of *Campylobacter* from clinical and food samples have been reported, which have been reviewed by Corry et al. (20). Some of the most commonly used ones are Skirrow, Preston, Karmali, modified charcoal cefoperazone deoxycholate agar (mCCDA), cefoperazone amphotericin teicoplanin (CAT) agar, Campy-CVA (cefoperazone vancomycin amphotericin), Campy-Cefex agar (CCA), modified CCA (mCCA), Campy-Line agar (CLA), *Campylobacter* agar plates (CAP), and *Campylobacter* selective chromogenic medium CASA. More recently, culture methods for recovery of more fastidious species from various sources including chicken meat have also been described (89, 103), which should be useful for isolation of *Campylobacter* species other than *C. jejuni* and *C. coli* in a variety of sample types. The selective media contain a variety of different combinations of antibiotics to which thermophilic campylobacters are intrinsically resistant, such as polymyxin, vancomycin, trimethoprim, rifampicin, cefoperazone, cephalothin, colistin, cycloheximide, and nystatin. The multidrug efflux pump CmeABC in *Campylobacter* contributes, at least partly, to the intrinsic resistance to these selective agents (83). Use of these antibiotics inhibits the growth of many background microbial flora present in samples and allow the isolation of slow-growing *Campylobacter* spp.

Because *Campylobacter* spp. are sensitive to oxygen levels above 5%, *Campylobacter* selective media often contain various oxygen-quenching agents in order to neutralize the toxic effect of oxygen radicals (20). The commonly used oxygen-quenching agents include blood (e.g., Skirrow and Campy-CVA media), a combination of ferrous sulfate, sodium metabisulfite and sodium pyruvate (e.g., CCA), charcoal (e.g., mCCDA agar), and hematin (as in Karmali agar).

Depending on the type of specimen, selective media can be used either for direct plating or for an enrichment step followed by plating for isolation of *Campylobacter*. An enrichment step in liquid medium followed by plating on solid agar plates is usually superior to direct plating alone for the isolation of *Campylobacter* from processed foods in which bacteria are usually in relatively low numbers and/or in an "injured" state (20, 60). However, the enrichment step may not always perform better than direct plating when culturing fecal samples. Musgrove et al. (95) compared enrichment and direct plating for isolation of *Campylobacter* from ceca and crops, which showed that direct plating of cecal samples on selective media resulted in a significantly higher recovery rate than the enrichment method. However, enrichment was slightly better than direct plating for the recovery of *Campylobacter* spp. from crop samples. When an enrichment step is used, it should be controlled for less than 24 hours because a prolonged incubation in enrichment broth may actually decrease the isolation rate.

To isolate thermophilic campylobacters from environmental water, 2 methods can be used to increase the detection sensitivity. A large volume of water can be filtered through a single membrane with a pore size of 0.2 μm. Subsequently the membrane can either be placed directly on a selective agar plate, or first cultured in an enrichment broth followed by selective plating (104, 107). Alternatively, water samples can be concentrated by high-speed centrifugation from which the supernatant is discarded, and the pellet is cultured by direct plating or enriched in broth followed by plating. More recently, 0.65 μm pore size membrane filters were used to facilitate the passage of *Campylobacter* organisms onto a selective agar medium following pre-enrichment in selective broth media of water and fecal samples as well as broiler meat with high specificity (67, 129).

Usually typical *Campylobacter* colonies are visible on solid media after 48 hours of incubation, but it may take up to 72–96 hours to observe some slow-growing strains (20, 117). Depending on the media used, colonies of *Campylobacter* spp. may appear differently. If the agar is

moist, the colonies may appear gray, flat, irregular, and thinly spreading. Round, convex, or glistening colonies may be formed when plates are dry (20). Presumptive identification of thermophilic *Campylobacter* spp. can be made according to colony morphology, typical cellular shapes (spiral or curved rods), and characteristic rapid darting motility as observed under a phase-contrast microscope. The phenotypic tests for identification of *Campylobacter* to genus or species level include biochemical tests (catalase, oxidase, nitrate reduction, hippurate hydrolysis, indoxyl acetate hydrolysis), antibiotic susceptibility patterns (nalidixic acid, cephalothin), and growth characteristics at different temperatures (25°C, 37°C, and 42°C) (102, 103). Differentiation between *C. jejuni* (hippurate-positive) and *C. coli* can be performed with the hippurate test. However, hippurate-negative *C. jejuni* isolates have been reported (130), emphasizing the need for further testing of hippurate-negative strains with other methods when species identification is considered important. Most recently, matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry has been successfully utilized for species level identification of *Campylobacter* with very high speed and specificity (93, 103).

Immunology-Based Diagnostic Methods

Enzyme immunoassays (EIA), based on antigen–antibody interaction, have been developed for direct detection of *Campylobacter* spp. in animal feces or processed food. These EIA assays are commercially available in a very similar format to sandwich-ELISA assays, which use 2 different antibodies, to detect *Campylobacter* spp. directly in crude samples (58) or after a selective enrichment step (81). Most of these assays have been evaluated with human stool samples, but their utility for detection of *Campylobacter* in chicken feces or on chicken carcasses remains to be determined.

Nucleic Acid-Based Diagnostic Methods

Molecular techniques, in particular PCR, has been utilized for direct detection of *Campylobacter* from various sources, but its main use has been for the confirmation/identification of *Campylobacter* isolates (32, 103). PCR assays can be used in conjunction with conventional culture methods to improve the speed and accuracy of *Campylobacter* detection and identification. Additionally, improvement of DNA purification from complex samples should also increase the utility of PCR-based detection methods. In addition to PCR, probe-based assays (e.g., DNA microarrays) have also been reported for detection of *Campylobacter* from chicken feces and carcasses at the abattoir and retail (30). With the use of multiple probes, probe-based assays allow simultaneous detection and differentiation of different species and strains.

A growing number of DNA-based methods have been developed for molecular subtyping of *Campylobacter* isolates from chickens and other animal reservoirs for epidemiological purposes. These include pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), comparative genomic fingerprinting (CGF), ribotyping, and sequence-based methods such as multilocus sequence typing (MLST) and whole genome sequencing (WGS) (27, 136). These molecular typing and subtyping methods are used for pure *Campylobacter* cultures and are not suitable for direct detection from feces and other complex samples.

Intervention Strategies

Poultry houses can be contaminated by *Campylobacter* in many different ways from various environmental sources, making the control of flock colonization by *Campylobacter* a very challenging task. In general, on-farm control strategies that have been examined for control of *Campylobacter* in poultry can be broadly divided in 2 approaches: (1) prevention of flock colonization by use of biosecurity-based interventions, and (2) prevention and/or reduction of *Campylobacter* colonization by nonbiosecurity-based measures such as vaccination, bacteriocins, feed additives, bacteriophages, and competitive exclusion (53, 66, 84). At present no single measure is completely effective in controlling *Campylobacter* infections on poultry farms.

Biosecurity

Several epidemiological investigations have found a correlation between decreased *Campylobacter* infection in broiler flocks and the employment of stringent biosecurity measures and hygienic practices on farms (2, 35, 53, 148). These practices include washing hands before engaging with the flocks, designating separate boots and personal gear for different broiler houses, deploying footbaths for disinfection, limiting access to the flocks only to essential personnel, training workers in best hygiene practices, controlling pests such as rodents and insects, thorough decontamination of drinking water delivery systems, maintaining the physical structure of broiler houses, and discontinuation of flock thinning. In most of these studies, adherence to biosecurity measures either reduced the colonization level or delayed the onset time of colonization of birds by *Campylobacter*, but was largely unsuccessful in totally preventing the introduction of *Campylobacter* into broiler flocks. In addition, stringent biosecurity measures are cost-prohibitive, hard

to maintain, and their effectiveness varies with production systems. Although on-farm biosecurity measures appear to be effective in reducing the incidence of *Campylobacter* infection in northern Europe in countries such as Norway, Sweden, and Finland, these measures have met limited success in other countries such as the United Kingdom, the Netherlands, and Denmark (53, 121, 140, 152). Because *Campylobacter* spp. are commonly present in the poultry farm environment and poultry flocks can be infected by multiple sources, it is rather difficult to completely eliminate *Campylobacter* from poultry houses by use of biosecurity practices alone. So far, use of fly screens on poultry houses has been found to be a consistently effective measure to reduce the introduction of *Campylobacter* into broiler houses during the summer months in some European countries (6), but their use in other countries such as in the United States remains to be examined because of prominent differences in the rearing practices (e.g., horizontal [tunnel] ventilation systems in the United States vs. vertical ventilation shafts in Europe) (6). Therefore, it is important to consider variations in production practices when developing and implementing control strategies.

Competitive Exclusion

A number of studies investigated competitive exclusion as a mean of preventing *Campylobacter* colonization in broiler chickens (53, 84). These studies used fecal or cecal mucus suspensions, intestinal homogenates, undefined cecal mucus culture, or defined flora from cecal mucus. Although some level of protection was observed in chickens challenged under laboratory conditions, the degree of reduction was inadequate for practical purposes. Ideally, competitive exclusion should use pure (defined) cultures instead of crude intestinal mucus suspensions. However, the effect of a defined competitive exclusion flora on *Campylobacter* was variable and inconsistent (53, 121). For example, in several studies assessing the effectiveness of the competitive exclusion product Broilact, a substantial reduction in *Campylobacter* colonization was observed in 1 study but was not reproduced by others. In another example, it was reported that the administration of *Bifidobacterium longum* PCB 133 in feed reduced *C. jejuni* by approximately 1 log in the feces of experimentally infected chickens, but this was not reproduced in a follow-up study (5). In general, the currently available competitive exclusion products are of low effectiveness and poorly reproducible under production conditions (53, 121). In a very recent study, the gut microbiota composition had a significant effect on *Campylobacter* colonization in broiler chickens (46); however, the key member(s) of the microbiota and the underlying mechanisms for this observation are currently unknown. With the advance of metagenomics technology and improved understanding of the poultry gut microbiome, it is possible that more effective products for competitive exclusion will be developed in the future.

Vaccination

Currently there are no commercial vaccines available for control of *Campylobacter* in poultry. The commensal nature of *Campylobacter* colonization, the extensive genetic/antigenic diversity among different *Campylobacter* strains, and our lack of understanding of protective immunity create a great challenge for developing an efficacious vaccination regimen for the induction of a strong and persistent mucosal immune response. The reported immunization studies used killed whole cells, live-attenuated cells, flagellin-based subunit vaccines, genetically engineered live vectors expressing *Campylobacter*-specific antigens, and chitosan-encapsulated DNA vaccines, most of which showed a somewhat protective effect in chickens (34, 84, 121). Very recently, Nothaft et al. (101) constructed and evaluated 2 glycoconjugate vaccines (based on the conserved N-glycan of *Campylobacter*) to prevent colonization by *C. jejuni* in layer chickens. Both vaccines resulted in up to a 10-log reduction in *C. jejuni* colonization in the ceca and induced specific antibodies, without altering the gut microbiota composition. These encouraging results suggest the high possibility of using vaccination to control *Campylobacter* infection on poultry farms in a practical and economically viable manner.

Other Intervention Strategies

Several other potential intervention strategies have been evaluated to reduce *Campylobacter* colonization in chickens, including phage therapy, litter treatment, bacteriocin-based treatment, and feed/water additives as well as genetic resistance (53, 66, 84, 121). *Campylobacter*-specific bacteriophages are commonly recovered from broiler chickens and the farm environment. Experimental challenge studies, in which broiler chickens were used to assess the prophylactic or therapeutic effect of bacteriophages against *Campylobacter* colonization, showed significant decreases (2–3 logs) in *Campylobacter* numbers in chickens treated with phages (28, 31, 147). However, the level of reduction was variable and was affected by the phage types and doses, and did not seem sustainable over time. At present, there are still several difficulties to overcome for phage therapy to become a practical intervention, which include the narrow spectrum of phages, the rapid development of resistance, and the low efficiency of phage production in *Campylobacter*. Despite these limitations, bacteriophages may still offer a viable complementary approach when applied immediately before chickens are due for slaughter (63).

Several feed and water additives (including short- and medium-chain fatty acids, plant-derived additives, egg yolk powder, chlorination, monocaprin, etc.) were found to be partially effective in controlling *Campylobacter* colonization and transmission in broiler chickens (121). Chlorination or acidification of drinking water with organic acids (e.g., formic acid, acetic acid, propionic acid, and lactic acid) lowered *Campylobacter* concentrations in broiler ceca and its transmission between birds in some studies (64, 138), but showed no significant effect in others (17). In a study by Skanseng et al., a combination of 2% formic acid and 0.1% potassium sorbate totally prevented colonization of broilers by *C. jejuni* (127); however, this approach still requires evaluation under field conditions on commercial farms. Supplementation of feed with prebiotics (e.g., lactose, fructooligosaccharide, mannose-oligosaccharide), immune response stimulators (selenium, beta-glucan), activated charcoal, and other compounds have had limited success in reducing *Campylobacter* colonization in chickens (26, 53). Similarly, although the acidification of litter (with aluminum sulfate and sodium bisulfate) has been shown to be successful in decreasing *Campylobacter* colonization and cecal load in broilers in laboratory trials, the effect on commercial broiler farms was found to be negligible (86).

Several bacteriocins (antimicrobial peptides of bacterial origin) purified from *Paenibacillus polymyxa*, *Lactobacillus salivarius*, and *Enterococcus* spp. have been evaluated for controlling *Campylobacter* colonization in chickens (84, 135). When given to chickens as feed supplements, these bacteriocins were highly effective in reducing *C. jejuni* infections. Notably, the bacteriocins had broad anti-*Campylobacter* activities and were effective against different strains of *C. jejuni* when tested in chickens. Also, treatment of market-age broiler chickens naturally colonized by *Campylobacter* with bacteriocins in feed or water a few days before slaughter significantly reduced intestinal colonization (135). These findings suggest that bacteriocin supplements are promising approaches for control of *Campylobacter* in poultry, but it is unknown if these bacteriocins can be produced in a cost-effective manner for commercial use and if they will meet regulatory approval.

Finally, genetic resistance may constitute another approach to control *Campylobacter* in poultry (59). In a recent study, the resistance of chickens to *Campylobacter* colonization was found to be associated with the inhibition of small GTPase-mediated signal transduction and the tumor necrosis factor receptor superfamily genes (80). In another study, *Campylobacter* was shown to be commensal in some breeds of broilers, but a pathogen in other breeds (59). These observations suggest the possibility of selective breeding of broilers that are more resistant to *Campylobacter* in future.

Acknowledgment

A large number of published studies are available on *Campylobacter* in poultry and it is impossible to cite every relevant reference because of the space limit. The authors regret not being able to include many other excellent publications in the reference list.

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18

Colibacillosis

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Summary

Agent, Infection, and Disease. Colibacillosis is a localized or systemic disease of production poultry associated with the bacterial pathogen avian pathogenic *Escherichia coli* (APEC). The disease may present in a number of forms including colisepticemia, air sac disease, peritonitis, swollen head syndrome, and salpingitis among others. The previous school of thought was that *E. coli* was a secondary pathogen to other pre-disposing factors such as viral infection, stress, ammonia, etc. Current thought is that APEC can also be a primary pathogen.

Diagnosis. Diagnosis is based on isolation and identification of *E. coli* from lesions typical of colibacillosis in affected birds. Classic microbiological culture of samples on media to select for *E. coli* are helpful followed by further characterization using molecular tools and techniques to type and subtype the organism. A rapid polymerase chain reaction protocol that targets genes of the APEC virulence plasmids has proven successful in providing routine screening, but may not provide 100% identification because of the diverse nature of strains implicated in disease.

Intervention. Current interventions for colibacillosis include hatchery and flock management practices, with emphasis on air quality, temperature, litter and housing environment, and sanitation. Vaccines have proven effective in controlling some strains of *E. coli*; however, the diversity of strains implicated in colibacillosis is large and vaccines may not prove effective for all strains. With new limitations on the use of antimicrobial agents in poultry production, the choice of drugs for use may be limited or not effective should resistant strains be present.

Introduction

Definition and Synonyms

Colibacillosis refers to any localized or systemic infection caused entirely or partly by avian pathogenic *Escherichia coli* (APEC). Syndromes of disease include colisepticemia, hemorrhagic septicemia, coligranuloma (Hjarre's disease), airsacculitis (chronic respiratory disease, CRD), swollen-head syndrome, venereal colibacillosis, coliform cellulitis (inflammatory or infectious process), peritonitis, salpingitis, orchitis, osteomyelitis/synovitis (including turkey osteomyelitis complex), panophthalmitis, omphalitis/yolk sac infection, and enteritis. Lesions alone should not be used to infer an *E. coli* infection without the descriptor "coli" or "coliform" being added, because other opportunistic bacteria can behave similarly to *E. coli* in secondary infections. Colibacillosis in mammals is most often a primary enteric or urinary tract disease, whereas colibacillosis in poultry is typically a localized or systemic disease often occurring secondarily when host defenses have been impaired or overwhelmed by virulent *E. coli* strains (31). Strains of *E. coli* that cause disease outside the intestinal tract of any species share common characteristics and are called extraintestinal pathogenic *E. coli* (ExPEC) (449). Most APEC are ExPEC and share characteristics with mammalian ExPEC.

Several reviews on APEC and colibacillosis in poultry have been published and the readers are referred to the previous edition of *Diseases of Poultry* for a more comprehensive review.

Economic Significance

There is general agreement that colibacillosis is the most common infectious bacterial disease of poultry and that collectively, *E. coli* infections in their various forms

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are responsible for significant economic losses. Often colibacillosis is among the most frequently reported diseases in surveys of poultry health or condemnations at processing. In the United States, 5.7 billion pounds of federal infected poultry were produced from January to October 2017, with condemnation rates of 0.23% antemortem and 0.90% postmortem. Airsacculitis was responsible for 17.3% of condemnations in chickens and 12% in turkeys, with septicemia responsible for 35% of condemnations in chickens and 50.8% in turkeys (375).

Flocks with airsacculitis at processing were found in general to have lower average body weights (84 g/bird), more processing errors, fecal and *Campylobacter* contamination (447). APEC infections were identified as a major factor in poultry disease in Belgium. Data collected at the East-Flanders regional laboratory between 1997 and 2000 on groups of healthy and sick broilers, layers, and breeders showed the incidence of APEC infection to be 17.7%, 38.6%, and 26.9% respectively (516). The most frequent cause of mortality in a study of organic layer flocks and confined flocks on deep litter in Denmark was *E. coli.* Coinfections with *Pasteurella multocida, Erysipelothrix rhusiopathiae,* other bacteria, and *Histomonas meleagridis* resulted in higher mortality (483). A study on *E. coli* peritonitis syndrome in egg-producing farms in the Netherlands found that total losses was 0.28 euros per hen housed in caged-layers and 1.87 euros per hen housed in meat-type breeders (276). However, in spite of its recognized importance, no studies accurately defining the economic significance of colibacillosis in poultry appear to have been done.

Public Health Significance

Though poultry has not been a significant source of shigatoxin producing *E. coli* (STEC) in human disease, continued vigilance is recommended because STEC, including *E. coli* O157:H7, an important enterohemorrhagic pathogen of humans, has been isolated from various types of birds and poultry products (104, 165, 176, 401). More concerning is the possibility that APEC-contaminated poultry and eggs are a foodborne reservoir of ExPEC that cause human urinary tract infections, meningitis, and other extraintestinal diseases (312, 485). This hypothesis is based on the remarkable similarities that some APEC and human ExPEC share, by contrast to other meat commodities, in their genomic sequences, serogroups, virulence genotypes, phylogenetic types, plasmid content, antimicrobial resistance patterns and abilities to cause disease in various *in vitro* and *in vivo* models of human disease (230–232, 239, 251, 288, 289, 312, 331, 335, 338, 339, 347, 352, 437, 443, 472, 485). Support for this hypothesis is also found in the fact that retail poultry meat harbors *E. coli* that are more like APEC and human ExPEC than commensal *E. coli* from the feces of birds at slaughter. These similarities are especially notable in regards to the virulence plasmid content of these organisms (232). Though ExPEC causing human urinary tract infections may harbor virulence plasmids (239), APEC and human neonatal meningitis *E. coli* are defined in great part by their presence (238, 299, 438). These plasmids have been shown to contribute to the pathogenesis of colibacillosis, urinary tract infection, and meningitis (230, 472), and are transmissible by conjugation from APEC to other pathogens of human health significance (233). Evidence that such transfer occurs in poultry production is found in the emergence of a highly virulent strain of *Salmonella* var *enterica* Kentucky, which contains APEC-like plasmids (236).

Also concerning is the fact that in APEC, these virulence plasmids may harbor multidrug-resistance (MDR)-encoding islands or co-transfer with large MDR-encoding R plasmids (233, 237). Such MDR islands or R plasmids can encode resistance to heavy metals, disinfectants and various antimicrobials. The potential for spread of resistance genes from APEC to organisms of human health importance must be considered. *S. enterica* subsp. enterica serovar Newport, a disease-producing organism in people, and another *E. coli* serotype readily acquired antibiotic resistance in the absence of antibiotic selection pressure through transfer of a large conjugative resistance plasmid from antibiotic-resistant *E. coli* in the intestine of turkey poults. The plasmid was transferred to over 25% of *Salmonella* serovar Newport strains following coinfection (418). Antibiotic resistance of fecal *E. coli* was greater in broilers and turkeys that received antibiotics relatively frequently compared with layers, which had little exposure to antibiotics (509). Similar antibiotic resistance patterns were found in *E. coli* isolated from poultry slaughter workers and birds, and in some instances specific strains were shared indicating that transmission of resistant organisms and/or plasmids from poultry to people is common. Although exposure could also occur with people who own backyard flocks, these may not be an important reservoir (417).

Thus, it is prudent to consider poultry as a reservoir of ExPEC strains and/or plasmid-linked virulence and resistance genes contributing to the pathogenesis and outcome of disease caused by ExPEC or other human pathogens.

History

Mortality of fowls and isolation of a bacterium from heart, liver, and spleen that was consistent with *E. coli* was first reported by Lignieres in 1894 (398). Following experimental inoculation, the isolate was virulent for pigeons and variably virulent for chickens depending on dose and route of administration. Subsequently diseases

in grouse, pigeons, swans, turkeys, quail, and additional chicken flocks associated with a similar organism were documented between 1894 and 1922 (398).

The first description of colisepticemia was published in 1907 based on chickens dying from a cholera-like disease while being transported (398).

Infectious enteritis and paralysis from which *E. coli* could be isolated was described in 1923 (398). In 1938, a pullorum-like disease associated with poor incubation was reported in chicks less than 10 days of age diagnosed with pericarditis, perihepatitis, and white spots on the liver with *E. coli* isolated from tissues (86). By 1965, *E. coli* had been isolated from a variety of lesions affecting virtually all bird organs, as well as eggs (474).

Etiology

The etiology of colibacillosis is *E. coli*. Other infectious agents and noninfectious factors usually predispose a bird to infection or contribute to the severity of the disease. Two additional *Escherichia* species, *E. fergusonii* and *E. albertii,* have been isolated from birds and are capable of causing disease or are of public health significance.

Escherichia fergusonii is a closely related species that has been isolated from poultry (128, 130), shown to cause disease in day-old chicks (130) and acute death in adult ostriches (194), and exhibit a broad range of antimicrobial resistances (130, 274). Whole genomic sequencing of a strain of *E. fergusonii*, isolated from a broiler chicken, localized a number of antimicrobial resistance genes and known and putative virulence genes to the chromosome of 1 of 5 plasmids. The similarities of one of its large plasmids to well-described APEC virulence plasmids is especially notable, because these plasmids are the defining characteristic of APEC and neonatal meningitis *E. coli* pathotypes (238, 239, 299, 437) and contribute to the pathogenesis of colibacillosis in poultry and meningitis, urinary tract infection, and sepsis in murine models of human disease (230). This report plus descriptions of the emergence of an APEC plasmid-containing strain of *Salmonella* var enterica Kentucky (131, 236) suggests that acquisition of APEC plasmids by commensal or pathogenic bacteria in poultry production may enhance their virulence and/or resistance and lead to the emergence of new disease problems among poultry.

Escherichia albertii is an *Escherichia* species that ferments d-mannitol but not lactose or d-xylose. Phenotypic and molecular tests are used to identify *E. albertii*, but conventional identification systems often incorrectly identify the organism as *E. coli, Hafnia alvei,* or other enteric bacteria (2). *E. albertii* causes gastroenteritis in people and in birds; infections range from asymptomatic to severe intestinal disease and mortality. Virulence factors include intimin (responsible for attaching-effacing lesions in the intestinal tract) and cytolethal distending toxin, also a cause of diarrheal disease (389, 390). Another study isolated 65 isolates from 27 poultry carcass rinses. They found that the majority of the isolates (89%) were positive for *clpX*, *lysP*, *mdh*, *eae*, and *cdtB* and negative for *staA*, *stx1*, *stx2*, and *stx2f* (297).

Classification

Escherichia is the type genus of the family Enterobacteriaceae, which is composed of organisms that can grow aerobically or anaerobically and utilize simple carbon and nitrogen sources (43, 126). *E. coli* is the type species of the genus *Escherichia*. Additional species have been assigned to the genus but *E. coli* occurs most commonly and is most important as a pathogen. Although *Shigella* is still recognized as a genus with 4 species, they actually group genetically with *E. coli* (374).

Name and Synonyms

Escherichia coli was initially named *Bacterium (Bacillus) coli* commune, which was shortened and modified to *B. coli* before being given its present name by Castellani and Chalmers in 1919. The genus is named for Theodor Escherich, a pediatrician who first identified and described the organism, which appeared in the feces of infants soon after they began nursing. It is typical of bacterial species within the family Enterobacteriaceae (374). Diagnostic characteristics of *E. coli, E. fergusonii,* and *E. albertii* are presented in Table 18.1.

Morphology and Staining

Escherichia coli is a Gram-negative, non-acid-fast, uniform staining, non-spore-forming bacillus, usually 2–3 × 0.6 μm. Organisms grown in culture are more variable in size and shape. Intracellular organisms are often smaller than extracellular bacteria. Most strains are motile and have peritrichous flagella.

Growth Requirements

Escherichia coli grow aerobically or anaerobically on ordinary nutrient media at temperatures of 18–44°C. It ferments carbohydrates, often producing gas. Generation time and growth rate are related to temperature (see Table 18.2).

Colony Morphology

On agar plates incubated for 24 hours at 37°C, colonies are low, convex, smooth, and colorless. Colonies are bright pink and surrounded by a precipitate on MacConkey's agar, have a dark green-black metallic sheen on

Table 18.1 Diagnostic characteristics of *Escherichia coli* (*Ec*), *E. fergusonii* (*Ef*), and *E. albertii* (*Ea*) (2, 374).

Gram-negative, rod (bacillus) shape, nonspore forming, oxidase negative, facultative anaerobe			
	Ec	***Ef***	***Ea***
MacConkey agar	+ Pink colonies, precipitate	Clear colonies	Clear colonies
Tergitol-7 agar	+ Yellow colonies	Red colonies	Red colonies
EMB agar	+ Dark colonies, metallic sheen	Clear colonies	Clear colonies
Motility	+	+	–
Catalase	+	+	+
Nitrates → nitrites	+	+	+
Gelatin	–	–	–
Hydrogen sulfide	–	–	–
Indole	+	+	–/+*
Methyl red	+	+	+
Voges-Proskauer	–	–	–
Citrate (Simmons)	–	–	–
Urease	–	–	–
KCN medium	–	–	–
Lysine decarboxylase	+	+	+/–*
Ornithine decarboxylase	v	+	+
Adonitol	–	+	–
Arabitol	–	+	–
Cellobiose	–	+	–
Dulcitol	v	v	–
Glucose	+	+	+
Inositol	–	–	–
Lactose	+	–	–
Malonate	–	v	–/+[1]
Mannitol	+	+	+
Xylose	+	+	–
Salicin	v	v	–
Sorbitol	+	–	–/+[1]
Sucrose	v	–	–

+ Growth or reaction occurs.
– Growth or reaction does not occur.
v Reaction or character is variable among isolates.
[1] Biogroup 1 (human-origin)/biogroup 2, formerly *Shigella boydii* 13, (human- and animal-origin).

eosin-methylene blue (EMB) agar, and are yellow on tergitol-7 agar. Although colony morphology may vary, they are usually 1–3 mm in diameter with granular structure and an entire margin. Rough colonies are larger with irregular margins. Mucoid colonies are raised, larger, appear wet, and are sticky when probed. In contrast to the frequent occurrence of hemolysis by mammalian pathogenic *E. coli* on blood agar, hemolysis is not a common characteristic of APEC (438). *E. coli* rapidly produces diffuse turbidity in broth cultures.

Biochemical Properties

Acid and gas are produced from fermentation of glucose, maltose, mannitol, xylose, glycerol, rhamnose, sorbitol, and arabinose, but not dextrin, starch, or inositol. Substituting sorbitol for lactose in MacConkey agar is useful for distinguishing *E. coli* O157:H7 from other *E. coli* because O157:H7 typically does not ferment sorbitol and will appear colorless to straw yellow compared with typical *E. coli* isolates which will appear pink. Most

Table 18.2 Effect of temperature on generation time and numbers of *Escherichia coli* that could develop within 24 hours in the absence of limits on growth (nutrition, accumulation of inhibitory substances, etc.).

Temperature			
(°F)	(°C)	Generation Time (Hours)	No. of *E. coli* in 24 Hours
32	0	20	2
40	4.4	6	8
50	10.0	3	128
60	15.6	2	2,048
70	21.1	1	8,388,608
80	26.7	0.75	3,435,973,800
90	32.2	0.50	24,073,749,000,000
100	37.8	0.30	236,118,320,000,000,000,000

E. coli isolates ferment lactose, but negative strains, which must be differentiated from *Salmonella*, are occasionally isolated. Fermentation of adonitol, sucrose, salicin, raffinose, and dulcitol is variable. Isolates that fermented raffinose and sorbose produced high mortality in an embryo lethality test (346). *E. coli* produces indole, a positive methyl red reaction, and reduces nitrate to nitrite. Voges-Proskauer and oxidase reactions are negative and hydrogen sulfide is not produced in Kligler's iron medium. *E. coli* does not grow in the presence of potassium cyanide, hydrolyze urea (urease negative), liquefy gelatin, or grow in citrate medium. Biochemical tests can be used to distinguish *E. coli* from other *Escherichia* species (43) and bacteria in the family Enterobacteriaceae (126). *E. fergusonii* does not ferment lactose, sucrose, raffinose, or sorbitol, which helps distinguish it from *E. coli* (Table 18.1). *E. albertii* does not ferment lactose, sorbitol, or xylose, which helps distinguish it from *E. coli* (Table 18.1).

Susceptibility to Chemical and Physical Agents

Escherichia coli have susceptibility patterns to chemical and physical agents typical of vegetative, Gram-negative bacteria. Inactivation of most strains will occur at temperatures ranging from 60°C for 30 minutes to 70°C for 2 minutes. Thorough precleaning and/or presence of a germicide enhance thermal inactivation. The organism survives freezing and persists for extended periods at cold temperatures. Thermal inactivation in litter to achieve a 90% reduction in the number of bacteria is dependent on time and temperature. Inactivation in litter is slower in the presence of high moisture, but more rapid when free ammonia is present (196).

Reproduction of most strains is inhibited by a pH of less than 4.5 or greater than 9, but the organism is not killed. Some virulent strains, e.g., O157:H7, are acid tolerant, which permits them to pass through the stomach. Organic acids are more effective than inorganic acids at inhibiting growth. Treatment with citric, tartaric, or salicylic acids significantly reduces coliform counts in poultry litter (219). Certain plasmids may also contribute to APEC's acid and bile tolerance affecting survival of APEC in the bird or elsewhere (332). A salt concentration of 8.5% prevents growth but does not inactivate the organism (39).

Stabilized chlorine dioxide is highly effective when used as a water disinfectant (404). Chlorate in feed selectively reduces the number of *E. coli* and related bacteria in the digestive tract by converting relatively nontoxic chlorate to highly toxic chlorite via the same pathway *E. coli* uses to convert nitrate to nitrite (12). Solar disinfection of water through the action of ultraviolet light and temperature is a low-cost method of treating drinking water for people that may have application in the poultry industry (40).

Drying is detrimental to the organism. When samples of flooring from broiler transport coops were contaminated with *E. coli* and allowed to dry for 24 or 48 hours, only very few organisms were still viable (41). Washing before drying completely eliminated the organism.

Resistance to Heavy Metals, Disinfectants, and Antibiotics

Escherichia coli have the ability to acquire resistance to a broad range of heavy metals (arsenic, copper, mercury, silver, tellurium, zinc) and disinfectants (chlorhexidine, formaldehyde, hydrogen peroxide, quaternary ammonium compounds). Specific strains can vary substantially in their susceptibility to heavy metals and disinfectants (1, 455). Strains develop resistance to disinfectants when subjected to environmental selection pressures, and such resistances are often encoded by large R plasmids (230, 233, 237). In addition to antibiotic resistance, the APEC IncHI2 plasmid, pAPEC-O1-R, conferred resistance to potassium tellurite, silver nitrate, copper sulfate, and benzalkonium chloride following transfer of the plasmid to a recipient strain by conjugation (237). Similarly, the APEC IncF plasmid, pAPEC-O2-R encoded resistance to quaternary ammonium compounds, silver and other heavy metals, as well as several antibiotics (230, 233). Such plasmids are readily transferred to other APEC, avian fecal commensal *E. coli*, *Salmonella* spp., and uropathogenic *E. coli* of human beings via conjugation (233) suggesting the possibility that APEC's R plasmids could be reservoirs of resistance genes for other bacteria of animal and human health importance.

Antigenic Structure and Toxins

Serotypes of *E. coli* are classified according to the Kauffmann scheme (126). Currently there are approximately 180 O, 60 H, and 80 K antigens (481); the numbers change as new ones are identified and previous ones that are duplicated or attributable to another bacterial species are removed. In most serologic typing schemes only the O and H antigens are determined, e.g., O157:H7. The O antigen determines serogroup; addition of the H antigen and sometimes K antigen determines serotype (246). Rough strains autoagglutinate and cannot be serotyped. Additional serotypes with O antigens that have not been recognized also are found in most surveys. Fimbrial (pilus) antigens are included in serotyping when considered important. Recent innovations have enabled assignment of *E. coli* to "serogroups" using polymerase chain reaction (PCR)-based schemes (97).

O (Somatic) Antigen

Lipopolysaccharide (LPS) in the cell wall, also known as endotoxin, is a polysaccharide–phospholipid complex that is released when the cell undergoes lysis. O antigen is the antigenic portion of LPS whereas the toxic portion of the molecule is lipid A. O antigen is resistant to boiling. Methods to prepare and use antisera, which typically agglutinate O antigen at high titers (usually over 1:2560) when antigen–antibody mixtures are incubated at 50°C for 24 hours, have been described (531).

H (Flagellar) Antigen

To examine for H antigens, isolates must be grown under conditions that promote motility. H antigens are proteins found in the different types of flagellin that comprise the flagella. Heating to 100°C destroys them. Tube agglutination tests are read after incubation at 50°C for 2 hours (531).

K (Capsular) Antigen

K antigens are polymeric acids containing 2% reducing sugars, are associated with virulence, are on the surface of the cell, interfere with O agglutination, and can be removed by heating for 1 hour at 100°C. A few strains require heating for 2.5 hours at 121°C. On the basis of heat stability, K antigens are subdivided into L, A, and B forms. Antisera are prepared in rabbits by inoculating live organisms intravenously. Tube agglutination titers are determined by incubating antigen–antibody mixtures at 37°C for 2 hours and overnight at 4°C. Titers are low (1 : 100–1 : 400). Most of these antigens can be identified by the slide agglutination test using appropriately diluted serum (531). Presently K antigens are not commonly included in serotyping.

F (Pilus) Antigen

F antigens are involved in attachment to cells. They are variably expressed depending on the environment in which the organism is growing both *in vitro* and *in vivo*. Pili are classified as being mannose sensitive or mannose resistant depending on whether or not agglutination is inhibited or unaffected respectively when mannose is present. A variety of tests have been developed for detecting fimbrial antigens (531).

Toxins

Avian pathogenic *Escherichia coli* are much less toxigenic than pathogenic *E. coli* in mammals and human beings. Although APEC do not commonly produce enterotoxins, other toxins are elaborated, but their roles in diseases of poultry are currently uncertain (see Virulence Factors: Toxins). Pigeons can be a source of shigatoxin producing *E. coli* strains (see Public Health Significance).

Strain Classification

Antigenicity

Even though molecular methods for identifying specific virulence genes are available, serotyping remains a useful tool for epidemiologic studies. Serotyping provides a means of relating previous work with new work. Also it is important to know the serotype of an APEC strain because the immune response in poultry primarily is directed against O antigens. Numerous surveys have been made in many parts of the world to determine serotypes most frequently associated with diseases in poultry caused by *E. coli* (444, 464). Variations according to geographic region occur, but in most studies the common serotypes have been O1, O2, O18, O35, O36, O78, and O111 (27, 310, 474). Many other serotypes have been found less frequently, whereas some APEC do not belong to known serotypes or are untypeable (542). Some outbreaks are consistently associated with a specific serotype, e.g., O111 causing mortality, septicemia, and polyserositis in egg-laying chickens (541).

Sixty-two different O types were found among typeable strains in a study comparing serotypes of 458 *E. coli* isolates from chickens with colibacillosis to 167 isolates from healthy chickens. Only 15% of the strains belonged to the serogroups O1, O2, O35, O36, or O78, which previously had been associated with avian colibacillosis. Several isolates from diseased birds belonged to 5 serogroups (O18, O81, O115, O116, O132), which had not previously been associated with colibacillosis. Although serotypes from diseased birds were significantly different to those of healthy birds, intestinal infection of healthy birds with serotypes isolated from diseased birds still occurred frequently (48).

Molecular Typing

In addition to phenotyping and serotyping, isolates of *E. coli* can be further characterized by antimicrobial susceptibility patterns, toxigenicity; presence of various virulence factors, cell attachment, invasiveness, hemagglutination, lysogeny (phage typing), plasmid profiling, phylogenetic typing, and virulence genotyping. DNA probes and PCRs have been developed to detect specific genes important in virulence (263, 270, 531). Detection of multiple genes that encode virulence factors by multiplex PCR is an efficient method for identifying several characteristics of APEC and commensal strains simultaneously (125, 438, 470).

Methods of "fingerprinting" isolates including pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), and random amplification of polymorphic DNA (RAPD) can be used in epidemiological studies (226, 323). PFGE was used to fingerprint *E. coli* isolates from chickens with cellulitis and was able to link specific fingerprint types with farms and successive flocks (468, 469).

Random amplification of polymorphic DNA is a rapid, cost-effective procedure for determining clonal types of *E. coli* in epidemiologic studies (60, 323). It is less costly and quicker than molecular fingerprinting using RFLP (323). However, it was not helpful for discriminating between pathogenic and nonpathogenic isolates (60) or for correlating with antimicrobial resistance profiles (323). It is less costly and quicker than molecular fingerprinting using RFLP (323).

Multilocus enzyme electrophoresis (MLEE) identified specific genotypes, which demonstrated that relatively few clonal types are responsible for different forms of colibacillosis in chickens and turkeys in widespread geographic areas. Virulence varied little among isolates within a clonal group but varied considerably between clonal groups (527). MLEE applied to a large number of *E. coli* isolates showed that they could all be placed into 1 of 4 clonal groups designated A, B1, B2, and D. Several clonal groups were identified among isolates from chickens with omphalitis, swollen head syndrome, septicemia, and intestines of healthy chickens in Brazil. MLEE permitted better discrimination of the isolates than ribotyping; most pathogenic isolates clustered together in 2 clonal subgroups whereas most commensal isolates occurred in other clonal groups (84).

Multilocus sequence typing (MLST) is based on the principles of MLEE but relies on nucleotide sequencing of alleles at each target locus. MLST can be used to construct phylogenetic trees, providing insight into the evolution of APEC and its zoonotic potential (289, 351).

Assignment of APEC to phylogenetic types can also be accomplished with a multiplex PCR-based method (71, 72). This method assigns isolates to A, B1, B2, C, D, E and F phylogenetic groups. Clonal groups B2 and D are considered to contain most of the pathogenic isolates. However, Rodriguez-Siek et al. (437) found that the majority of 524 APEC isolates fell into so-called nonpathogenic clonal groups. With the revised Clermont scheme (72), significant designation changes were observed from A to C and D to E and F among APEC. With the new scheme, clonal groups B2 and F are considered to contain most of the pathogenic isolates. These studies suggest that the new scheme has a significant impact on APEC classification (300).

Genomes of *E. coli* contain multiple sequences, which can be identified by PCR and used to characterize individual isolates of the organism and determine their relatedness to each other. The procedure is known as enterobacterial repetitive intergenic consensus (ERIC). ERIC can be combined with repetitive extragenic palindromic (REP) PCR, which determines repetitive sequences outside of the genome. Using these methods dendrograms based on the different patterns revealed extensive genetic diversity among avian *E. coli* strains. Pathogenic and nonpathogenic isolates tended to group in different clonal groups whereas serotypes were distributed among all groups. No specific genotype or serotype could be identified as being the cause of colibacillosis (91). In a subsequent study, commensal and omphalitis isolates grouped together whereas APEC from poultry with septicemia or swollen head syndrome occurred in different clonal groups indicating the opportunistic nature of the omphalitis isolates (83).

Comparative genomic analysis (112, 231, 240), signature-tagged transposon mutagenesis (STM) (291), selective capture of transcribed sequences (SCOTS) (105), genomic suppression subtractive hybridization (85, 250), virulence genotyping (125, 238, 239, 438), transcriptomics (292), proteomics (288), and recombination-based *in vivo* expression technology (RIVET) (506) are methods that have been useful in identifying previously unknown putative virulence genomic sequences in APEC (250, 339, 459, 482). Also, methods identifying gene regulation of virulence expression have become common (29, 77, 195, 544).

These recognized genetic sequences did not occur in commensal strains suggesting their importance in virulence, whereas mutant strains lacking these putative novel virulence factors were less virulent than the originating APEC strains. Furthermore, these novel virulence factors were more frequent in other avian and mammalian pathogenic strains than commensal strains. The specific nature of the genes, and their roles in the pathogenesis of colibacillosis in poultry, remain to be determined.

When 2 virulent avian strains (O2 and O78) from colisepticemia cases were compared, they were found to be very different with only a few shared genes. Analysis of

additional strains of each serogroup of human and animal origin showed similar genetic diversity leading to a "mix-and-match" theory, i.e., different virulence factors can combine to provide an ability to cause septicemia (339).

The molecular tool ribotyping has been used to subtype *E. coli* and has been used to classify *E. coli* isolates from species of origin (55, 177). Riboprints of fecal *E. coli* provided the most accurate determination of source when comparisons were made among no more than 3. For example, the source of *E. coli* from geese, turkeys, or chickens was correctly determined for approximately 96% of the isolates using ribotyping (55)

Pathogenicity

The ability to cause mortality in embryos or chicks differentiates APEC from commensal *E. coli* strains (146, 147, 346, 382). An embryo lethality test can be used to test avian *E. coli* isolates for virulence. Eleven 12-day-old chicken embryos are inoculated via the allantoic cavity with 100 cfu of the test organism. Two-day mortality is less than 10% for nonvirulent strains, 10–29% for intermediate strains, and more than 29% for virulent strains (530). Extending the postinoculation observation time resulted in higher mortality, but the pattern of mortality among various strains remained essentially unchanged (346). Intravenous and subcutaneous inoculation of chicks correlated with embryo lethality, whereas intratracheal inoculation did not (147). Compared with the embryo lethality test, virulence of an isolate correlated with complement resistance and the presence of the ColV plasmid, but neither of these tests conclusively identified all isolates as virulent strains. Efforts to differentiate APEC from commensal *E. coli* using *in vitro* tests have proven effective in some strains. Efforts to differentiate APEC from commensal *E. coli* using *in vitro* tests have proven effective in some strains (125, 238, 460, 470).

Virulence Factors

The idea that avian colibacillosis is a secondary disease and APEC are opportunists is widely accepted. However, increasing evidence indicates that most APEC are well equipped for a pathogenic lifestyle, suggesting that APEC infections might not always be opportunistic or secondary to some predisposing condition. Certainly APEC, like other pathogenic *E. coli*, have acquired genes by horizontal transfer that encode virulence factors, which serve to distinguish APEC from commensal strains (231, 238, 240, 438, 482). These virulence genes may be clustered into chromosomal- or plasmid-located pathogenicity islands (PAIs). Because APEC usually cause extraintestinal disease, they are commonly classified as a subpathotype of the ExPEC pathotype (246, 449). The ExPEC pathotype also includes uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC) that cause disease in humans and other hosts (246). ExPEC share certain virulence attributes enabling their extraintestinal lifestyle, including adhesins, toxins, protectins, iron acquisition mechanisms, and invasins (246, 437, 438). Identification of these traits among APEC has fostered development of a rudimentary definition of an APEC pathotype (438) and led to interest in APEC's zoonotic potential (231, 239, 289, 352, 437, 443, 472).

Despite the fact that most APEC infections are extraintestinal, some APEC contain traits associated with intestinal *E. coli* pathotypes, including enteropathogenic *E. coli* (EPEC) (247, 270), enterotoxigenic *E. coli* (ETEC) (247, 273), enteroinvasive *E. coli* (EIEC) (444), enterohemorrhagic *E. coli* (EHEC) (273, 477), and enteroaggregative *E. coli* (EAEC, EAggEC) (221, 394). Furthermore, APEC strains causing the same disease may differ substantially in their gene content (339). In view of this high level of genomic plasticity, it is not surprising that no single virulence factor has been identified that will distinguish all APEC from all commensal *E. coli* strains. However, APEC's plasmid PAIs occur so commonly among APEC that they are considered a defining trait of the APEC pathotype (438), whose presence in an avian *E. coli* isolate has been used diagnostically (238). Genes encoding virulence factors in APEC are summarized in Table 18.3.

Adhesins

Adhesins may be fimbrial or nonfimbrial. The role of fimbriae in the pathogenesis of avian colibacillosis is unclear, although it seems likely that these virulence factors would be important in APEC's colonization of the host (22). Fimbriae can undergo phase variation depending on the types present on the organism and tissue being colonized. Several fimbrial types have been described among APEC, including AC/I (avian *E. coli* I) (22, 376), P (F11) (273), type 1 (F1) (16, 273), Stg (301), curli (162, 268, 270), and ExPEC adhesion I (13). Also, a ColV plasmid, encoding type 4 pili, has been found in an APEC O78 strain. Although type 4 pili are known to contribute to host cell adherence of some bacteria, their roles in APEC adherence and virulence, if any, are unknown (163). Also, a ColV plasmid, encoding type 4 pili, has been found in an APEC O78 strain. Although type 4 pili are known to contribute to host cell adherence of some bacteria, their roles in APEC adherence and virulence, if any, are unknown (163).

F1 fimbriae are expressed during initial colonization of tracheal epithelial cells, whereas P fimbriae are expressed later when the organism is in the lower respiratory tract or body tissues. Bacteria are rapidly killed by macrophages when they express F1 fimbriae (420, 422).

Table 18.3 Putative APEC virulence and virulence-associated genes and regions.

Gene, Operon or Region	Description
Iron-Related Genes	
feoB	Primary gene mediating ferrous (Fe^{2+}) iron uptake
ireA[1]	Iron regulated, siderophore receptor, outer membrane protein
Yersiniabactin operon[1]	
irp2	Encodes iron repressible gene associated with yersiniabactin synthesis
fyuA	Encodes ferric yersiniabactin uptake receptor
Sit operon[1,2]	
sitA	Putative iron transport operon
Aerobactin operon[2]	
iutA	Ferric aerobactin outer membrane receptor gene
iucC	Involved in aerobactin synthesis
	Aerobactin operon is involved in iron uptake and transport
Salmochelin operon[2]	
iroN	Catecholate siderophore receptor gene
Eit operon[2]	
eitA	ABC iron transporter; periplasmic-binding protein
Toxin/Bacteriocin-Related Genes	
stx1, stx2[3]	Shiga toxins; inhibit protein synthesis
hlyD	Transport gene of the α-hemolysin operon
hlyF[2]	Avian *E. coli* hemolysin
cdtB	Cytolethal distending toxin; DNase I activity; blocks mitosis
vat[1]	Vacuolating autotransporter toxin
cnf1	Cytotoxic necrotizing factor 1; altered cytoskeleton, necrosis
usp	Uropathogenic-specific protein (bacteriocin)
CoIV operon[2]	
cvaC	Structural gene of the CoIV operon
CoIB operon[2]	
cbi	Immunity gene of the CoIB operon
CoIM operon[2]	
cma	Structural gene for CoIM activity
Adhesins	
Type 1 fimbrial adhesin operon (Fim)	
fimH	D-mannose-specific adhesin of Type 1 fimbriae
Pap pilus operon[1]	
papC	Acts as a molecular usher in P pilus assembly
papA	Encodes the major structural subunit
papG	Encodes the pilus tip adhesin
S fimbrial operon	
S fimbrial family of adhesins differ in receptor specificity S fimbriae (Sfa)	
sfaS	Encodes pilus tip adhesin of S fimbriae; S fimbriae interact with glycoproteins containing sialic acid
F1C-fimbriae (Foc)	
focG	Encodes a component of F1C fimbriae
focA	Encodes the major fimbrial subunit
S/F1C-related fimbriae (Sfr)	
AC/I fimbriae (Fac)	
facA	Encodes major subunit of avian *E. coli* I (AC/I) fimbriae
Curli operon	
crl	Gene cluster encoding for curli fibers; involved in cell adhesion and internalization
iha	IrgA homologue adhesin
afa	Afimbrial adhesin, a member of the Dr family of adhesins
gafD	G fimbrial adhesin
bmaE	Blood group M-specific adhesin
Stg operon	
stgA	On the C-terminus of the Stg fimbrial operon
tsh[2]	Temperature-sensitive hemagglutinin gene
bfp	Bundle-forming pilus, initiates attaching and effacing lesions in typical AEEC isolates
eae	*E. coli* attaching and effacing gene that encodes intimin
Protectins	
iss[2]	Encodes an outer membrane protein involved in increased serum survival and surface exclusion
traT[2]	Encodes an outer membrane protein involved in serum resistance and surface exclusion

(*Continued*)

Table 18.3 (*Continued*)

Gene, Operon or Region	Description
bor	Virulence determinant encoded by λ bacteriophage; involved in serum resistance
ompA	Outer membrane protein A is involved in serum resistance
kps cluster	Involved in encoding capsular (K) antigens
Invasins	
ibeA[1]	Promotes invasion of brain microvascular endothelial cells
ipa	Cell penetration and intracellular survival
tia[1]	Tia invasion determinant
Miscellaneous	
ompT[1,2]	Encodes a protease able to cleave colicin
maIX	Pathogenicity island marker from UPEC CFT073
fliC (H7)	Produces flagellin protein associated with the H7 antigen group
Ets operon[2]	
etsA	Encodes ABC transporter, efflux pump protein

Source: Modified from (228, 246, 438).
[1] Localized to an APEC chromosomal PAI.
[2] Localized to an APEC plamid-linked PAIs.
[3] Encoded by phage.

Although the F1 fimbrial adhesin, FimH, is required for adhesion to cultured chicken epithelial pharyngeal or tracheal cells, lack of FimH favors *in vivo* colonization of the trachea of chickens (16). Curli may contribute to bacterial invasion of eukaryotic cells (162) and APEC's persistence in the cecum (268).

Intimin is a non-fimbrial adhesin encoded by the *E. coli* attaching and effacing (*eae*) gene, which is found in EHEC and EPEC. It permits the bacterial cell to adhere to the surface of the enterocyte, which initiates a characteristic attaching and effacing (AE) lesion. Several genetic variants of intimin, denoted by letters of the Greek alphabet, have been identified. The most common type in APEC is ß-intimin followed by γ-intimin (263, 484). A highly virulent APEC (O86:K61) that caused mass mortality of passerine birds in Britain produced γ-intimin (270) and on further analysis was reclassified as *E. albertii* (389).

Organisms producing AE lesions are known as attaching and effacing *E. coli* (AEEC). In mammals, a specific pilus (bfp, bundle-forming pilus) occurs together with intimin to cause the AE lesion. AEEC that do not have bfp are referred to as "atypical AEEC." Avian AEEC are usually atypical AEEC as isolates usually lack bfp. AEEC either are absent or found infrequently in most surveys of poultry (263, 484) except for pigeons infected with shigatoxin strains (176, 477). However, a high prevalence of eae+ isolates were obtained from dead-in-shell embryos and chicks with yolk sac infections in Mexico (30%) (444) and the feces of healthy chicks in Kenya (60%) (247), suggesting there may be certain geographic areas where AEEC commonly infect chickens.

A novel avian respiratory soluble lectin, distinct from pulmonary collectins and ficolins, which binds with surface polysaccharides of pathogenic *E. coli* (serogroups O2 and O78) has been discovered in air sac fluids of turkeys. Its role, if any, in colibacillosis has yet to be defined (523).

Also, temperature-sensitive hemagglutinin (Tsh), the first described serine protease autotransporter of the Enterobacteriaceae (SPATE) (423), is secreted by some APEC strains. It is a bifunctional protein that acts as an adhesin and protease (260) and mediates colonization of the host's respiratory tract during early infection (106). The contribution of Tsh to the pathogenesis of colisepticemia appears to be restricted to the early stages of infection, as *tsh*$^-$ mutants cause less severe and less frequent lesions in air sacs. Tsh does not appear to be required for high levels of APEC virulence (501). Reports of its prevalence among different APEC populations vary widely (7, 90, 98, 99, 124, 125, 221, 321, 327, 437, 438, 514, 536, 542). Another autotransporter, AatA, which has a role in APEC adherence and virulence, has recently been described (289). *aatA*, is strongly associated with *E. coli* from avian sources but not with *E. coli* isolated from human hosts. In some APEC, *aatA* is plasmid-linked (289), but in others, it is chromosomally encoded (85). Also, a Type VI secretion system has been described in APEC, which contributes to APEC adherence, invasion of cultured HeLa cells, biofilm formation on abiotic surfaces, motility, survival within macrophages, and virulence (92, 93).

Toxins

Avian pathogenic *Escherichia coli* tend to be less toxigenic than mammalian pathogenic *E. coli* (221). This difference may be because of the lack of toxin production or that toxins produced by avian strains are not detectable with tests for toxins produced by mammalian strains. In addition to endotoxin, a structural component of the organism's cell wall, APEC can elaborate several toxins that are important in disease (401, 402, 452–454); low-level occurrence of certain toxin genes among APEC has been reported. These genes include those encoding cytolethal distending toxin (*cdt*) (273, 437, 438, 444), cytotoxic necrotizing factor 1 (*cnf1*) (437, 438) and various hemolysins (7, 229, 234, 327, 347, 355, 432, 437, 438).

Some of the genes encoding toxins occur in a substantial number of APEC. A *Salmonella* virulence homologue, *hlyF*, was first described in an avian *E. coli* isolate in 2004 (347). It shares significant homology with the *E. coli* K12 "silent" hemolysin gene, *she,* and occurs commonly among APEC. The gene is found within a virulence cluster of large, conjugative ColV and ColBM plasmids (229, 234). Its role in APEC suggests that *hlyF*-induced autophagy in eukaryotic cells, coincides with an enhanced production of outer membrane vesicles (OMVs) by bacteria expressing HlyF (355).

Vacuolating autotransporter toxin (Vat), which is encoded by the *vat* gene, also occurs commonly among APEC (124). Vat is a 148.3-kDa protein, which has a structure typical of SPATE. It causes cytotoxic effects in cultured cells similar to those caused by *Helicobacter pylori* VacA toxin. Vat appears to be a virulence factor for APEC, because deletion of the *vat* gene results in attenuation of virulence (400).

Iron Acquisition Mechanisms

The ability of APEC to obtain iron is well documented and likely because of various iron-acquisition mechanisms (aerobactin, yersiniabactin, sit, and iro systems) (56, 57, 105, 451). Genes of these operons occur frequently among APEC, but are significantly less common in commensal *E. coli* strains (438). APEC frequently contain several of these operons, 1 or more of which may be found on large plasmids (105, 234, 237, 437, 438, 451, 501). This apparent redundancy in iron acquisition mechanisms and widespread distribution of these systems among APEC suggest that the ability to obtain iron is important in the pathogenesis of avian colibacillosis.

The sit operon was originally described in a *Salmonella enterica* serovar Typhimurium isolate (543) and more recently identified in APEC using genomic subtractive hybridization and signature-tagged mutagenesis (291, 459). The sit operon encodes an ABC transport system involved in metabolism of iron and manganese, and resistance to hydrogen peroxide (451). In at least 1 APEC, this operon occurs in both chromosomal and plasmid-located PAIs (229, 231). In its plasmid location, sit is closely associated with the aerobactin siderophore operon and iro locus (229). The yersiniabactin operon in at least 1 APEC is found in a chromosomal PAI (GenBank accession no. NC 008563) (231).

Protectins

The ability to resist complement is a common characteristic of APEC, regardless of the syndrome or avian host species of origin (383). Resistance of *E. coli* to complement is related to several structural factors including K1 capsule (79, 143, 330) or other capsule type (450, 487), a smooth LPS layer (79, 154) or particular LPS type (330), and certain outer membrane proteins (OMPs), including TraT, Iss, and OmpA (67, 68, 340, 525). When 294 APEC were compared with 75 fecal *E. coli* isolates from clinically healthy birds for possession of a capsule, smooth LPS layers, *ompA*, *traT*, and *iss*, only *iss* was found to occur significantly more often in APEC relative to commensal strains (415).

The increased serum survival gene (*iss*), first described by Binns and coworkers in 1979 for its role in complement resistance associated with a ColV plasmid, increased the virulence of an *E. coli* 100-fold for day-old chicks (44) and its complement resistance over 20-fold (67, 68). The gene *iss* encodes Iss, a lipoprotein exposed on the outer membranes of *E. coli* (303). It occurs frequently among APEC (437, 438, 536, 542) compared with a low rate of occurrence in commensal strains. Although it has been proposed that the substantial difference in distribution of *iss* between APEC and commensal strains might reflect its importance in APEC virulence (383), Mellata et al. (330) reported that *iss* did not play a major role in resistance of APEC strain χ7122 to serum. In contrast, Tivendale et al. (501) found a strong association between APEC virulence and carriage of *iss* and/or *iucA*, a gene of the aerobactin operon. From such disparate observations, it is evident that much remains unknown about complement resistance in APEC, its mediators, and its role in disease pathogenesis. Recently, Li et al. (288) used a global microarray approach to assess serum resistance in APEC O1. The organism quickly adapted to growth in serum by significantly upregulating 311 genes and downregulating 299 genes. The extent of these changes demonstrates the complexity of APEC's response to serum. Upregulated genes were involved in biosynthesis of cofactors, prosthetic groups, and carriers; and downregulated genes were involved in energy metabolism. Of the upregulated genes, only 39 were localized to chromosomal islands, whereas most were found in APEC O1's three plasmids with 56 of these localized to APEC O1's large virulence plasmid, pAPEC-O1-ColBM.

The ability of APEC to resist the detrimental effects of heterophils and macrophages is likely another important determinant that contributes to successful infection. Resistance to phagocytosis or its effects may be related to complement resistance or possession of other traits. Kottom et al. (261) reported that a complement-sensitive mutant bound significantly more C3 subunits and was phagocytosed significantly more often than the wild-type APEC strain from which it had been derived. It was hypothesized that the mutant's decreased virulence resulted from its increased sensitivity to complement-mediated bacteriolysis or enhanced susceptibility to complement-opsonized phagocytosis. However, subsequent studies by Mellata et al. (330) showed that nonopsonized APEC were eliminated by phagocytes to the same or greater extent than serum-opsonized bacteria.

Phagocytosis of APEC by avian phagocytes is promoted by presence of type 1 fimbriae and absence of P fimbriae, K1 capsule, O78 antigen, and an uncharacterized pathogen-specific chromosomal region. Presence of type 1 and P fimbriae, O78 antigen, and the 0-minute chromosomal region contributed to the protection of APEC against the bactericidal effect of phagocytes, in particular, heterophils (330).

Certain strains of APEC can survive within macrophages and cause their destruction through apoptosis (35, 436). Caspases, enzymes essential for apoptosis, were activated by a strain of APEC (APEC17), which resulted in cytotoxicity within 8 hours of infection (35).

Invasins

The *ibeA* gene contributes to invasion of brain microvascular endothelial cells (BMEC) by neonatal meningitis ExPEC. It is significantly more likely to be found in APEC than in avian commensal strains (144, 250, 437, 438). The abilities of APEC strain BEN2908 to invade human BMEC, adhere to BMEC and cause avian colibacillosis are significantly reduced when *ibeA* is inactivated (75, 144). These results indicate that *ibeA* is a virulence attribute of APEC. *ibeA* occurs in 14%–20% of APEC (144, 437, 438). *ibeA* and *ibeT*, which is located downstream of *ibeA* in APEC BEN2908, may affect adherence by modulation of type 1 fimbrial expression (75). *ibeA* is found in chromosomal PAIs (APEC O1). APEC BEN2908 also usurps cellular endocytic pathways to invade cultured human pneumocytes and avian hepatocytes (61).

Other

Formation and residence within a biofilm could enhance the ability of APEC to resist cleaning and disinfection and to acquire virulence and resistance genes by horizontal gene transfer. When 105 APEC and 103 avian commensal *E. coli* strains were compared for their ability to form biofilms on plastic surfaces, formation of biofilms by APEC was induced by nutrient poor conditions. In contrast, commensals formed biofilms in both nutrient poor and rich conditions (472). There are a few genes known to contribute to biofilm formation in APEC (*icmF*, *upaB*, *ibeA*, *epcA*, *waaL*, *ychO*, and *yfcO*) but our understanding of how and when biofilm formation occurs in APEC is far from complete. For instance, these genes do not occur in all APEC biofilm producers, and some APEC have them but do not produce biofilms. Thus, there is still much to learn about this important disease mechanism (92).

Genomic Location of Virulence Genes

Much progress has been made on localizing various virulence genes in the APEC genome, providing insight into their organization, regulation, and evolution. These insights have been accelerated with the completion of the first genomic sequence of an APEC strain, APEC O1 (231). Such genomic sequences are often "starting points" for high-throughput studies of APEC pathogenesis and gene function under host conditions (288, 290, 292) and have been greatly enhanced with the public release of additional genomic sequences in GenBank including APEC O2(242); APEC O18 (379); an O7 strain (311) and a number of seroytype O78 strains (112, 313, 440, 521); O113, O38 and OR (439) because APEC are highly diverse in their genomic composition (438).

Sequences of several APEC chromosomal PAIs and multiple APEC virulence (PAI-containing) plasmids are currently available. Common features of most PAIs include encoding 1 or more virulence factors, being between 10 and 200 kb in size, usually being flanked by small direct repeat sequences, and bearing traces of their introduction into the genome via horizontal transfer, including deviation of G-C ratios and codon usage from the organism's typical pattern (245). PAIs may contain mobility elements, such as integrons, transposons, and insertion sequences. If they themselves move they are likely carried on plasmids, conjugative transposons, or phages, whose loss may spontaneously convert a virulent organism into an avirulent one (245).

Several chromosomal PAIs have been identified among APEC including the VAT-PAI (400), PAI $I_{APEC\text{-}O1}$ (249), AGI-3 (66), the *tkt1*-containing PAI (290) and several other putative PAIs found in APEC O1 (231, 240). VAT-PAI is a 22-kb PAI that includes the *vat* gene, which encodes Vat (see Virulence Factors: Toxins). Another chromosomally located APEC PAI, PAI $I_{APEC\text{-}O1}$ (249), is 56-kb in size and harbors the complete pap operon and other *E. coli* genes (*tia* and *ireA*). Also, PAI $I_{APEC\text{-}O1}$ lies immediately upstream of the *kps* gene cluster, which is required for biosynthesis of the polysialic acid capsule. Although the role of this PAI in virulence has yet to be elucidated, a study of 95 APEC and 95 avian commensal isolates for possession of 6 genes of this PAI revealed that they occurred more often in APEC of high and intermediate virulence than in isolates of low virulence. None of the commensals contained all 6 of these targets, whereas 7.2% of APEC strains had all of the genes (248). Another chromosomal PAI, AGI-3 (66), is 49.6 kb in size and is arranged in 5 modules. Deletion analysis of module 1 demonstrated its contributions to APEC's carbohydrate uptake and virulence for chickens. Studies of its prevalence among 249 ExPEC strains, including 205 APEC and 36 nonpathogenic strains of avian origin, showed that about 12% of all strains tested contained this region. Also, all 15 APEC strains of the O5 serogroup contained this PAI, suggesting that it might be serogroup-associated. The 16-kb *tkt1*-containing PAI, found in the chromosome of APEC O1, is strongly associated with human and avian ExPEC of the B2 phylogenetic group but not avian fecal commensal *E. coli*. Tkt1 seems to contribute to APEC's bipeptide metabolism (290).

Avian pathogenic *Escherichia coli* PAIs also have been found on large transmissible virulence plasmids, some of which have been sequenced to date. These plasmids, and more specifically, their PAIs, are the defining trait of the APEC subpathotype (438). APEC plasmid PAIs share remarkable similarity in their conserved regions (229, 230, 234, 333). However, these plasmids also may have important differences allowing their host bacteria to exploit various niches. For instance pAPEC-O2-ColV harbors the ColV operon (234), whereas, pAPEC-O1-ColBM contains only remnants of the ColV operon and has ColBM operons (229). Still, other APEC virulence plasmids may contain a MDR-encoding island in addition to the PAI (230). As more APEC plasmid sequences are released, added "variations on the theme" are likely to be identified, because genotyping of large collections of APEC has suggested that these plasmids are very diverse (238, 239, 437, 438).

A 180-kb ColV plasmid, known as pAPEC-O2-ColV, found in an APEC O2 strain, was shown to harbor many of the genes associated with APEC virulence and be similar in genetic make-up to a plasmid and chromosomal PAI of human uropathogenic *E. coli* (472). When transferred by conjugation along with a large R plasmid into a commensal avian *E. coli* strain, the resulting transconjugant showed enhanced virulence for chick embryos and abilities to grow in human urine and cause urinary tract infection in the murine model of human disease. pAPEC-O2-ColV was sequenced and analyzed (234), and its role in virulence was further evaluated (471). In addition to regions devoted to plasmid transfer, maintenance, and replication, pAPEC-O2-ColV contained a 94-kb cluster of putative virulence traits, including *hlyF*, *ompT*, *iss*, *tsh*, the ColV operon, and several iron-related systems. The iron-related systems included those encoding aerobactin and salmochelin, and the sit ABC transport system. Also, this PAI contained another putative ABC transport system known as ets. A study of the distribution of these PAI genes in 595 APEC and 199 avian fecal commensal *E. coli* isolates revealed that a portion of this PAI was highly conserved among APEC and that the genes of the conserved region occurred more often in APEC than in commensal strains. This conserved portion, which occurred in nearly 80% or more of APEC examined, included: the sit, salmochelin, aerobactin, and ets operons; *hlyF*; *iss*; *ompT*; the RepFIB replicon; and the 5' end of the ColV operon. The variable portion of this PAI contained the 5' end of the ColV operon, *tsh*, and the eit operon. The split between conserved and variable portions occurred within the *cvaB* gene of the ColV operon with the 5' end of *cvaB* and many of its upstream genes occurring significantly more often among APEC than the 3' end of *cvaB* and many of its downstream genes. This difference in prevalence between conserved and variable portions of the PAI among APEC suggested that there must be an alternative location for the conserved portion in APEC. Indeed, a very similar PAI was found in a 174-kb ColBM-encoding APEC plasmid, known as pAPEC-O1-ColBM (229), which was isolated from an APEC O1 strain. This F-type plasmid shares remarkable similarities with pAPEC-O2-ColV, except that it encodes for production of colicins B and M rather than ColV. Similarly, Tivendale et al. (502) described the sequence of a 151-kb plasmid, pVM01, whose conserved PAI region contributed to the virulence of its host APEC, an O nontypeable: H28 APEC field isolate. Mellata et al. (333) also described the complete sequences of several APEC plasmids, found in an APEC O78:K80:H9 strain, and their contributions to APEC virulence and fitness (329, 332, 333), again confirming the importance of these plasmids in the pathogenesis of avian colibacillosis. Johnson et al. (230) sequenced pAPEC-O103-ColBM, a 124.7-kb hybrid RepFIIA/FIB plasmid harboring components of the ColV PAI and an MDR-encoding island. Acquisition of this plasmid conferred the abilities to cause colisepticemia in chickens, bacteremia resulting in meningitis in the rat model of human disease, and resistance to the bacteriostatic or killing effects of human serum, streptomycin, sulfisoxazole, tetracycline, erythromycin, and trimethoprim-sulfamethoxazole.

At least portions of these plasmid-linked PAIs appear to occur widely among APEC isolated from different parts of the world (7, 98, 99, 124, 221, 321, 327, 437, 438, 514, 536, 542), various avian host species (7, 327, 437, 438), and different syndromes (90, 438). These observations suggest that the conserved region of these plasmid-linked PAIs is a defining characteristic of the APEC pathotype (238, 438) that could be exploited in colibacillosis control. Protocols for rapid characterization of APEC, based on detection of certain virulence genes, including some from this cluster, show promise (125, 238, 470).

During conjugation, these virulence plasmids may co-transfer from donor to recipient strains with large MDR-encoding R plasmids (233, 237), and, as noted above, genes encoding virulence and resistance can be found on the same APEC plasmids (230). This close association between resistance and virulence genes in APEC suggests that use of any of a number of antibiotics, heavy metal compounds or disinfectants in the poultry production may select for APEC with enhanced ability to cause disease, resist therapy and disinfection, and persist in the environment (235).

Thus, APEC virulence plasmids are found in most APEC (238, 438) and are the defining trait of the APEC subpathotype, whose presence can be exploited in colibacillosis control. They contribute to the fitness of *E. coli* for different environments and enhance its abilities to cause colibacillosis in avian hosts, and UTI, sepsis, and meningitis in murine models of human disease. They

also are found in the *E. coli* that contaminate retail poultry meat (232), suggesting the possibility that retail poultry could be a foodborne reservoir of plasmid-linked virulence or resistance genes in human disease. Though APEC-like virulence plasmids occur at relatively low prevalence in human uropathogenic *E. coli*, they occur in a majority of human NMEC and are considered a defining characteristic of the NMEC subpathotype (73, 299).

Regulation of APEC Virulence

Mutational analysis of the specific phosphate transport system (Pst) operon of an APEC strain resulted in deregulation of phosphate sensing and changes in the composition of the bacterial surface. These changes were accompanied by increased susceptibility to serum, acid shock, and polymyxin, and resulted in decreased virulence, suggesting that a functional Pst system is required for full virulence of APEC O78 strain χ7122 (275). Also, the Pho regulon in APEC is controlled by the two-component regulatory system PhoBR and modulated by the Pst system (42). Also, the BarA-UvrY two-component system has been shown to regulate APEC virulence. Mutants lacking *barA* or *uvrY* had impaired adherence, invasiveness, persistence in tissues, survival in macrophages, and serum resistance (195). Another two-component system has been shown to regulate APEC virulence, AutA-AutR. It controls the expression of K1 capsule and acid resistance systems in AFI during host–pathogen interaction (544). FNR (fumarate and nitrate reduction) is a global regulator that works as an oxygen sensor. It was observed that FNR regulates the type I fimbriae, a plasmid-encoded outer membrane protein (ompT), and aatA, encoding an autotransporter, acting in adherence and invasion, type VI secretion, survival during oxidative stress, and growth in iron-restricted environments (29). It is likely that completion of the APEC genome will facilitate experimentation that will provide insight into the critical issue of virulence regulation.

Pathobiology and Epidemiology

Incidence and Distribution

Escherichia coli have a cosmopolitan distribution. The various serotypes of *E. coli* are normal intestinal inhabitants and occur in high numbers in most animals, including humans. The presence of *E. coli* in the lower intestinal tract is beneficial, aiding in growth and development (458) and inhibiting other bacteria including *Salmonella* (322, 419). *E. coli* occurs in most mammals and birds although healthy psittacines may be an exception (24, 486). It is a common inhabitant in the intestinal tracts of poultry at concentrations up to 10^6 *E. coli*/g of intestinal contents. Higher numbers are found in younger birds, birds without an established normal microbiota, and in the lower intestinal tract (283, 529). A diversity of *E. coli* serotypes colonize the cecal mucosa, which may shift abruptly as birds age (225). Among normal chickens, 10%–15% of intestinal coliforms may belong to potentially pathogenic serotypes (186) although intestinal strains may not be the same serotype as those from extraintestinal sites in the same bird. Intestinal *E. coli* provide a reservoir for virulence and antimicrobial resistance factors (381).

Egg transmission of pathogenic *E. coli* is common and can be responsible for high chick mortality (149, 413, 444). Fluoroquinolone-resistant *E. coli* were vertically transmitted from clinically normal breeders and caused high mortality in chicks (413). Pathogenic coliforms are more frequent in the intestine of newly hatched chicks than in the eggs from which they hatched (187), suggesting rapid spread after hatching. The most important source of egg infection is fecal contamination of the egg surface with subsequent penetration of the shell and membranes.

Coliform bacteria can be found in litter and fecal matter. However, *E. coli* accounts for only a small number of total bacteria in litter (371). Environmental isolates often constitute a distinctly different population from APEC occurring in the birds (223). Dust in poultry houses may contain 10^5–10^6 *E. coli*/g. These bacteria persist for long periods, particularly under dry conditions (185). Wetting dust inside houses with water resulted in an 84%–97% reduction within 7 days. Feed and feed ingredients are often contaminated with pathogenic coliforms and are a common source for introducing new serotypes into a flock (315). Rodent droppings frequently contain pathogenic coliforms. The intestinal tract of the mouse is a suitable environment for transfer of genes from resistant to susceptible strains. Exposure of mice to an antibiotic accelerates the process (188). Pathogenic serotypes also can be introduced into poultry flocks through contaminated well water (362).

Natural and Experimental Hosts

Most, if not all, avian species are susceptible to colibacillosis. The various forms of colibacillosis are considered to be the most common infectious bacterial disease of broiler chickens and turkeys. Natural infections of other avian species occur including quail (54), pheasant (491), pigeons (429), guinea fowl (298), waterfowl (45, 78, 336), ostriches (257), emus (197), peacocks (28), and partridge (102), especially if they are kept intensively in confined conditions. The disease is less common in wild birds (200).

Age of Host Commonly Affected

All ages are susceptible to colibacillosis, but young birds are more frequently affected and disease severity is

greater in young birds, including developing embryos (183, 228, 344). Outbreaks can occur in caged layers (514, 541) and coliform salpingitis/peritonitis is a common cause of mortality in breeders (241). Colibacillosis in older birds is often manifested as an acute septicemia.

Host Susceptibility Factors

Compared with bacterial virulence factors, host susceptibility and resistance factors are probably an equal or greater determinant of colibacillosis occurrence (Tables 18.4, 18.5). Normal, healthy birds with intact defenses are remarkably resistant to naturally occurring *E. coli* exposure including virulent strains. Infection occurs when skin or mucosal barriers are compromised (e.g., unhealed navel, wounds, mucosal damage from viral, bacterial, or parasitic infections, lack of normal microbiota, etc.), the mononuclear-phagocytic system is impaired (e.g., viral infections, toxins, nutritional deficiencies), there is immunosuppression (e.g., viral infections, toxins), exposure and/or stress are overwhelming (e.g., environmental contamination, wide temperature variation, poor ventilation, contaminated water). Effective control of colibacillosis depends on identifying and eliminating the predisposing cause(s) of the disease.

Table 18.4 Factors known or suspected to increase host susceptibility to *Escherichia coli* infections in poultry. See also (32).

Factor	References
Viruses	
Adenovirus (Type 1)	(197, 445, 520)
Avian influenza virus	(380)
Avian metapneumovirus	(508)
Chicken infectious anemia virus	(428)
Duck enteritis virus (low virulent)	(465)
Hemorrhagic enteritis virus	(416)
Infectious bronchitis virus	(319, 365)
Infectious bursal disease virus	(445)
Infectious laryngotracheitis virus	(296, 365)
Marek's disease virus	(135)
Newcastle disease virus	(118, 172, 416)
Pigeon paramyxovirus 1	(534)
Reovirus	(445)
Turkey coronavirus	(178)
Pigeon circovirus	(429)
"Stunting syndrome"	(132)
Bacteria	
Bordetella avium	(198, 416)
Erysipelothrix rhusiopathiae	(483)
Pasteurella multocida	(483, 486)
Campylobacter jejuni	(153)
Clostridium perfringens	(354)
Mycoplasma gallisepticum	(172, 370)
M. meleagridis	(380)
M. synoviae	(308)
Chlamydiophila psittaci	(511)
Parasites	
Ascaridia (larvae)	
A. dissimilis	(386)
A. galli	(411)
Eimeria brunetti	(191, 362)
Eimeria tenella	(262)
E. tenella/whole wheat diet	(139)
Cryptosporidium baileyi	(80)
Histomonas meleagridis	(325, 483)
Toxins	
Ammonia	(361)
Cyclophosphamide	(100, 115)
Iron – parenteral	(51)
Mycotoxins	
Ochratoxin	(264, 265)
Fumonison/Moniliformin	(294)
Physiologic	
Age – young	(228, 344)
Stress – minimal or severe	(204, 317)
Sex – male	(206)
Fast-growing strains	(540)
Obesity	(388)
High antibody response	(175)
High inflammatory response	(36)
Environmental	
Contaminated water	(363)
Dry, dusty conditions	(185)
Feed/water restriction	
Inadequate ventilation	
Overcrowding	
Poor litter conditions	
Temperature extremes	
Nutrition	
Hypervitaminosis E	(133)
Hypervitaminosis A	(134)
Vitamin A deficiency	(134)

Table 18.5 Factors known or suspected to decrease host susceptibility to *Escherichia coli* infections in poultry. See also (32).

Factor	Nutrition
Immunity	Protein
Passive	Vitamin A
Active	Vitamin C
Immunostimulants	Vitamin D
Phagocyte priming	Vitamin E
Physiologic	ß-carotene
Genetics	High iron – oral
Age – older	Selenium
Sex – female	
Moderate stress	
Socialization	
Deoxycorticosterone	
Short heat stress	
Intestinal microbiota	

Colibacillosis often occurs concurrently with other diseases making it difficult to determine the contribution of each agent to the overall clinical disease. For example, colibacillosis, paratyphoid, and histomoniasis caused high mortality in a broiler flock maintained free of antibiotics where high ambient temperatures and humidity may have been additional factors contributing to the disease (140). High mortality in Japanese quail with signs of respiratory disease was associated with *Mycoplasma gallisepticum*, *Pasteurella multocida*, *Staphylococcus sp*., *Streptococcus sp*., *Cryptosporidium sp*., and *E. coli*; relatively high ammonia levels likely also contributed to the clinical disease (353). Coliform peritonitis was more severe in commercial layers challenged with *Mycoplasma synoviae* and *E. coli* than in those challenged with *E. coli* alone (430).

Infection with infectious bronchitis virus (IBV) in chickens (365), infection with hemorrhagic enteritis virus in turkeys (416), and exposure of avian species to ammonia (361) are the most commonly reported factors that predispose to colibacillosis. Interactions between IBV and *E. coli* have been studied extensively and used to determine virulence of both organisms, efficacy of IBV vaccination programs, and effect of IBV vaccination on subsequent colibacillosis (319). IBV vaccination of chicks at 1 day of age by spray reduced the occurrence and severity of airsacculitis following challenge with virulent IBV and *E. coli*. In contrast, IBV vaccination by eye-drop reduced systemic infection and improved uniformity, but did not protect against airsacculitis (319).

Moderate stress increases resistance, possibly resulting from development of immunity following contact of organisms with the immune system (283), or because of developing and exercising defense mechanisms and maintaining them in a state of readiness (169). Similarly, provoking mild, nonspecific inflammation of the respiratory system increases resistance to subsequent respiratory *E. coli* infection (503). Individual survival is likely promoted by diversion of feed-derived nutrients from growth and development to antibacterial defenses (173). Protein does not accumulate at the same rate in muscles of infected birds once they recover and they do not match the weight-for-age of uninfected birds (498). Inhibition of prostaglandin E2 by naproxen restored normal growth (499), which is consistent with the earlier finding that inhibition of prostaglandins with aspirin and vitamin E decreased the severity of disease resulting from *E. coli* challenge (295).

Genetic lines of chickens and turkeys vary in their resistance to *E. coli* infections (20, 540). Variations among genotypes in growth rate, nutritional interactions, and immune responsiveness that relate to *E. coli* susceptibility also have been identified. Consistent among studies in both chickens and turkeys is an inverse relationship between growth rate and resistance to colibacillosis (175, 203, 540). Selection for rapid growth is believed to require redirection of nutrients towards growth at the expense of bacterial resistance (173). However, no correlation between body weight at market for broilers or chick production of breeders with high early antibody response to *E. coli* vaccine was found, indicating the feasibility to select for both immune responsiveness and desirable production traits (285). Immune responses to other vaccines and antigens paralleled response to *E. coli* vaccine in selected lines (539). In general, chickens and turkeys that are more immunologically responsive (e.g., high early antibody lines) are more susceptible to colibacillosis unless they have been vaccinated or otherwise exposed prior to challenge (36, 110). When 5 broiler lines, a slow-growing line, and 2 line crosses were examined using a standardized pure *E. coli* challenge, substantial differences in mortality, lesion occurrence, and growth depression were found. These results indicated that selection for resistance would be feasible, but that heterosis was either negative or negligible making test crossings essential (20, 21). Evaluation of 4 broiler strains for their response to endotoxin revealed differences in weight gain and changes in bone breaking strength. Response of the strains to endotoxin with regard to changes in liver size and bone breaking strength were highly correlated with mortality prior to endotoxin exposure. Strains that had a greater loss of bone breaking strength because of inflammation were more likely to have higher overall mortality (337). Variations in physiological and behavioral responses to endotoxin also occur among egg-laying strains (64).

Transmission, Carriers, and Vectors

Escherichia coli are present in the intestinal tracts of most animals and shed in the feces, often in high numbers. Direct or indirect contact with other animals or feces can introduce new strains into the poultry flock. Free-living birds are especially important because they are colonized with strains that are already adapted to avian species. *E. coli* is readily isolated from free-living waterfowl, especially ducks (127), and passerine birds, including European starlings (141). A particularly virulent O86 APEC, recently reclassified as *E. albertii* (389), caused significant mortality in free-living finches in Britain, but has yet to be found in poultry (410).

Trachea, ceca, and oviduct of recovered laying hens remained persistently colonized for at least 21 weeks after either oral or intra-air sac inoculation with pathogenic *E. coli*. Hens with colonized oviducts continued to lay eggs of which 2.7% contained the organism. Interestingly, *E. coli* was not isolated from the shell surface, even when the oviduct was heavily colonized (15).

Larval and adult darkling beetles (*Alphitobius diaperinus*) likely contribute to *E. coli* transmission and its spread among poultry houses and farms following consumption of infected larvae or beetles or contact with their feces by the birds (161). Following exposure, larvae and adults were positive for *E. coli* both externally and internally for up to 12 days. The organism was shed in their feces for 6–10 days. Chicks became colonized with *E. coli* after eating infected larvae or adults, but the number of infected chicks was higher when the birds ate larvae (324).

Adult houseflies (*Musca domestica*) serve as mechanical vectors of *E. coli*, and fly larvae develop digestive tract infections with *E. coli* following ingestion of bacteria-laden material. Once infected, *E. coli* persists through the pupal and adult stages making it possible for flies to serve as a reservoir for virulent strains (435). The gut of the housefly provides a suitable environment for horizontal transfer of *E. coli* antibiotic resistance and virulence genes (414).

Incubation Period

The time between infection and onset of clinical signs varies with the specific type of disease produced by *E. coli*. The incubation period is short, generally between 1 and 3 days, in experimental studies in which birds are exposed to high numbers of virulent organisms. In the field, it is more common to see colisepticemia 5–7 days after infection with a predisposing agent such as infectious bronchitis virus in chickens or hemorrhagic enteritis virus in turkeys.

Clinical Signs

Clinical signs vary from inapparent to total unresponsiveness just prior to death depending on the specific type of disease produced by *E. coli*. Localized infections generally result in fewer and milder clinical signs than systemic diseases. Coliform cellulitis is typically not detected until the birds are processed. Lameness and retarded growth are seen in birds with skeletal lesions that develop as a sequel to sepsis. Affected birds are typically undersized for the flock and found at the ends of the house, along the side walls, or under feeders or waterers. They may be victims of persecution ("cannibalism") by other birds. When joints or bones of 1 leg are affected, birds walk with a characteristic hopping motion to keep weight off the affected leg. Birds with lesions in both legs are either nonambulatory or have great difficulty in standing and walking. When the thoracolumbar spine is affected, the birds have an arched back, sit on their hocks, and bear little or no weight on their feet. Occasionally they will sit back on their tail and hocks with their feet elevated off the ground. Birds with chronic lameness have caking of droppings around the vent and on abdominal feathers. Feces are green with white to yellow urates because of anorexia and dehydration. Young birds with omphalitis and infected yolk sacs also may have difficulty in walking because of abdominal distention, which alters weight distribution and impairs balance.

Birds with colisepticemia are often terminally moribund or very lethargic. Decreased water consumption is associated with a poor prognosis. Severely affected individual birds are unresponsive when approached, do not react to stimuli, and are easily caught and handled. They sit with their eyes closed in a hunched position with drooping of the head, neck, and wings. The beak may be inserted into the litter to support the head. Dehydration is indicated by dark dry skin, which is especially noticeable in the shanks and feet. Dehydrated young chicks typically have prominent raised folds of skin along the medial and lateral sides of the shanks and toenails that appear black. Although, technically, death is not a clinical sign, this may be the main indication of an outbreak of colibacillosis in a flock.

Clinical signs of predisposing or compounding factors often are seen concurrently with signs of *E. coli* infections.

Morbidity and Mortality

Both morbidity and mortality are highly variable depending on the type of disease produced by *E. coli*. It is probable that most, if not all, commercial flocks experience some degree of morbidity, mortality, or condemnation caused by *E. coli* infections.

In flocks with highly virulent colisepticemia, it is occasionally possible to watch a bird sicken and die within a few hours. A flock that appears clinically normal when examined during the day but has an excess number of dead birds the following morning, is a common finding in mildly affected flocks. This pattern is typical for egg

Table 18.6 Classification of the different types of pathological manifestations of colibacillosis (adapted from (31)).

Localized Infections
Coliform omphalitis/yolk sac infection
Coliform cellulitis (inflammatory process)
Swollen head syndrome
Diarrheal disease
Venereal colibacillosis (acute vaginitis)
Coliform salpingitis/peritonitis/salpingo-peritonitis (adult)
Coliform orchitis/epididymitis/epididymo-orchitis
Systemic Infections
Colisepticemia
Respiratory origin (air sac disease, chronic respiratory disease)
Enteric origin
Hemorrhagic septicemia
Neonatal
Layer
Duck
Colisepticemia sequelae
Meningitis/encephalitis
Panophthalmitis
Osteomyelitis
Spondylitis
Arthritis/polyarthritis
Synovitis/tenosynovitis
Sternal bursitis
Chronic fibrosing pericarditis
Salpingitis (juvenile)
Coligranuloma

layer and breeder flocks experiencing coliform salpingitis/peritonitis.

Pathology

Several localized and systemic types of colibacillosis affect poultry. Often the name is based on the tissue(s) affected or disease process (Table 18.6). APEC responsible for different forms of colibacillosis (septicemia, omphalitis, swollen head syndrome) form subpathotypes with different pathogenic, genotypic, and phenotypic traits (320).

Localized Forms of Colibacillosis

Coliform Omphalitis/Yolk Sac Infection. Omphalitis is an inflammation of the navel (umbilicus). In birds the yolk sac is also usually involved (yolksacculitis) because of its close anatomic relationship to the umbilicus. Infection follows contamination of the unhealed navel with APEC. Fecal contamination of the egg shell and unsanitary conditions in the hatchery are considered the most important sources of infection. Bacteria may be acquired *in ovo* if the hen has oophoritis or salpingitis or via contamination following artificial insemination (184, 344). Yolksacculitis also can result from translocation of bacteria from the chick's intestine or from the bloodstream. In these cases the navel is not affected. Similarly, peritonitis can occur without involvement of the umbilicus.

It is common to recover low numbers of *E. coli* from normal yolk sacs. Between 0.5% and 6% of eggs from normal hens contain *E. coli*. Experimentally inoculated hens may shed *E. coli* in up to 26% of their eggs. Pathogenic strains accounted for 43 of 245 isolates from dead embryos (183). About 70% of chicks with "mushy chick disease" had *E. coli* in their yolk sacs (183). Other types of bacteria also can cause omphalitis, although *E. coli* is most common. *E. coli* and *Enterococcus faecalis* infections accounted for approximately half of the mortality that occurred in layer chicks during the first week after hatching. First week mortality was significantly correlated with total mortality in the flock but not flock uniformity. A variety of *E. coli* genotypes indicated different sources of infection. For good flock performance, first week mortality needs to be less than 1% (393).

Adhesin factors characterizing omphalitis isolates of *E. coli* include type 1 (F) fimbriae in 96%, P fimbriae in 8%, and afimbrial adhesins in 16%. Afimbrial adhesin occurred more frequently in omphalitis isolates compared with isolates from cases of salpingitis, swollen head syndrome, or respiratory disease (258). When genotyped, omphalitis isolates tended to be more similar to commensal isolates than they were to isolates from swollen head syndrome or septicemia (9, 83). A high percentage of *E. coli* isolates from eggs, dead embryos, and chicks that died between placement and 7 days of age possessed the virulence genes *ipaH* (invasion and persistence in cells), *eae* (attaching and effacing lesions), and *cdt* (cell distension and death) compared with other APEC (444).

Some embryos may die before hatching, particularly late in incubation; whereas others die at or shortly after hatching. Surviving infected chicks can be a source of *E. coli* for other chicks in the same hatch (344). The incidence of birds with omphalitis increases after hatching and declines after about 6 days with occasional losses continuing up to 3 weeks. As few as 10 organisms of serotype O1a:K1:H7 caused 100% mortality in day-old chicks following yolk sac injection (466). When birds become infected with low virulent strains there may be no embryo or chick mortality or some may survive although hatchability, chick livability, and relative yolk weights may be affected (344); the only pathologic finding is retention of infected yolk sacs containing caseated yolk (174).

Swelling, edema, redness, and possibly small abscesses characterize acute inflammation of the navel. The abdomen is often distended and blood vessels are hyperemic (Figures 18.1A, 18.1B). In severe cases, the body wall and overlying skin undergo lysis and are wet and dirty leading to the term "mushy" chicks or poults. Other nonspecific changes such as dehydration, visceral gout, emaciation, vent pasting, and enlarged gall bladder may be seen. The yolk sac is typically distended because yolk has not been

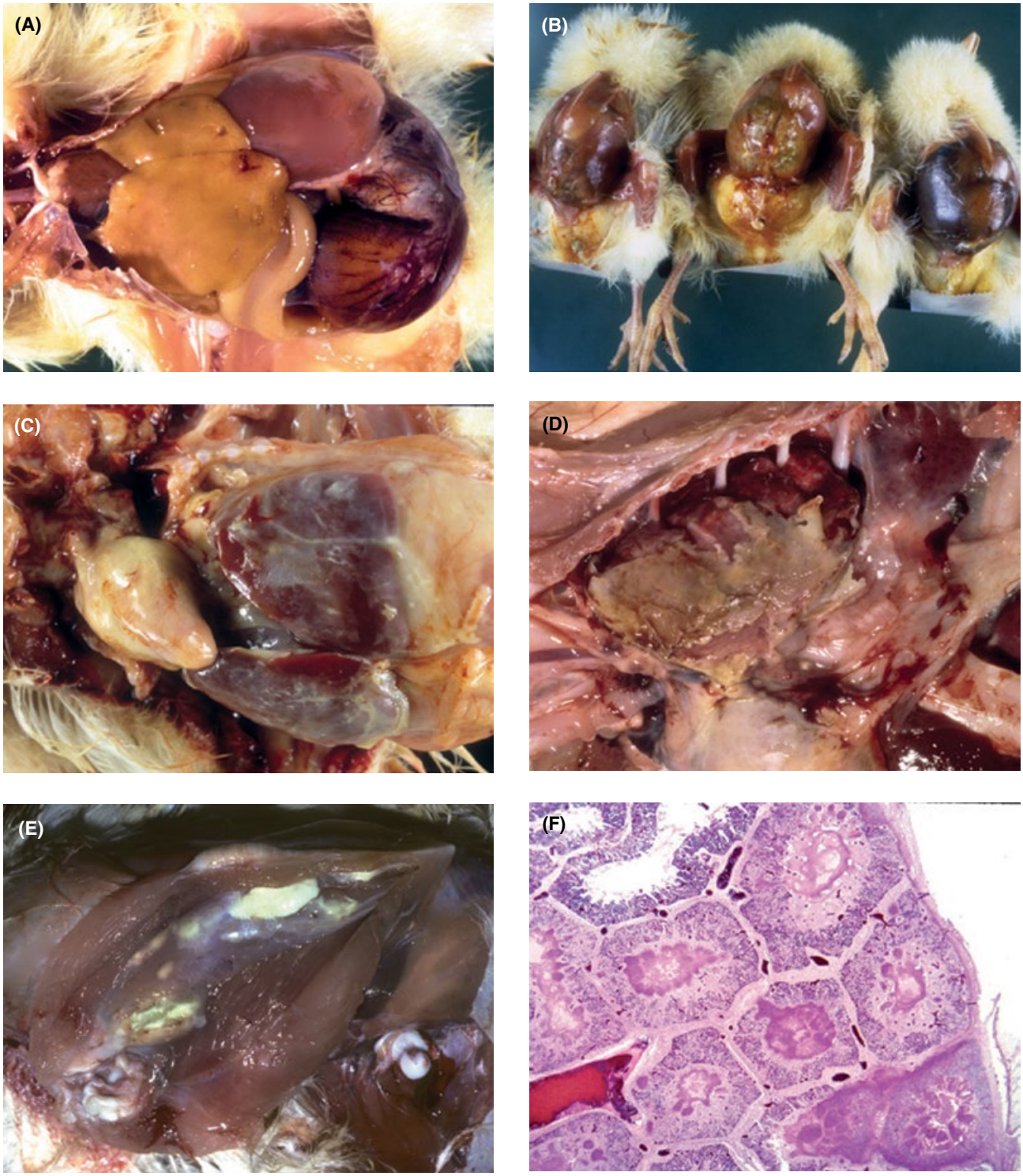
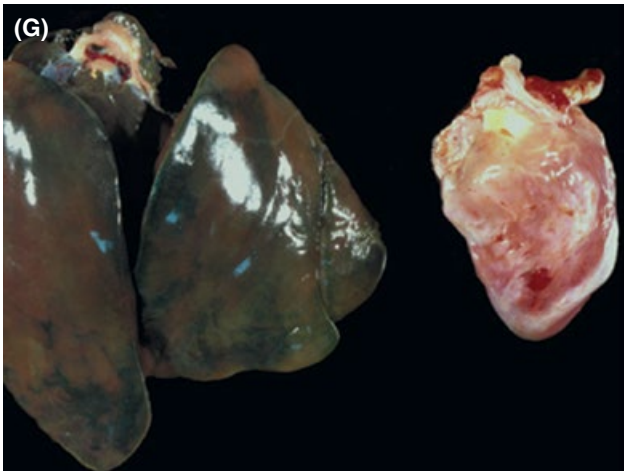


Figure 18.1 Colibacillosis. (A) Yolk sac infection in a 4-day-old leghorn chick. Yolk sac is distended, hyperemic (note prominent vessels), and filled with abnormal brown, watery contents. (B) Omphalitis and yolk sac infection in a group of 3-day-old leghorn chicks. Navels are inflamed and yolk sacs are distended with abnormal contents. (C) Advanced air sac disease in a 20-day-broiler chicken. Polyserositis (pericarditis, perihepatitis, peritonitis, airsacculitis) has occurred as a result of systemic spread of *Escherichia coli*. (D) Pleuropneumonia and airsacculitis in a broiler chicken caused by *E. coli i*nfection. (E) Experimental colibacillosis in a turkey. Extension of inflammation between superficial and deep pectoral muscles from airsacculitis involving the interclavicular air sac. Detecting this type of lesion is important during inspection at processing. (F) Microscopic appearance of pneumonia caused by *E. coli* in a broiler chicken. Exudate fills the lumen of several affected parabronchi (compare with unaffected parabronchi at the top left of the figure). Exudate has expanded some atria. Some atria have ruptured, permitting extension of the inflammatory process through the air capillary bed into the interstitium. The process involves almost an entire lobule with extension to the adjacent pleural surface. ×10. (G) Pericarditis and green discoloration of the liver in a turkey that survived the acute septic phase of colibacillosis. Pericardium is thickened and exudate in the pericardial sac is beginning to undergo fibrosis. Green discoloration of the liver can indicate inflammation elsewhere in the bird, especially in turkeys. (H) Salpingitis in a young bird caused by *E. coli*. This lesion occurs infrequently but is often associated with airsacculitis involving the left abdominal air sac. (Figures A–H courtesy of L. Munger) (*For color detail, please see the color section*.)



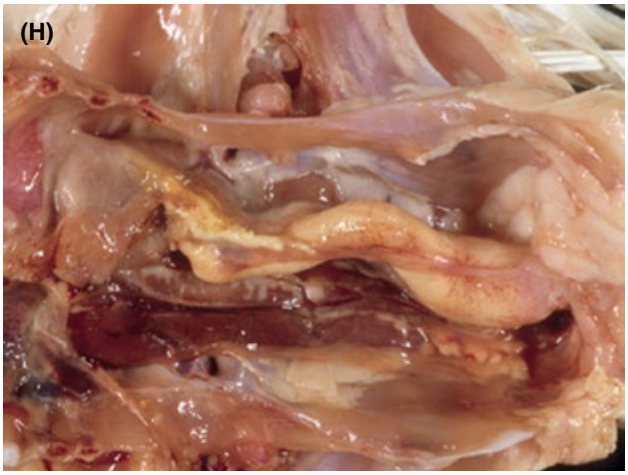


Figure 18.1 (Continued)

absorbed and inflammatory products have been added. Yolk is abnormal in color, consistency, and smell, and may contain visible exudate. Blood vessels of the yolk sac are often prominent. Chicks or poults with infected yolk sacs that live more than 4 days also may have peritonitis, pericarditis, or perihepatitis, indicating local and systemic spread of the organism from the yolk sac.

Microscopically the wall of the infected yolk sac is edematous with mild inflammation. There is an outer connective tissue zone adjacent to a layer of inflammatory cells containing heterophils and macrophages, a layer of giant cells, a zone of necrotic heterophils and masses of bacteria, and then the inner, abnormal yolk contents. A few plasma cells may be found in some yolk sacs.

Consequences of yolk sac infection include deprivation of nutrients and maternal antibodies, absorption of toxins, and spread of *E. coli* by extension into the body cavity (peritonitis) or systemically to produce colisepticemia and its sequelae (polyserositis, arthritis). Survivors are usually stunted and do poorly. Birds that survive the acute infection have small, firm, persistent yolk sacs (often referred to as "retained" yolk sacs) that contain inspissated exudate and yolk material. *E. coli* persists in these chronically inflamed yolk sacs and can be isolated from them for weeks to months after hatching. Adhesions to intestines, especially the tip of the duodenal loop, or other visceral organs are common. Rarely the elongated stalk of the yolk sac will wind around the intestine and cause strangulation.

Coliform Cellulitis (Avian Cellulitis, Inflammatory Process, Infectious Process). Coliform cellulitis is characterized by sheets of serosanguineous to caseated, fibrinoheterophilic exudate in subcutaneous tissues. Lesions, often referred to as "plaques," are located in the skin over the abdomen or between the thigh and midline. Other colibacillosis lesions, or reduced productivity, occasionally accompany coliform cellulitis (121, 158, 395, 497), but usually lesions are discovered at processing when inspectors open the thickened yellow abdominal body wall of an otherwise normal carcass.

Coliform cellulitis has emerged as a significant disease since its description in 1984 (434) because of increased condemnations, downgrading at processing, and higher labor costs to process affected flocks. Between 1986 and 1996, condemnations for coliform cellulitis increased almost 12-fold in Canada. Estimated annual losses to the US broiler industry caused by coliform cellulitis have increased from $20 million in 1991 to more than $80 million in 1998 (469). In Brazil, cellulitis lesions are estimated to cause the loss of 0.14%–1.4% of poultry meat production, leading to losses of at least 18 thousand tons of meat in 2011 (25).

Escherichia coli are the most frequently isolated organisms from cellulitis lesions. Other bacteria that have been isolated from cellulitis lesions include *Pseudomonas aeruginosa, Proteus vulgaris, Enterobacter agglomerans, Pasteurella multocida, Streptococcus dysgalactiae, Aeromonas spp., Staphylococcus aureus, Actinomyces pyogenes,* etc., but they are not considered significant (152, 334, 384, 427, 468).

Cellulitis isolates of *E. coli* belong to the same serogroups as those that cause other forms of colibacillosis. They usually produce colicin and aerobactin (407). Virulence properties and molecular characteristics are similar among isolates from cellulitis and colisepticemia lesions and normal birds (25, 159, 222, 378). However, isolates from cellulitis lesions have a greater ability to produce cellulitis in experimentally exposed birds than *E. coli* isolates from airsacculitis lesions or feces of healthy chickens (228, 406). By looking for significant associations between the presence of virulence-associated genes and the cellulitis pathogenicity, it was found that the presence of genes for invasins *ibeA* and *gimB* and group

II capsule KpsMTII resulted in increased ability of APEC to cause cellulitis (25).

A vacuolating cytotoxin produced by cellulitis *E. coli* isolates is also produced by isolates from chickens with colisepticemia and swollen head syndrome but not by isolates from healthy chickens. The cytotoxin is similar to one produced by *Helicobacter pylori*, except that *H. pylori* cytotoxin is specific for mammalian cells whereas the avian *E. coli* cytotoxin is specific for avian cells (454).

Initially, isolates of *E. coli* from litter and lesions could not be differentiated based on biotyping, suggesting that litter was the source of *E. coli* in cellulitis lesions (120). However, genotyping has shown that the prevalence of pathogenic *E. coli* in a broiler house is independent of the prevalence of other *E. coli* in the environment (223). DNA fingerprinting identified the presence of endemic populations of specific cellulitis-associated *E. coli* existing in the broiler house environment. These organisms persist for at least 6 months, irrespective of partial or complete cleaning and disinfection as performed in the field, and cause coliform cellulitis in successive flocks (468, 469).

Regional differences in the prevalence of coliform cellulitis emphasize the important roles of environmental and management factors in occurrence of the disease. Increased condemnation rates caused by coliform cellulitis during the past 25 years indicate that changes have occurred in either the occurrence or characteristics of risk factors associated with coliform cellulitis. The most notable change during this time has been in the genotype and phenotype of the bird being raised, so it is not surprising that bird-related factors contribute significantly to the increased incidence of scratches and subsequent coliform cellulitis.

Fast-growing, heavy broiler strains are more likely to have an increased prevalence and severity of skin scratches, which predispose to coliform cellulitis (Figure 18.3). Several reasons may explain this association. The strength of the skin in broilers is related to genetics. The lack of association between scratches and abdominal circumference suggests that strain of bird *per se* could be a better predictor than body characteristics (121). Aggressiveness or nervousness of chickens may also be strain dependent. Birds from a more nervous strain could be more active, increasing the chances of being injured or scratched. If aggressiveness is a problem, the source could be farm dependent (e.g., behavioral studies have demonstrated the importance of socialization of the flock by the grower on the birds' behavior) (109). Rapid growth by modern broiler breeds results in a higher stocking density sooner in the life of the flock, at a time when feathering is not well developed. Poor feathering and crowded conditions could have a significant impact on the incidence of coliform cellulitis. The major histocompatibility complex (MHC) affects the likelihood of an individual chicken developing cellulitis, although not the severity of the lesion (304). Commercial broilers with MHC type B21 are more susceptible to cellulitis than ones with MHC type B13. However, the severity of lesions was not related to their MHC (305).

Feather cover helps to protect the skin from damage. A positive association exists between scratches and poor feathering (119). Although little is known about nutritional and environmental factors that affect feather growth and development, birds kept in warm temperatures tend to feather less rapidly than birds kept in cooler temperatures.

Coliform cellulitis occurs more frequently in males than females (121, 497). The gene responsible for sexing regulates feather growth. Slower feathering males may be more vulnerable to skin injuries because of greater exposure of the skin to potential physical damage. Sex may also contribute to coliform cellulitis because of its association with weight, aggressiveness, or management practices. In addition, production time is longer for roasters than for broilers.

Stocking density plays a dual role as a risk factor. It leads to an increase in skin scratches (119) and stress, but it also contributes by increasing the level of contact between birds. Cellulitis lesions occurred more readily when birds were palpated daily to simulate close contact among birds (334).

Flocks grown on straw were 2.8 times more likely to experience coliform cellulitis than flocks grown on shavings (121). Physically, straw consists of sharp, pointed pieces that may inflict minor injuries to the skin. Straw may also provide a good medium for growth and multiplication of *E. coli* because of its ability to hold more moisture than shavings. Similarly, in Brazil, occurrence of coliform cellulitis was greatest in broilers on *Brachiaria* grass litter compared with corncob, rice shells, or sawdust litter (532). A positive association also exists between the number of flocks raised on the same litter and cellulitis (532). However, this association could not be explained by an increase in litter bacterial load. Furthermore, litter environmental variables (water activity, pH, moisture content, and ammonia levels), as measured in this study, were also not significant. Nevertheless, litter quality should be considered an important factor by those working on reducing this condition in the field. Total down time is negatively associated with coliform cellulitis (i.e., the longer the down time, the lower the incidence of the disease) (223, 461).This supports the hypothesis that the bacterial load in the environment is associated with disease prevalence.

In a prospective study, a positive association between ambient temperature during early grow-out and cellulitis was found. The predictive model, after controlling for other significant variables, indicated a 40%– 60 % increase in cellulitis as temperatures increased over a

range of approximately 60°F (15.5°C) from 29°F (–1.7°C) to 94°F (34.4°C). Low cellulitis prevalence flocks would increase from 0.5% to 0.8 %, whereas high prevalence flocks would increase from 1.2% to 1.9 %. Similar to ambient temperature, increased relative humidity at mid-grow-out correlated with increased occurrence of cellulitis. An increase in relative humidity from 36% to 93 % was predicted to increase cellulitis from 0.3% to 0.9 % in low-prevalence flocks and from 1.0% to 1.9 % in high-prevalence flocks (461).

A positive association was observed between coliform cellulitis and feed company in a prospective study (121). The effect of nutrition on the pathogenesis of the disease is not well known. Amino acid levels in the feed may be important. Feed deficient in cysteine and methionine can cause nervousness and affect feathering (403, 457). A relative deficiency occurs in feeds with high energy to total protein ratios. High-energy feeds may also contribute to coliform cellulitis by increasing fat deposition in the skin, which may result in the skin being more susceptible to scratches and injuries (457).

The occurrence of coliform cellulitis was higher in vegetarian broilers compared with broilers fed feeds containing animal products. Condemnation rates for birds fed a standard diet, which contained growth promotants, antibiotics, and anticoccidials, was substantially lower (0.26%) than for birds fed a vegetarian or organic feed without additives (1.18%) (193).

Providing vitamin E at 300 mg/kg or vitamin A at 60,000 IU/kg improved the resistance of 6-week-old broilers against *E. coli* infection (495). Supplementation with vitamin E had a variable impact on development of coliform cellulitis. Intermediate levels were superior to both lower and higher levels of the vitamin (306). Birds fed both vitamin E at 48 IU/kg and a zinc-protein complex at 40 ppm of zinc decreased the occurrence of coliform cellulitis (307). Improved wound healing and immune system potentiation by the supplements were considered responsible for the beneficial effect.

Older chickens are more likely to develop lesions of cellulitis following inoculation of scratches or subcutaneous injection than young chickens, which tend to develop systemic disease and experience high mortality (25, 160, 227, 228, 307, 385). Cellulitis has also been described in quail (54). In turkeys, cellulitis is similar to gangrenous dermatitis and differs from coliform cellulitis in chickens (69).

Cellulitis lesions are primarily unilateral and located on the abdomen or thigh. Skin color varies from normal to yellow or red-brown, and the skin may be swollen at the site of inflammation (Figure 18.3). The size of the lesion normally varies between 1–10 cm (121). Scratches and scabs on the skin overlying the lesions often can be identified (Figure 18.4). Beneath the skin, there is subcutaneous edema, exudate, and muscle hemorrhage. A fibrinous to caseous plaque between the muscle and subcutis is the characteristic lesion (Figure 18.2H).

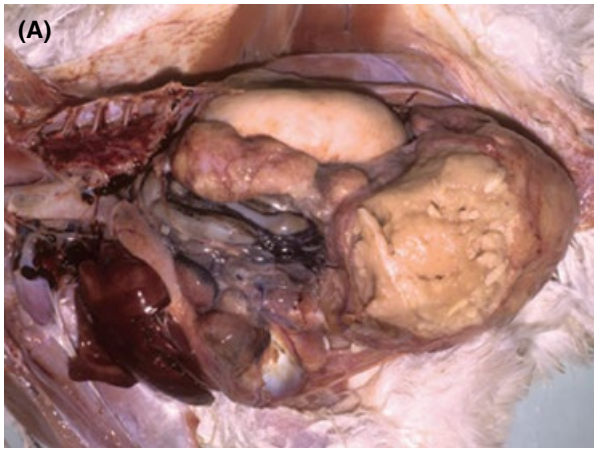
Lesions develop rapidly; exudate is visible as early as 6 hours postinfection, and the caseous plaque could be experimentally produced within 18–24 hours postinfection. Rapid lesion development suggests that events occurring late in the life of the flock could be important in the development of lesions found at processing (160, 385). When birds were inoculated experimentally with *E. coli* strains isolated from coliform cellulitis lesions, the highest percentage of birds developing typical lesions had been challenged only 3 days prior to processing (387). Lesions were still present 3 weeks postinoculation (385).

High coliform cellulitis condemnation rates are not of hatchery origin. In Canada, only 1.7% of coliform cellulitis lesions were consistent with a primary navel infection (121).

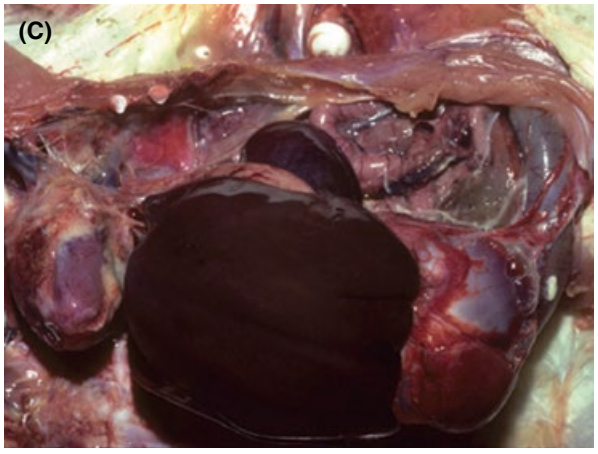
Experimental exposure of young chickens to cellulitis isolates of *E. coli* results in septicemia, death, or marked stunting, indicating that most birds affected by *E. coli* in the hatchery would either die or be culled before reaching the processing plant (227, 228). No association between cellulitis and the source of eggs, age of parent flocks, total bacterial count, and coliform count in the hatchers was found (120).

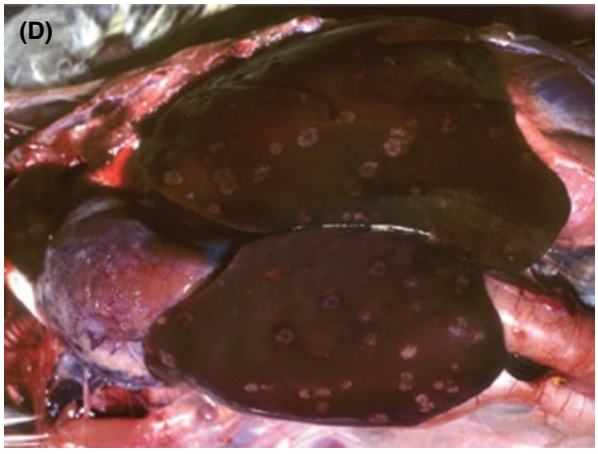
Skin trauma, especially scratches, provides the main portal of entry into the host for specific cellulitis-type *E. coli* present in the litter. Applying bacteria to feather follicles from which the feather had been pulled did not

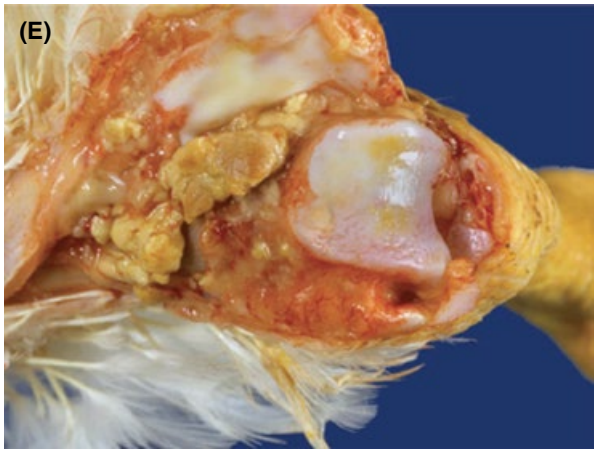
Figure 18.2 Colibacillosis. (A) Large caseated masses distending the oviduct of this mature laying hen are characteristic of salpingitis caused by *Escherichia coli*. Salpingitis in the adult female most likely results from an ascending infection from the cloaca. (B) Goose breeder with acute peritonitis. Yolk was demonstrated in the peritoneum and *E. coli* was isolated. (C) Acute *E. coli* septicemia in a turkey. Spleen is markedly enlarged and congested. Note that it is approximately the same size as the proventriculus. Liver is also enlarged and congested, and there is evidence of early pericarditis and peritonitis. (D) Experimental colibacillosis in a turkey. Liver from a bird that survived the acute septicemic phase has multiple pale foci, which were determined microscopically to be focal areas of early heterophilic, granulomatous hepatitis. (E) Advanced tenosynovitis/arthritis involving the hock joint and flexor tendons of a lame commercial broiler. E. coli and Staphylococcus spp. were isolated from the lesion. (F) Panophthalmitis affecting the eye of a turkey that survived an earlier episode of colisepticemia. This lesion is uncommon and typically unilateral. The organism can be isolated from the eye for an extended period after it is no longer present in other tissues. (G) Swollen-head syndrome in a broiler chicken. There is conjunctival inflammation and periorbital swelling due to cellulitis. Evidence of exposure to high ammonia levels and infection with infectious bronchitis virus and E. coli were found in this flock. (H) Avian coliform cellulitis (inflammatory process). Subcutaneous yellow, caseous exudate is present over the abdomen of this affected bird. (Figures E and G courtesy of L. Munger) (*For color detail, please see the color section*.)


(A)


(B)

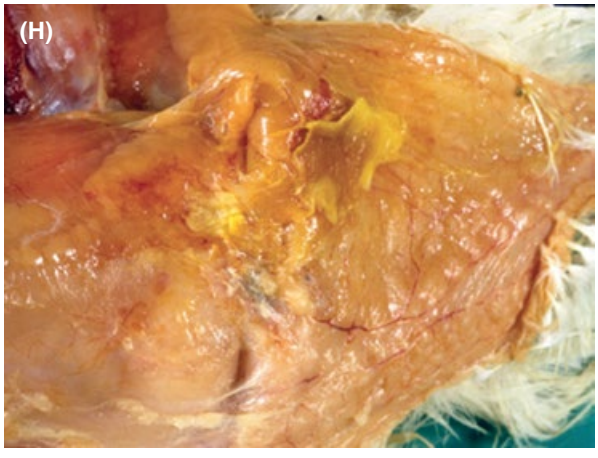

(C)


(D)


(E)


(F)


(G)


(H)

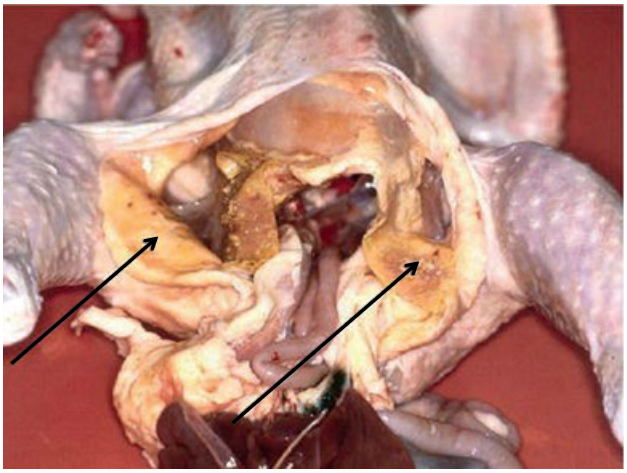


Figure 18.3 Coliform cellulitis lesion at processing. A caseous sheet of exudate, often referred to as a plaque, is located in the subcutaneous tissues beneath an area of thickened, yellow discolored skin.



Figure 18.4 Five-week old broiler chicken with a skin laceration (scratch). Scratches often go unrecognized but are the most important risk factor for development of coliform cellulitis. Taking measures to reduce scratches reduces cellulitis condemnations at processing.

cause coliform cellulitis. Oral feeding or swabbing the navel of young chickens did not produce cellulitis but did result in mortality, depressed growth, and other types of colibacillosis, which was dose dependent (227). The disease is reproduced readily by swabbing damaged skin with broth cultures or subcutaneous inoculation (25, 160, 227, 228, 307, 385).

When a fast-growing strain of broiler chicken was compared with a strain of leghorns, the broiler strain was more predisposed to cellulitis because of an inferior first line of defense of their skin. In the broilers, wound-healing was slower, lesions were more severe and covered a larger area, and mobilization and functionality of phagocytic cells were inferior (391).

Usually, an affected bird has only skin lesions, but concurrent lesions of systemic colibacillosis occasionally can be found suggesting that cellulitis may result from systemic spread or, conversely, that localized lesions in the skin can be a source for systemic disease. The latter is inversely correlated with age (i.e., the younger the bird, the more likely it is to develop systemic disease) (160, 228). Lesions have been correlated with other categories of condemnation in which *E. coli* would be expected to play a significant role (septicemia, airsacculitis, etc.) (120, 121, 158, 159, 497).

A positive association between cellulitis and ascites has been shown (120, 497). Ascites is a common condition in broiler chickens characterized by an abnormally large abdomen. Because most cellulitis lesions are located in the abdominal area, it may be that ascites is a biological predisposing factor for cellulitis. It also is possible that both conditions may share common risk factors such as rapid growth.

Valgus-varus leg deformity, characterized by lateral or medial deviation of the distal tibiotarsus with a corresponding deviation of the tarsometatarsus, occurred more frequently in carcasses condemned for cellulitis (120, 497). Valgus-varus deformity is considered to be the most frequent cause of leg weakness and lameness in broiler chickens (434). However, the association between valgus-varus deformity and coliform cellulitis needs to be interpreted with caution because of potential confounding with sex and breed. Most valgus-varus deformity affects male birds, and the incidence of coliform cellulitis can vary with breed. Birds with valgus-varus leg deformity spend more time lying on the floor (244), which results in greater contact exposure between the skin and the *E. coli* present in the litter. Also, prolonged resting by lame birds may result in skin damage as other birds tread on them (120).

Cellulitis lesions are identified readily at processing, normally making it possible to use condemnation results to assess control strategies. However, an epidemiological study in Ontario has found that 30% of the variation in cellulitis prevalence was dependent on the slaughter plant (478). Therefore, the possibility of misclassification may exist, and should be considered in an investigation. Lesions should be cultured aseptically to determine the presence of *E. coli*.

There is no treatment for coliform cellulitis, and eradication of the disease will not be possible because of the ubiquitous occurrence of *E. coli*. Advances in the development of immunoprotective agents or immunomodulators suggest that a molecular approach to cellulitis control is possible, although not currently practical or economical (6, 145). However, by carefully managing the environment and nutrition of the modern, fast-growing, heavy broiler, it is possible to reduce substantially the incidence and impact of the disease. A key aspect of any control strategy is its cost–benefit. Adequate monitoring to

ensure implementation and compliance of control strategies will be needed to determine their effectiveness. The following are some recommendations.

Very early lesions consist mainly of serosanguineous fluid in contrast to the caseous lesions observed after 24 hours postinfection. A high prevalence of acute lesions indicates that events occurring just prior to or during transportation should be investigated, especially if at least 10 hours exists between load-out and processing. In contrast, a majority of chronic lesions would indicate the need to focus on earlier events that occurred during the grow-out period.

Problem flocks should be compared with flocks that did well during the same time period within the same company and the risk factors determined for each type of flock. Any management or environmental factors that affect the birds' resistance or contribute to skin scratches should be identified. Pay special attention to stocking density, feeder and waterer space (effective space, i.e., in some houses, the space is available, but the drinkers or feeders are not all functional), migration fencing, type of litter, quality of litter, and feed restriction and lighting programs. Any intervention must first focus on improving the environment of the birds. This includes good sanitation to reduce the bacterial load of the environment.

Swollen Head Syndrome. Swollen head syndrome (SHS) is an acute to subacute cellulitis involving the periorbital and adjacent subcutaneous tissues of the head (Figure 18.2G). SHS was first described in broilers in South Africa associated with *E. coli* and an unidentified coronavirus infection (350). The disease has subsequently been described in most intense poultry-producing areas of the world. The disease also affects turkeys and guinea fowl (298, 508).

Swelling of the head is caused by inflammatory exudate beneath the skin that accumulates in response to bacteria, usually *E. coli*, following upper respiratory viral infections (e.g., avian metapneumovirus, infectious bronchitis virus). Ammonia aggravates the disease (108). The portal of entry is considered to be the conjunctiva or inflamed mucous membranes of the sinuses or nasal cavity (367). Possible infection via the Eustachian tube also has been suggested (108). Microscopic lesions include fibrinoheterophilic inflammation and heterophilic granulomas in the air spaces of the cranial bones, middle ear, and facial skin. Lymphoplasmacytic conjunctivitis and tracheitis with formation of germinal centers have also been observed (218).

Although the pathogenesis of SHS has not been established, conjunctival-associated lymphoid tissue inflamed from virus infection and/or ammonia irritation may serve as the site through which bacteria gain access to subcutaneous tissues. Periorbital inflammation is typically seen early in the disease and hyperplastic lymphoid tissue has been shown to be a site where *E. coli* penetrates mucosal surfaces (172). Scarifying the conjunctival mucosa and instilling a pure culture of *E. coli* (350), or inoculation of *E. coli* into submucosal or subcutaneous tissues (368), will reproduce the disease. Intranasal inoculation of avian metapneumovirus and *E. coli* failed to reproduce the disease (368). Swollen head syndrome did not occur when day-old chicks were inoculated supraconjunctivally with either avian metapneumovirus or *E. coli*, but they did develop clinical disease, which was most severe when the chicks received both agents (4).

Escherichia coli isolates from SHS cases possess several virulence factors including fimbrial adhesins, colicin production, aerobactin, and complement resistance. In general, SHS isolates have virulence attributes similar to isolates from cases of septicemia (83), except colicin production and iron-acquisition siderophores were more frequent in SHS isolates. The colicins produced by SHS strains often differed from ColV (82). The majority of strains were motile, but presence of K1 capsule was infrequent (399). Similar results were found in a subsequent study in which SHS isolates were more similar to isolates that cause septicemia than isolates that cause omphalitis or commensal isolates except type 1 and curli fimbriae, and temperature-sensitive hemagglutinin (*tsh*) were more frequent in SHS isolates (9). A transferable 60 MDa plasmid from an SHS *E. coli* isolate contained genes for cell adhesion, colicin production, and *tsh* (480). Adhesin factors occurring in SHS *E. coli* isolates included type 1 in 94% and P fimbriae in 28%. The P-fimbrial adhesin factor occurred more frequently in SHS isolates compared with isolates from cases of salpingitis, omphalitis, or respiratory disease (258). A unique Shiga toxin (VT2y) (453) that may be involved in the pathogenesis of SHS was identified in a high percentage of SHS *E. coli* isolates (402). Additionally another toxin, similar to one produced by *Bacillus cereus* that is highly lethal for mice following injection, was identified in SHS isolates (452). Other work showed that *flgE* (flagellar hook), *tyrR* (transcriptional regulator), *potF* (putrescine transporter), *yehD* (putative adhesin) and *bfr* (bacterioferritin) were attenuated compared with a wild-type strain in a 1-day-old chickens infection model (94).

Diarrheal Disease. Primary enteritis is a common manifestation of *E. coli* infections in mammals including humans, but is considered rare in poultry. Diarrhea results from infections with enterotoxigenic (ETEC), enterohemorrhagic (EHEC), enteropathogenic (EPEC), or enteroinvasive *E. coli* (EIEC); each type possessing certain virulence factors that determine the characteristics for each type of enteric disease (see Virulence Factors). EHEC and EPEC strains produce attaching and effacing lesions on intestinal mucosal surfaces. Collectively, these strains are called attaching and effacing

E. coli (AEEC). Similar lesions are also produced by the closely related species, *E. albertii* (389, 390). Intestinal *E. coli* in poultry have been poorly studied, except as commensal strains for comparison with APEC or reservoirs of virulence genes that occur in human strains, so our knowledge of the role that *E. coli* may play in intestinal disease is limited.

Enterotoxigenicity caused by ETEC strains is uncommon in APEC. Most surveys for heat-stable and heat-labile enterotoxins either fail to find any positive isolates or identify only a few (122). ETEC that elaborated toxins capable of causing fluid accumulation in ligated intestinal loops of chickens were recovered from chickens with diarrhea (243), and an O15 APEC strain that produced heat-labile toxin II was isolated from ostrich chicks experiencing severe diarrhea and high mortality (373).

Natural and experimental infections with AEEC or presence of *eae* gene have been reported in chickens (138, 247, 263, 444, 484, 488), turkeys (397, 484), pigeons (520), ducks (484), psittacines (462), and other avian species (269). Infections with infectious bursal disease (IBD) virus in chickens and adenovirus infection in the pigeon were considered possible predisposing factors to AEEC infection. In turkey poults, coinfection of EPEC and turkey coronavirus (TCV) resulted in severe stunting and very high mortality (178). Clinical disease was most severe when poults were infected with TCV prior to inoculation with EPEC (397). Ten of 12 commercial turkey flocks experiencing high mortality because of poult enteritis mortality syndrome (PEMS) were infected with EPEC, confirming the importance of natural EPEC infection as a cause of mortality in young turkeys (397). Infections with IBD virus in chickens and adenovirus infection in the pigeon were considered possible predisposing factors to AEEC infection. In turkey poults, coinfection of EPEC and TCV resulted in severe stunting and very high mortality (178).

Birds infected with AEEC may be clinically normal or have diarrhea and be dehydrated. In clinically affected birds, the intestines are pale and distended with fluid, which may contain visible flecks of mucus and exudate. Ceca are often the most obviously affected part of the digestive tract. They are typically distended with pale brown fluid and gas. Bacteria intimately attach to the surface of enterocytes causing effacement of microvilli, pitting, and pedestal formation, which are best seen by electron microscopy (Figure 18.5). Lesions are most common in the ceca. Organisms are readily identified in tissue sections using Giemsa stain or by immunohistochemical methods.

Experiments to define a role for *E. coli* in malabsorption syndrome of chickens have not been successful (345, 476). In contrast, specific strains of *E. coli* have been associated with PEMS (115). Turkey astrovirus, an agent involved in PEMS, impairs macrophage function, which could explain the enhanced susceptibility of affected poults to secondary bacterial infections such as colibacillosis (424).

Diseases resulting from infection with EIEC have not been described but are likely, especially in the case of neonatal septicemia. EIEC possess genes such as *ipa*, which encodes a virulence factor that provides the organism with the ability to penetrate and survive within cells. The most frequently identified virulence gene in *E. coli* isolated from eggs, dead embryos, and chicks with omphalitis/yolk sac infections was *ipaH*. Most of the *ipaH*$^+$ isolates (62 of 80; 77.5%) came from liver or yolk sac of chicks that died between 3 and 7 days of age, which corresponded to a period of increased mortality (444). Further characterization of the *ipaH*$^+$ isolates revealed properties that did not match those of typical EIEC and

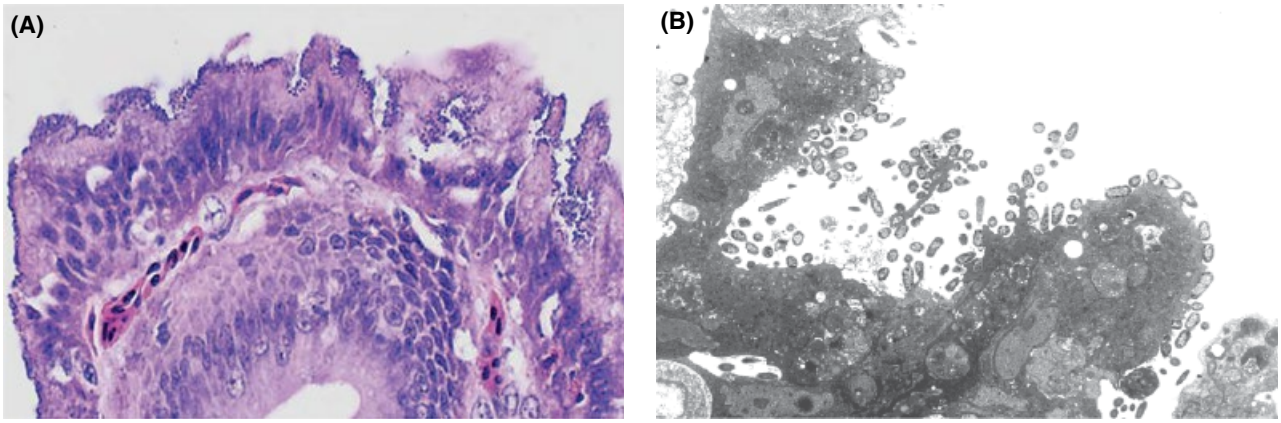


Figure 18.5 Attaching effacing *E. coli* bind tightly to the apical surface of enterocytes, destroying the normal brush border. (A) On light microscopy the surface epithelium appears irregular and numerous bacteria can be seen attached to affected cells. (B) By electron microscopy the organisms characteristically occupy small pits in the cell surface or are on pedestals. The number of bacteria and extent of brush border effacement is apparent. (Courtesy of H.L. Shivaprasad)

the existence of specific EIEC clone complexes among avian isolates. Cell invasion was confirmed *in vitro* (444).

Venereal Colibacillosis (Acute Vaginitis). Venereal colibacillosis is an acute and frequently fatal vaginitis that affects turkey breeder hens shortly after they are first inseminated usually in the first weeks of egg production. Puncturing the hymen of young turkey hens can lead to a severe localized *E. coli* infection characterized by vaginitis, cloacal and intestinal prolapse, peritonitis, egg binding, and internal laying. The affected mucosa is markedly thickened, ulcerated, and covered with a diphtheritic, caseonecrotic membrane, which causes obstruction of the lower reproductive tract. The thickness of these membranes posed an obstruction to egg passage leading to internal laying and egg peritonitis. The upper oviduct is grossly and histologically normal. Flocks can have losses of up to 8% because of increased mortality and culling. Egg production is decreased and there is an increased number of cull eggs because of small size. Swabs from cloaca and vagina produced numerous colonies of only *E. coli*. No other infectious agents have been identified as contributing to the disease (142). Anecdotal reports indicate a similar disease can affect young broiler breeders as they come into production.

Coliform Salpingitis/Peritonitis/Salpingoperitonitis (Adult). Inflammation of the oviduct caused by *E. coli* results in decreased egg production and sporadic mortality. It is one of the most common causes of mortality in commercial layer and breeder chickens (46, 241) and also affects other female birds, especially ducks, geese (45), and quail. Accumulations of caseating exudate in the body cavity resemble coagulated yolk, which is the reason for the common name "egg peritonitis." Yolk peritonitis is a mild to moderate diffuse peritonitis without exudate resembling coagulated yolk that results from free yolk in the body cavity. Yolk peritonitis is usually associated with bursting atresia that occurs during acute ovarian regression. Marked exudation, extensive inflammation, and positive cultures characterize coliform peritonitis and serve to distinguish it from yolk peritonitis.

Salpingitis and egg binding may occur concurrently, which can cause confusion because both result in an obstructive mass within the oviduct. If an egg is not grossly visible, cutting through the mass in the oviduct and finding a shelled egg in the center indicates egg binding.

Infection occurs when *E. coli* ascends the oviduct from the cloaca (505). Injecting large (10^9) numbers of bacteria into the reproductive tract reproduces the disease. Mucosal infections with viruses (e.g., infectious bronchitis virus) or mycoplasmas also may predispose a bird to salpingitis. *M. synoviae* infection increased the occurrence of coliform peritonitis (430). Coinfection with *E. coli* and *Tetratrichomonas* occurred in Pekin duck breeders with salpingitis (78). Heavy egg production and associated estrogenic activity predispose hens to salpingitis by relaxing the sphincter between the vagina and cloaca. Spread of *E. coli* to the oviduct from an airsacculitis is also possible, but this form of salpingitis occurs more frequently in young birds as part of a systemic infection.

Isolates of APEC from birds with salpingitis have similar virulence characteristics to those that cause airsacculitis. In a study of 30 isolates, 11 belonged to serogroups O2 and O78 whereas 10 were untypeable. Twenty-seven of the isolates were of either high or intermediate virulence in a day-old chick assay. Most isolates possessed type 1 fimbriae and adhered to oviductal epithelium, especially from adult breeders, and they had the ability to acquire iron when grown in iron-deficient medium. Isolates were resistant to serum from young breeders, but sensitive to serum from older breeders (343). In a separate study, type 1 fimbriae also were identified in 49 of 50 isolates from broiler breeders with salpingitis; few isolates possessed other fimbrial types (258). Presence of type 1 fimbriae can be considered a characteristic of APEC salpingitis isolates.

Escherichia coli was isolated from the cloacae of clinically normal birds and dead birds with peritonitis, and from the oviducts and peritoneal cavities of birds with peritonitis, but not from these sites in clinically normal birds. *E. coli* from the oviducts and peritoneal cavities of each of the dead birds had identical characteristics. Isolates from one flock belonged to serogroup O78, phyologentic group A, and contained virulence genes *iroN*, *sitA*, *iutA*, *tsh*, and *iss*. Isolates from a second flock belonged to serogroup 111, phylogenetic group D, and contained virulence genes *iroN*, *sitA*, *iutA*, *traT*, *iss*, and *ompT*. The similarity among isolates from hens in each flock indicated a single clone of *E. coli* affected that specific flock (505). Molecular characteristics of *E. coli* isolated from layers with salpingitis, peritonitis, and salpingoperitonitis (241) also were clonal and possessed several of the same virulence factors including *iss*, *sfa*, *tsh*, *iucC*, *ibeA*, and *sitA* (500). Specific clones caused colibacillosis in free-range layer flocks indicating they were primary pathogens that did not require predisposing factors to cause disease (394).

In chronic cases, the oviduct is markedly distended and thin-walled with single or multiple masses of caseous exudate in the general form of the oviduct (Figure 18.2A). The mass of exudate may expand to the point that it fills most of the body cavity (88). Rupture of the oviduct is possible. Exudate is laminated, often contains a central egg, shells, and/or membranes, and is malodorous. Spread of the organism into the body cavity through the compromised oviduct wall or the open end of the infundibulum leads to concurrent peritonitis, which is

termed salpingoperitonitis when there is involvement of both the oviduct and peritoneum. Peritonitis in the absence of salpingitis also can occur but is uncommon. Acute cases have less exudate in the oviduct or peritoneum that tends to be soft and not as caseated (Figure 18.2B). Affected birds are incapable of laying eggs although they typically continue to ovulate. Repeated ovulations and albumen secretion are responsible for the laminated appearance of the oviductal masses. Abdominal laying and misovulated ova may accompany salpingitis and contribute to peritonitis.

Microscopically, the tissue reaction in the oviduct is surprisingly mild in view of the marked gross lesions. It primarily consists of multifocal to diffuse heterophil accumulations subjacent to the epithelium and caseous exudate in the lumen, which often contains bacterial colonies. Lymphoid foci develop in the mucosa with time and indicate chronicity.

Coliform Orchitis/Epididymitis/Epididymo-Orchitis. An ascending *E. coli* infection of the male reproductive tract, analogous to that resulting in salpingitis in the hen, occurs infrequently in roosters. Testicles are swollen, firm, inflamed, irregularly shaped, and have a mosaic of necrotic and viable tissue when opened. Heavy growth of *E. coli* can be obtained from the testicle and epididymis (342).

Systemic Forms of Colibacillosis

Colisepticemia. Presence of virulent *E. coli* in the blood stream defines colisepticemia. Virulence and number of organisms balanced against efficacy of host defenses determine duration, degree, and outcome of the disease, as well as the pattern and severity of lesions (420, 421). Colisepticemia progresses through the following stages: acute septicemia, subacute polyserositis, and chronic granulomatous inflammation (65). Whereas lesions are typical of colisepticemia, other bacteria capable of producing septicemia also can cause similar changes. Characteristic features of colisepticemia at necropsy are tissues that develop a green discoloration following exposure to air and a characteristic odor, possibly related to indole produced by the organism. The bursa of Fabricius is often atrophic or inflamed as a result of colisepticemia. It should not be interpreted that a small bursa is evidence of a prior immune-suppressing disease such as IBD (103, 366).

Pericarditis is common and is a characteristic of colisepticemia. It is usually associated with myocarditis, which results in marked changes in the electrocardiogram (170), often before gross lesions appear. Vessels in the pericardium become increasingly prominent because of hyperemia and the pericardium becomes cloudy and edematous. Initially, fluid and soft masses of pale exudate accumulate within the pericardial sac followed by fibrinous exudate (Figure 18.1C). Exudate can be seen loosely adhering to the epicardium when the pericardial sac is opened. As the disease progresses, exudate increases, becomes more cellular (fibrinoheterophilic), and undergoes caseation. The pericardial sac adheres to the epicardium with chronicity.

Microscopically, the same progression of lesion development is seen. Serous and serofibrinous exudate is seen initially followed by increasing numbers of heterophils and subsequently macrophages. Within the myocardium, particularly close to the epicardium, there are accumulations of lymphoid cells and by 7–10 days there also are many plasma cells. Subsequently, exudate in the pericardial sac undergoes organization (Figure 18.1G), which, in survivors, eventually results in constrictive pericarditis and liver fibrosis caused by chronic passive congestion. Cardiac lesions reduce arterial blood pressure from a norm of about 150 mmHg to about 40 mmHg just before death.

Several distinct clinical forms of colisepticemia can be distinguished depending on how the organism gains access to the circulation and the age and type of bird.

Respiratory-Origin Colisepticemia. Respiratory-origin colisepticemia affects both chickens and turkeys and is the most common type of colisepticemia. *E. coli* gains access to the circulation following damage to the respiratory mucosa by infectious or noninfectious agents (148, 167). IBV and Newcastle disease virus (NDV), including vaccine strains, mycoplasmas, and ammonia, are the most common predisposing agents. Avian metapneumovirus infection increases susceptibility of turkeys to colisepticemia (4, 508). Severity of the resulting disease, which is commonly referred to as airsacculitis, CRD, or multicausal respiratory disease (198), is directly related to the number of agents that are involved. A diversity of *E. coli* serotypes can be identified in a disease outbreak. Those in the tissues are usually different from those in the intestinal tract of the same bird but can be found in the intestinal tracts of other birds and the environment.

Susceptibility is increased when IBV, IBD or NDV infection occurs. Five days after administration of a vaccine strain of NDV, clearance of aerosol-administered *E. coli* is reduced. Microscopically, 3–8 layers of immature, nonciliated cells replace the pseudostratified, columnar epithelium of the trachea (129). Mixed IBV-*E. coli* infections are more severe than those caused by either agent alone (365). Antibodies against *E. coli* produced by chickens infected with IBD virus and/or IBV had a significant decrease in opsonizing ability compared with antibodies produced by normal chickens. Reduced opsonization of the organism resulted in decreased macrophage function, which may help explain the frequent infection with *E. coli* that follows IBV infection (372).

Mycoplasmal infection increases susceptibility to *E. coli* about 12–16 days postinoculation, and susceptibility

persists for at least 30 days. Coinfection with IBV or NDV in addition to mycoplasma further decreases resistance to *E. coli* and the period of increased susceptibility begins earlier and persists longer.

Inhaled coliform-contaminated dust has been implicated as one of the most important sources for infecting air sacs of susceptible birds. Exposure to chicken-house dust and ammonia results in deciliation of the upper respiratory tract of birds (361) permitting inhaled *E. coli* to colonize and cause respiratory infection.

Lesions are prominent in respiratory tissues (trachea, lungs, air sacs), pericardial sac, and peritoneal cavities and are typical of the subacute polyserositis stage of colibacillosis (65, 421) (Figures 18.1C, 18.1D, 18.1F). Infected air sacs are thickened and often have caseous exudate on the respiratory surface. Microscopically, the earliest changes consist of edema and heterophil infiltration. Mononuclear phagocytes are frequently seen 12 hours after inoculation. Later, macrophages become common, with giant cells along margins of necrotic areas. There is fibroblast proliferation and accumulation of vast numbers of necrotic heterophils in caseous exudate. Bacterial colonies are often present in caseous exudate and contain numerous organisms. *E. coli* colonies have a typical appearance in histologic sections. They are usually circular with concentrated bacilli forming a distinct smooth perimeter with fewer bacilli and spaces centrally. They stain negative with tissue Gram stain. Lesions of predisposing respiratory disease are usually present in the trachea and lungs and consist of lymphoid follicles, epithelial hyperplasia, and epithelium-lined air passages that may contain heterophils. Pneumonia and pleuropneumonia are more common in turkeys whereas chickens usually have pleuritis or pleuropneumonia with less lung involvement. Extension of the disease process into the oviduct from the left abdominal air sac may occur and cause salpingitis in juvenile birds (Figure 18.1H).

Inoculating pathogenic *E. coli* or bacteria-free culture filtrates into the air sac readily reproduces lesions of uncomplicated coliform infection (21, 100). Airsacculitis occurs within 1.5 hours. Bacteremia and pericarditis develop within 6 hours. In birds that survive, lesions are well-developed by 48 hours postinoculation. Most mortality occurs during the first 5 days. Recovery is usually rapid if birds survive the initial infection, although a few with persistent anorexia become emaciated and die. Ask et al. (21) have developed a defined method for determining susceptibility to colibacillosis that is reproducible and has been used to determine the relative innate (genetic) susceptibility of various broiler chicken genotypes to the disease (20). Another study observed that preceding infection with IBV vaccine does not impair the clearance of *E. coli* in the respiratory tract of broilers, but rather induces an exaggerated inflammatory response only in the airsacs (111).

Enteric-Origin Colisepticemia. Enteric-origin colisepticemia is most common in turkeys. *E. coli* gain access to the circulation and tissues following damage to the intestinal mucosa by infectious agents. The most common predisposing agent is hemorrhagic enteritis virus (416). Usually only 1 or 2 types of *E. coli* are involved in the disease outbreak, and those in the tissues and intestinal tract of each bird tend to be the same.

Lesions are typical of the acute septicemic stage of colibacillosis (65). Affected birds are in good physical condition and often have full crops containing feed and water. The most characteristic lesions are congestion or green discoloration of the liver, marked enlargement and congestion of the spleen, and congested muscles (Figure 18.2C). Microscopically the spleen is congested with proteinaceous fluid in sinuses and has multifocal necrosis, often containing intralesional bacteria. Fibrin thrombi are present in liver sinusoids and occasionally renal glomeruli. In some cases, multiple, pale foci in the liver are seen. Microscopically these are areas of acute necrosis initially, but with time they evolve into granulomatous hepatitis in survivors (Figure 18.2D). After a few days birds eventually develop lesions similar to those of respiratory-origin colisepticemia.

Hemorrhagic Septicemia. This form of colibacillosis occurs in turkeys and is characterized by generalized circulatory disturbances, discoloration of subserosal fat, bloody fluid on serosal surfaces, pulmonary edema and hemorrhage, and enlargement of the liver, spleen, and kidneys. Generalized necrosis of the liver and multifocal necrosis of the spleen are seen on cut surfaces. Pure cultures of *E. coli* are obtained from the liver, spleen, and pericardium. Characterization of isolates from 7 affected flocks showed they belonged to multiple serogroups (O1, O2, O18, O78, and O111). For each flock, isolates were clonal based on serotype, plasmid profiling, ribotyping, and MLST. Virulence factors were similar among all isolates in spite of differences in serotype and phenotype, and included F11 pili, *iucD*, *iss*, *vat*, *tsh*, and colicin V (392).

Neonatal Colisepticemia. Chicks are affected within the first 24–48 hours after hatching. Mortality remains elevated for 2–3 weeks and usually totals 10%–20%. Up to 5% of the flock may be stunted and require culling. Unaffected birds grow normally and the disease does not appear to spread. Initial lesions consist of congested lungs, edematous serous membranes, and splenomegaly. The proventriculus and lungs develop a dark color that can approach black as the interval between death and necropsy increases. Microscopically, bacteria are numerous in affected tissues and easily identified. After a few days the typical pattern of acute, fibrinoheterophilic polyserositis involving the pericardial sac, pleura, air

sacs, and peritoneum becomes evident. Lesions are often extensive and severe in birds that survive into the second week. Occasionally birds with arthritis or osteomyelitis may be found late in the disease. Most affected birds have yolk sac abscesses suggesting the navel is the portal of entry. Alternatively, *in ovo* infection may be responsible (344).

Layer Colisepticemia. Colisepticemia is usually a disease of young birds, but occasional outbreaks of acute *E. coli* infection resembling fowl typhoid or fowl cholera occurs in mature chickens and turkeys (32, 101, 512, 541). Acute colibacillosis in layers is being seen with increasing frequency (512). The majority of outbreaks are associated with onset of egg production, but less frequently they occur at an older age, or may continue as the flock ages and potentially spread to older flocks on the same farm. The disease may reoccur in the same flock or subsequent flocks placed on farms or in houses where affected flocks had been previously (512). Death usually occurred suddenly without premonitory signs, although depression and/or dirty vents were observed in some affected hens in approximately half of the flocks. Weekly mortality was significantly higher in affected flocks than age-matched control flocks (0.26%–1.71% vs. 0.07%–0.30%). Cumulative mortality ranged up to 10% and mortality remained elevated for 3–10 weeks. Polyserositis (perihepatitis, pericarditis) and peritonitis associated with free yolk in the peritoneal cavity were present in most birds at necropsy. Oophoritis and salpingitis occurred less frequently.

Isolates of *E. coli* from outbreaks in Italy were lactose-negative, nonmotile, and belonged to serogroup O111. Intramuscular inoculation of the O111 APEC reproduced the disease whereas it developed in only a few birds following oro-nasal administration (541). In contrast, the majority of isolates from outbreaks in Belgium were serogroup O78. Outbreak isolates were more likely to have P fimbriae (F11), especially if they were serogroup O78 and recovered from the heart. Serogroup O78 isolates also had the lowest percentage of motile strains (512). A number of virulence factors were significantly more frequent in outbreak isolates compared with control isolates. However, when cecal isolates and extraintestinal isolates within either the outbreak or control groups were compared, they were not significantly different. Collectively no virulence factors or combination of factors were found only in outbreak isolates (514).

The pathogenesis of the disease is unknown, but stress associated with onset of egg production is believed to be an important contributing factor (541). Ascending infections via the oviduct have been suggested as a means by which *E. coli* gain access to systemic tissues, but in a recent study, higher colonization rates of the trachea, but not the oviduct, in affected flocks suggests layer colisepticemia may be aerogenous (512). Lack of recognized stressors or indications of diseases known to predispose chickens to colisepticemia suggest layer colisepticemia results from a primary *E. coli* infection (512).

Risk factors for developing layer colisepticemia include close proximity to other poultry farms and higher stocking density (513). Control has been through chlorination of water or treatment of the flock with appropriate antibiotics.

Coliform Septicemia of Ducks. Coliform septicemia of ducks is characterized by moist, granular to curd-like exudate of variable thickness causing pericarditis, perihepatitis, and airsacculitis. A characteristic odor is often noted at necropsy. Liver is frequently swollen, dark, and bile stained, and spleen is swollen and dark. *E. coli* (usually O78) can be recovered from any internal organ (282, 336). *Riemerella anatipestifer* causes similar lesions, but it can be identified by appropriate cultural procedures.

Coliform septicemia occurs throughout the growing season but is most frequent in late fall and winter. All ages of ducklings are susceptible. Distribution of losses suggests individual farms, rather than hatcheries, are the source of infection (282). Poor husbandry and marked contamination of pond water used by ducklings were contributing factors to an outbreak of colisepticemia in captive mallards (336).

Colisepticemia Sequelae. Death is the usual outcome of colisepticemia, but some birds may completely recover or recover with residual sequelae. If the bird does not control *E. coli*, it can localize in poorly protected ("immunologically privileged") sites including the brain, eyes, synovial tissues (joints, tendon sheaths, sternal bursa), and bones. In immature females, salpingitis can occur when there is involvement of nearby air sacs. IBV infection of the oviduct may also be an important predisposing factor in juvenile salpingitis. After *E. coli* is no longer present, constrictive pericarditis develops as exudate in the pericardial sac undergoes organization, and liver becomes fibrotic (Figure 18.1G). Ascites may develop because of residual pulmonary damage from combined *E. coli*–IBV infection (504). It also is possible for ascites to develop from the direct action of endotoxin on the pulmonary vascular system. Endotoxin causes vasoconstriction leading to pulmonary hypertension and the potential to develop ascites (pulmonary hypertension syndrome) (62).

Meningitis and Meningoencephalitis. *E. coli* localization in the brain, although uncommon, has been reported (255). Meninges are affected (meningitis) but in some birds there also is involvement of the brain (encephalitis) and ventricles (ventriculitis). Meningeal lesions are evident

at necropsy as areas of discoloration, often adjacent to major blood vessels. Fibrinoheterophilic to heterophilic exudate is seen microscopically early in the infection; the lesion becomes more granulomatous with time. Bacteria are usually numerous within lesions but may not form distinct colonies.

Panophthalmitis. As with the brain, involvement of the eye is uncommon. However, if it is infected the resulting panophthalmitis is severe (171, 364). Typically there is hypopyon and hyphema, and infection is unilateral (Figure 18.2F). The eye is swollen, cloudy to opaque, and may be hyperemic initially. Later the eye shrinks as it undergoes atrophy. Fibrinoheterophilic exudate and numerous bacterial colonies are present throughout the eye. Inflammation, especially adjacent to necrotic tissue, becomes granulomatous with time. Varying degrees of retinal detachment, retinal atrophy, and lysis of the lens also may be seen. The organism persists in the diseased eye for long periods of time.

Osteoarthritis and Synovitis. Localization of *E. coli* in bones and synovial tissues is a common sequel to colisepticemia (Figure 18.2E). The term osteoarthritis is used when a joint is inflamed and 1 or more bones making up that joint have osteomyelitis. Polyarthritis refers to involvement of more than 1 joint. Bacterial chondronecrosis with osteomyelitis (BCO) is another name that has been used (326).

Affected birds likely have insufficient resistance to clear bacteria completely. Hematogenous spread of *E. coli* following hemorrhagic enteritis virus infection of turkeys resulted in synovitis, osteomyelitis, and green liver discoloration (107). Intravenous inoculation to simulate hematogenous spread to bones and joints has been used to reproduce the lesions, but mortality from the initial septicemia is often high (37). A preferable method is to inoculate the air sac of birds with low numbers of *E. coli* after a pretreatment with dexamethasone to immunosuppress the birds (209).

Mild to severe lameness and poor growth are seen clinically and affected birds are more likely to be victims of persecution ("cannibalism"). Often multiple sites are involved. Bacteria colonize the vascular sprouts that invade the physis of a growing bone provoking an inflammatory response that results in osteomyelitis. Transphyseal blood vessels in birds serve as conduits for the process to spread into the joint and surrounding soft tissues. Compared with clinically normal turkeys, lame turkeys had the following: higher splenic and liver weights, lower body and bursal weights, decreased cellular immunity, normal to increased humoral immunity, decreased circulating lymphocytes, increased circulating total leukocytes, monocytes, and heterophils, normal to marginally depressed phagocyte function, increased serum protein, uric acid, and blood urea nitrogen (BUN), and decreased hemoglobin, iron, alkaline phosphatase, and gamma-glutamyl-transferase (38). Bones most often affected are the tibiotarsus, femur, thoracolumbar vertebra, and humerus (358). Proximal physes of long bones are more frequently affected than distal physes. Lesions typically form where endochondral ossification is occurring and extend proximally to involve the adjacent physeal cartilage. It is common to find both osteomyelitis and tibial dyschondroplasia together, but this is most likely because of their occurrence at the same location rather than a cause and effect relationship. Osteomyelitis is easily recognized on gross examination of bones opened to expose the physes, but small lesions that can only be seen microscopically also occur (326).

Hock, stifle, hip, and wing joints and articulations of the free thoracic vertebra are sites where arthritis is most likely to occur (358). Lesions in other joints are less common. Trauma to joints and growing bones may predispose to the development of lesions. Tenosynovitis frequently accompanies arthritis. Less commonly, spread of the inflammatory process from a joint into the periarticular tissues occurs. An infectious sternal bursitis is also common but must be distinguished from traumatic sternal bursitis in which fluid is seen but not exudate. When inflammatory lesions involve the shoulder joint or proximal humerus, extensive exudate can accumulate between the superficial and deep pectoral muscles (Figure 18.1E). Lesions that develop in joint spaces of the articulating free thoracic vertebra are characterized by spondylitis, which results in progressive paresis and paralysis (136) (Figure 18.6). Lesions in the distal articulation of the free thoracic vertebra occur more commonly than lesions in its proximal articulation or both articulations.

Turkey Osteomyelitis Complex. Turkeys with turkey osteomyelitis complex (TOC) have infectious, inflammatory lesions in bones, joints, or periarticular soft tissues and enlarged, green discolored livers that are used at processing to indicate the possible presence of intraosseous lesions (70, 107, 205, 209). Green liver discoloration is rarely identified in the field even in lame turkeys. Most often green discolored livers are detected at processing and result in downgrading and, depending on severity, partial or whole bird condemnation. It is likely green discoloration of the liver is more evident in processed birds because they have bled out in contrast to birds in flocks that either die or are euthanized.

In TOC, osteomyelitis and arthritis occur as described previously. Spondylitis of the articulating free thoracic vertebra can be associated with green liver discoloration. Not all birds with green discolored livers have TOC, but generally birds with TOC have discolored livers (70). Feed and water withdrawal do not cause green liver



Figure 18.6 Spondylitis involving articulating free thoracic vertebrae of 2 lame turkeys (fixed tissues). Pressure from the lesion on the spinal cord has caused demyelination of the ventral tracts. *Escherichia coli* is a common cause, but the lesion also can result from infection with other bacteria that can localize in bones and synovial tissues.

discoloration (211), but infection with *M. synoviae* can be associated with a high percentage of carcasses with green discolored livers in the absence of TOC. The Food Safety Inspection Service in the United States uses green discoloration of the liver at processing to identify carcasses that may have TOC so that any abnormal tissue can be removed from the food chain. Osteomyelitis lesions can also be detected by ultrasonography (357). Tom turkeys are more affected than hens and affected birds have decreased cell-mediated immunity (38, 209).

Escherichia coli are frequently isolated from the lesions (107), but other bacteria may also cause TOC lesions, especially *Staphylococcus aureus*, or *S. hyicus* (493) (see Staphylococcosis). Culturing bones and livers from affected and unaffected birds in 2 turkey flocks resulted in recovery of pleomorphic, Gram-variable bacteria consistent with L-forms (cell-wall-deficient forms). Positive cultures were obtained more frequently from affected birds and bones than from unaffected birds or livers. The significance of these organisms in the disease is unknown, but the high number of isolates suggests these bacterial forms may be more common in turkeys than has been generally realized (37).

Exposure of turkeys to low levels of *E. coli* via air sac inoculation increases the occurrence of TOC (209). The current hypothesis is that TOC is related less to the virulence of the infecting bacteria than it is to an inappropriate response of a subpopulation of male turkeys to stress, which increases their susceptibility to opportunistic bacterial infections. The greater susceptibility of turkeys selected for rapid growth and higher body weights to experimental TOC further supports the concept of genetic susceptibility that is most likely mediated by how the birds respond to stress (203). The protective effect of vitamin D_3 (205) also suggests a possible genetic basis for TOC susceptibility related to vitamin D receptors and their function (209). However, when vitamin D metabolites were administered, TOC was reduced as with vitamin D treatment, but there were toxic effects in dexamethasone-treated turkeys challenged with *E. coli* (208).

Coligranuloma (Hjarre's Disease). Coligranuloma of chickens and turkeys is characterized by multiple granulomas in liver, ceca, duodenum, and mesentery, but not spleen (Figure 18.7). The disease also has been described in quail (81). Coligranuloma is an uncommon form of systemic colibacillosis that usually occurs sporadically in individual birds but can cause mortality as high as 75% when a flock is affected. Serosal lesions resemble leukosis tumors. Early in the disease there is confluent coagulation necrosis involving as much as half the liver. Only scattered heterophils are seen, and at the edge of the necrotic areas there are a few giant cells. Subsequently, typical heterophilic granulomas are present in the affected tissues. Pyogranulomatous typhlitis and hepatitis, which may be related to coligranuloma, have been described in turkeys with cecal cores and ruptured ceca (349). Recent studies attempting to replicate the disease using *E. coli* have failed to fulfil Koch's postulates (277).

Pathogenesis

Escherichia coli enters host tissues following mucosal colonization or directly through breaks or openings in the skin. Mucosal colonization is dependent on adhesin factors that permit the bacterium to attach to receptors and subsequently reproduce. A variety of fimbrial and nonfimbrial adhesins are produced by *E. coli*, which facilitates their attachment to host cells (see Virulence Factors). There is good evidence that 2 fimbriae (Type 1 [F] and P fimbriae) are important in the initial stages of infection. Type 1 fimbriae are expressed by *E. coli* that attach to upper tracheal epithelium (422), oviductal epithelium (343), and digestive tract mucosa (121). P fimbriae are expressed in deeper tissues (422). Type 1 fimbriae bind to mucus in the digestive tract but not to goblet cells producing the mucus. In contrast, AC/I



Figure 18.7 Coligranuloma in a market-age turkey. Numerous nodular lesions are located in gastrointestinal tissues including liver, but they do not involve the spleen. A mucoid *Escherichia coli* was isolated.

fimbriae bind poorly to mucus but attach to goblet cells (113). Flagella aid in penetrating the mucous layer in order to reach the cell surface, and curli, another adhesin factor, aids in attachment to the cell surface (272).

Virulent strains are capable of traversing the mucosa, especially if an injurious agent has compromised it, and surviving within the internal milieu of the body. Exactly how *E. coli* crosses mucosal barriers is poorly understood. Bacteria may penetrate between damaged cells. Air sac epithelial cells round up and become vacuolated following exposure to virulent strains, which causes them to separate from each other providing bacteria access to systemic tissues (100, 421). The ability of APEC to bind with fibronectin and laminin, 2 components of basement membranes, would aid in penetration through the damaged mucosa into host tissues (426). Toxins in cell-free culture filtrates, most likely endotoxin, produce the same acute inflammatory response as the living organism (100).

Alternatively, the initial portal of entry into the host's tissues, an essential first step in colisepticemia, may be transcellular through nonphagocytic cells. Certain strains of APEC have the ability to invade fibroblasts, much like virulent *S. typhimurium* (318). *E. coli* have been identified within air sac epithelial cells by electron microscopy (421). An APEC strain was able to readily adhere to and invade tracheal epithelial cells in primary cell culture and tracheal explants (425). Factors involved in cell penetration remain to be identified.

Once *E. coli* becomes extramucosal, the environment it has entered is extremely hostile. Unless the organism is equipped with survival capabilities (e.g., "virulence" factors), it is rapidly destroyed by phagocytic cells such as heterophils, thrombocytes, and macrophages (181, 182, 201). Macrophages located primarily in the spleen and liver phagocytize bacteria that gain access to the circulation (19). Complement and antibodies to O antigens (endotoxin), outer-membrane proteins (siderophores), and fimbriae serve as opsonins to promote phagocytosis and destruction of the organism (17, 18). Endotoxin also decreases the bacteriocidal ability of pulmonary macrophages (123), which may aid in survival and dissemination. *In vitro* results suggest that the dying cells in the lung may be macrophages as well as heterophils (201).

Immediately after *E. coli* contacts host tissues, there is an acute inflammatory response. Acute phase proteins produced in the liver and cytokines IL-1, IL-6, and tumor necrosis factor increase rapidly following exposure to endotoxin or *E. coli*, which can serve as nonspecific indicators of early disease (59, 369, 533). Acute phase effects of endotoxemia include hypothermia followed by hyperthermia, hypotension, decreased circulating heterophils associated with increased apoptosis and sequestration in the lung, and increased inflammatory mediators, TL1A, IL-1β, and IL-6 (89). Increasing amounts of endotoxin in the circulation causes decreased feed consumption and efficiency, decreased body weight and breast meat yield, decreased tibial bone size, weight, calcium content, and breaking strength, and increased mortality, liver weight, plasma ionized calcium, and antibody responses (337). Vascular permeability increases leading to the accumulation of fluid and protein in the tissues. Serous membranes become wet and edematous and liquid begins to accumulate in body cavities. Chemotactic factors attract heterophils, which marginate in postcapillary venules and emigrate into surrounding tissues (309). Between

6 and 12 hours, soft, gelatinous exudate becomes grossly visible. Heterophils can kill *E. coli* extracellularly by substances such as ß-defensins released as they degranulate and die (181, 489). After 12 hours there is a progressive shift in inflammatory cells from heterophils to macrophages and lymphocytes.

Exudate continues to accumulate and eventually undergoes caseation to form a firm, dry, yellow, irregular, cheese-like mass. Microscopically, caseous exudate consists of heterophilic granulomatous exudate containing variable numbers of embedded bacterial colonies. A palisade of multinucleated giant cells and macrophages surrounds the exudate (65). Depending on the size of the mass of exudate, an extended period of time will be required for the exudate to be slowly eroded away by the action of the surrounding phagocytic cells. Viable bacteria persist as microcolonies within the exudate. Epithelial tissue may be restored if damage has not been too severe, but usually there is some degree of fibrosis, which may be complete (scarring) if tissue destruction has been extensive. Exudate containing fibrin undergoes organization and is eventually converted to scar tissue.

Gross lesions are inversely related to virulence. Highly virulent strains cause mortality so quickly that gross lesions have little time to develop, whereas birds infected with less virulent strains survive longer and develop more extensive lesions.

Infections with APEC serve as a model for studying the molecular aspects of host–pathogen interactions of ExPEC infections. Both pulmonary and systemic infection models have been established using a serogroup O2 strain (14).

Diagnosis

Isolation and Identification of Causative Agent

Diagnosis is based on isolation and identification of *E. coli* from lesions typical of colibacillosis. Care must be taken to avoid fecal contamination of samples. Isolation of the organism from visceral organs of birds undergoing decomposition must be interpreted cautiously as *E. coli* rapidly spreads from the intestinal tract into surrounding tissues of dead birds. Bone marrow cultures are easy to obtain and are generally free of contaminating bacteria. The brain is another site to culture, but it is less easily accessed compared with bone marrow.

Material should be streaked on EMB, MacConkey, or tergitol-7 agar, as well as noninhibitory media. A presumptive diagnosis of *E. coli* infection can be made if most of the colonies are characteristically dark with a metallic sheen on EMB agar, bright pink, with a precipitate surrounding colonies on MacConkey agar, or yellow on tergitol-7 agar. Strains of *E. coli* can be slow or nonlactose fermenters and appear as nonlactose-fermenting colonies. Definitive identification of *E. coli* is based on the organism's characteristics (see Etiology). A flow chart for the isolation and identification of *E. coli* has been published (281). A number of manual and automated systems are available for identification of bacteria, including *E. coli*.

Antigenic identification, determination of virulence factors, or fingerprinting of the isolate might be helpful, particularly when done as part of an epidemiologic investigation. The correlation between virulence and complement resistance suggests this may be a good method for screening isolates for possible disease association. A relatively simple rapid turbidimetric assay has been described (408). Also, analysis of the results of an extensive virulence genotyping assay identified 5 genes that typified a large majority of APEC (238). From these data, a pentaplex PCR "quick test" was designed to distinguish APEC from commensal *E. coli* isolated from the feces of healthy poultry without having to resort to *in vivo* virulence models (238). Also, a recent and extensive study identified 4 patterns of virulence genes for identifying APEC (460) that could be exploited diagnostically. Despite the promise of these *in vitro* predictors of APEC identity, it may be unrealistic to expect them to identify all *E. coli* capable of causing avian colibacillosis. Most APEC are well equipped for a pathogenic lifestyle and are easily identified by their constellation of virulence genes; however, a few strains are not. Such "minority" isolates are likely opportunistic, causing disease in immunocompromised hosts and lacking the traits that gene-based assays target (238).

Serology has not been used as a diagnostic method. However, survival after challenge correlated better with antibody titers detected by an enzyme-linked immunosorbent assay (ELISA) than by the standard indirect hemagglutination procedure (284). Procedures to detect acute phase proteins (59, 337, 369, 533) or shifts in the heterophil/lymphocyte ratio (168) can serve as nonspecific markers of the inflammation and stress that accompany colibacillosis.

Differential Diagnosis

Acute septicemic diseases may result from pasteurellae (*Pasteurella*, *Ornithobacterium*, *Riemerella*), salmonellae, streptococci, and other organisms. *Chlamydophila*, pasteurellae, or streptococci (*Streptococcus*, *Enterococcus*) can cause pericarditis or peritonitis, and other bacteria, mycoplasmas, and *Chlamydophila* can cause airsacculitis. Many organisms including viruses, mycoplasmas, and other bacteria can cause synovial lesions similar to those resulting from *E. coli* infection. A variety of organisms including *Aerobacter* spp., *Klebsiella* spp., *Proteus* spp.,

salmonellae, *Bacillus* spp., staphylococci, enterococci, or clostridia are frequently isolated (often as mixed cultures) from yolk sacs of embryos and chicks (183). Liver granulomas have many causes, including anaerobic bacteria belonging to the genera Eubacterium and Bacteroides.

Intervention Strategies

Management Procedures

Reducing the numbers of *E. coli* through water, feed, environmental sanitation, and good air quality, and protecting the flock from factors, especially viral infections that decrease host resistance, will sharply reduce the likelihood of colibacillosis.

Fecal contamination of hatching eggs is the most important way that *E. coli* are transmitted between flocks. Collecting eggs frequently, keeping nest material clean, not using floor eggs, discarding cracked eggs or those with obvious fecal contamination, and fumigating or disinfecting eggs within 2 hours after they are laid can reduce transmission. *E. coli* on the shell surface can be reduced or eliminated with sanitizers (463).

Application of sanitizers by electrostatic spraying improves efficacy (448). Ultraviolet irradiation can reduce or eliminate *E. coli* and other bacteria on the surface of hatching eggs without altering conductance or hatchability (76). If infected eggs are broken during incubation or hatching, the contents are a serious source of infection to other chicks, especially when personnel and egg-handling equipment are contaminated. Eggs are particularly susceptible just before hatching. Venting incubators and hatchers to the outside and having as few breeder flocks as possible represented in each unit will help reduce cross-contamination and losses. Contaminated chicks survive better if kept warm and are not deprived of feed and water for an extended period of time (286).

Survival tends to be better in birds fed high protein diets, increased selenium (280), and increased vitamins A and E (207, 494). However, high levels of vitamin E can be detrimental to resistance to coliform cellulitis, colibacillosis, and antibody production (133, 287). Response to vitamin E is likely interrelated with the genotype of the bird (537). Feeding can have an impact on severity of colibacillosis. Chickens fed on alternate days were more resistant to *E. coli* challenge than full-fed chickens (49).

There are no known methods for reducing the level of pathogenic *E. coli* in the intestinal tract and feces, although the following considerations should not be overlooked: (1) pelleted feed has fewer *E. coli* than mash, (2) rodent droppings are a source of pathogenic *E. coli*, and (3) contaminated water can contain high numbers of the organism. Hot pelleting processes destroy *E. coli* (116), but care must be taken not to recontaminate finished feed. Adding 5–10% egg yolk powder to feed effectively reduced or eliminated *E. coli*, and other bacterial foodborne-illness pathogens in layers (254). Commercial broiler chickens and breeders were less productive when their water contained *E. coli* and nitrates (166). Chlorination of drinking water and use of closed (nipple) watering systems have decreased the occurrence of colibacillosis and condemnations for airsacculitis (50, 101, 404). Pathogenic strains of *E. coli* can be competitively excluded from intestines of chicks by seeding them with native microbiota from resistant chickens (524), commercial competitive exclusion products (199), or *Bacillus subtilis* spores (267, 496). A similar effect was achieved following *in ovo* inoculation of *Lactobacillus reuteri* (114).

Controlling or preventing immunosuppressive agents such as Marek disease virus (MDV), chicken infectious anemia virus (CIAV) and IBD virus as well as ensuring manageable levels of mycotoxins in the feed or feed ingredients (473) can contribute positively to reducing the impact of *E. coli* infection in broilers and growing turkeys. Colibacillosis can be indirectly controlled by controlling other respiratory agents such as NDV, IBV, avian metapneumovirus (aMPV), low pathogenic avian influenza virus (LPAIV), *M. gallisepticum* or *M. synoviae*.

Birds acquire short-term nonspecific resistance to colibacillosis following moderate stress and socialization with people (317). However, higher stresses will favor the development of colibacillosis, such as when breeders are pushed into peak production too quickly. Control of vent pecking in egg layers, particularly free-range layers, will also reduce the incidence of colibacillosis.

Maintaining good air and litter quality is fundamental to reducing risk of a flock developing colibacillosis (87). Proper ventilation minimizes respiratory tract damage from ammonia and reduces the levels of bacterial and aerial endotoxin exposure. Ammonia, even at levels below those that can be detected by human smell, impairs mucociliary clearance of inhaled particulates (361). Degree of damage to the respiratory mucosa correlates with the level of ammonia exposure (360). Dust also increases the risk of colibacillosis (185). Bacteria preferentially adhere to dust particles because of electrostatic charges. The combination of dust and ammonia results in birds inhaling high numbers of bacteria and being unable to clear them from their respiratory tract.

Wet litter provides an excellent environment for *E. coli* to persist and reach high numbers. Higher numbers of *E. coli* and *Salmonella* were found in litter that had water activity greater than 0.9% and moisture content above 35%. Air velocities across the surface of the litter of at least 100 feet/minute produce drier litter and decrease the number of *E. coli* (433). The incidence and severity of footpad dermatitis (often termed "paw score") at

processing can be used as an indicator of litter condition and air quality that were present during the production cycle (189). Nutrition may also affect footpad dermatitis by influencing moisture levels in litter (492). Maintenance of waterers is essential in eliminating wet spots in the house. Daily raking of soiled litter that builds up around feeders and waterers, removing heavily soiled litter, tilling, and replacing or covering wet litter with fresh dry litter are useful procedures for maintaining good litter quality. However, once a flock is diagnosed with colibacillosis, it is advisable to stop tilling in order to minimize environmental spread. Monitoring of fever/huddling is needed to adjust ambient temperature, if necessary. Treatment should be based on antibiotic sensitivity because of variable sensitivity profiles, even on the same farm. In layers and breeders, first consider using acids and other nonantibiotics. In mature birds with a well-established microbiota, supportive treatment to avoid bird suffering should be considered for a short period while considerable efforts are made to diagnose the primary causes of a secondary *E. coli* infection. The choice of antibiotics should take into account the possibility of resistance genes being transferred to the progeny and thus the multiplicative effect on antibiotic resistance.

Vaccination

Before considering any *E. coli* vaccination, it is important to ensure that a timely and appropriate vaccination program is in place for likely primary agents, such as NDV, IBD, or hemorrhagic enteritis virus. A variety of *E. coli* vaccines and vaccination methods have been developed including passive and active immunization, use of inactivated and live products, recombinant and subunit vaccines, and immunization against specific virulence factors. No vaccination procedure has proved to be highly efficacious for multiple serotypes in the field at the present time. This is why broilers are rarely vaccinated. It is best to prevent the primary issue. However, in breeder hens, a killed autogenous *E. coli* vaccine is effective. Live vaccines against *E. coli* are popular amongst egg layer producers and have contributed to reducing *E. coli* peritonitis in layers.

Types of Vaccines

Inactivated Vaccines. Effective inactivated vaccines against various serotypes including O2:K1 and O78:K80 have been produced (18, 95, 96). They provide protection against homologous serogroups, but no cross-protection against heterologous serogroups. An inactivated O78 vaccine protected ducks (456). Both homologous and heterologous protection were provided by a vaccine prepared by ultrasonic inactivation of the organism followed by irradiation (328). A vaccine containing bacterial membrane vesicles was effective in protecting turkey poults against challenge with pathogenic *E. coli* by stimulating antibody production, bacterial-lysis activity of complement, T cell proliferation, and cytotoxic T cell activity (58). Use of a liposomal inactivated vaccine given by either eye drop or coarse spray stimulated humoral and mucosal antibodies. The number of bacteria in the blood was decreased and clinical signs were less severe in vaccinated birds following APEC challenge (535). El Jakee et al. (117) reported that an outer membrane protein vaccine offered a superior protection rate compared with other types of autogenous vaccines. Inactivated *E. coli* autogenous vaccines (aqueous and water-in-oil types) against *E. coli* peritonitis syndrome (EPS) in layers administered at 14 and 18 weeks induced an almost complete protection against homologous challenge. In laying hens, EPS is likely caused by non-opportunistic virulent *E. coli* strains, making vaccination a good control option. The protective effect observed may be explained by the fact that vaccine-induced systemic antibodies likely impede the access of *E. coli* to the bloodstream and/or favor its rapid clearance (278).

Live Vaccines. A live vaccine prepared from a naturally occurring, nonpathogenic, piliated *E. coli* strain (BT-7) was efficacious when used in chickens older than 14 days of age. Protection against both homologous and heterologous strains was demonstrated (137). *E. coli* J5, a mutant strain that has incomplete endotoxin in the cell wall exposing Gram-negative core antigen, was both safe and effective for protecting chicks (3). Antibody titers to Gram-negative core antigens that develop in commercial chickens peak during the pullet period (446). In Japan, the productivity of field laying hens improved when Δcrp *E. coli* live vaccine was used (507).

Recombinant and Mutant Vaccines. A *carAB* mutation of a virulent O2 serotype caused defective utilization of arginine and pyrimidines, increasing the requirements by the mutant. Because low levels of these substances are generally available *in vivo*, the organism was unable to sustain itself, which resulted in a self-limiting infection. The mutant strain was found to be stable, immunogenic, and attenuated. Turkeys orally vaccinated with the mutant were protected against colibacillosis in a hemorrhagic enteritis virus-parent wild-type strain challenge model (266). Mutant O2 and O78 APEC with deletions of the genes *cya* or *crp*, which are involved in energy production, were used as a spray vaccine to immunize broiler chickens. The mutants were safe and immunogenic but provided only limited protection against airsacculitis following challenge (405). Similarly, strain O78 mutants with deletions of *galE*, *purA*, and *aroA* genes were found to be safe and immunogenic, but provided only moderate protection against homologous challenge with no protection against heterologous challenge (252).

In contrast to high mortality caused by the parent and wild-type strains, attenuated streptomycin-dependent (str-dependent) mutants derived from a virulent APEC did not cause mortality in challenged birds. No protection against cellulitis or systemic lesions resulted when birds were vaccinated with high numbers of the mutant strains by aerosol and oral routes. However, systemic lesions were significantly reduced when birds were given three vaccinations on days 1 (aerosol), 14 (oral), and 28 (oral) (11).

A recombinant vaccine using *S. typhimurium* was constructed to produce homologous group B determinants and *E. coli* O78 antigen. Vaccinated birds seroconverted and were protected against subsequent challenge with a pathogenic *E. coli* O78 strain (441). A similar vaccine constructed to express *E. coli* type 1 fimbrial antigen in addition to O78 LPS provided protection against homologous challenge. The O78 LPS was responsible for most of the efficacy of the vaccine although presence of the fimbrial antigen did decrease the severity of air sac lesions. The fimbrial antigen did not provide cross-protection following challenge with O1 and O2 APEC serogroups (442).

A modified-live vaccine containing an *aroA* gene deleted *E. coli*, type O78, is licensed in the United States for use in chickens and turkeys by administration via drinking water or coarse spray (Poulvac *E. coli*, Pfizer). The vaccine significantly reduces mortality and lesions associated with *E. coli*, and provides cross-protection for airsacculitis caused by *E. coli* serotypes O1, O2, and O18. A small study performed in Thailand did not find differences in mortality or morbidity between vaccinated and unvaccinated chickens, but lesions were significantly reduced in the vaccinated birds (431). A recombinant multiantigen vaccine has recently been tested *in vitro* and under experimental conditions, and appears to offer broad protection potential against avian pathogenic *E. coli* (510).

Molecular Vaccines. Immunization of chickens with Iss, a surface protein common to APEC, but not commensal *E. coli*, is important in complement resistance, suggesting the potential to achieve cross-protection among different serotypes (302). Chickens immunized with Iss produced humoral and mucosal antibody responses and provided good protection against colibacillosis following challenge with O1, O2, and O78 APEC serotypes. Challenged birds had significantly lower lesion scores compared with unvaccinated, challenged controls (302).

Multivalent vaccines made from pili containing low levels (180 μg) of protein per dose reduced the severity of challenge infection (179). Absorbed sera indicate pili of serotypes O1, O2, and O78 are antigenically different (490).

Fimbrial vaccines containing FimH, the adhesin of F1A (type 1) fimbriae, or PapGII, the highly conserved portion of P fimbrial adhesion, were immunogenic but did not provide protection against APEC challenge (515, 517). The results of PapGII immunization differ from the finding that passive immunization with PapG yolk-derived antibodies was protective (see Passive Immunization).

Passive Immunization. Passive immunization results in increased resistance to aerosol challenge and clearance of bacteria from blood (359). Use of inactivated vaccines in breeders provided passive protection against homologous challenge in progeny, which was complete for 2 weeks and partial for several additional weeks posthatch (192).

Antiserum prepared in rabbits against iron-regulated outer membrane proteins of *E. coli* protected turkeys against mortality following challenge. Frequency of bacteremia at 96 hours after challenge, recovery of *E. coli* from air sacs, and severity of gross lesions were significantly reduced in immunized birds compared with control birds given normal rabbit serum or saline solution (52).

Antibodies extracted from the yolk of eggs laid by immunized hens provided homologous protection against an O78 APEC. Partial protection against heterologous challenge with O1 and O2 serotypes was provided by immunizing hens with P pilus adhesin (PapG) or the aerobactin outer membrane receptor IutA. Immunizing with PapG provided the best overall protection. Breeder hens immunized by this method may provide immunity to their progeny (253).

Immunopotentiation. A problem with recombinant vaccines is low immunogenicity, which could potentially be solved by using effective immunopotentiators. Inoculation of chickens by intramuscular or subcutaneous routes and chicks by intramuscular or *in ovo* routes with cytosine-phosphodiester-guanine (CpG) oligodeoxynucleotides improved livability and reduced cellulitis lesion size following challenge with APEC (155, 157). Use of CpG as an adjuvant in an inactivated *E. coli* vaccine improved the efficacy of the vaccine (156). CpG motifs are present in high numbers in bacterial DNA and enhance innate immune responses (23, 155).

Modification of *E. coli* heat-labile enterotoxin (LT) resulted in a nontoxic protein (nLT) that stimulated antibody production in chickens following either oral or parenteral co-administration of an antigen to chickens (518).

Mixtures of antimicrobial/host defense peptides and gamma-amino-butyric acid promoted production of proinflammatory cytokines following subcutaneous injection. Chickens challenged with a virulent O2 APEC 24 hours after receiving the mixture had lower mortality and bacteremia, but there was no effect on cellulitis lesions. Intramuscular administration was less effective (6).

Treatment

Antimicrobial Drugs

Antimicrobial drugs have been used extensively for reducing losses from colibacillosis since their first introduction for treatment of poultry in the mid 1950s. Occurring in parallel with use of an antimicrobial has been a progressive development of resistance, which was initially identified following introduction of tetracyclines (475). Antimicrobial resistance is determined genetically and usually transferable within a species or between different types of bacteria via mobile genetic elements – plasmids, integrons, and transposons (34, 279, 536). The greatest reservoir for transferable antimicrobial resistance factors in the poultry flock environment is not *E. coli*, or even Gram-negative bacteria, but Gram-positive bacteria that comprise over 85% of the bacteria in poultry litter (371). The intestinal tract of the chicken is a suitable environment for transfer of genes from tetracycline-resistant to susceptible *E. coli* strains. Adding tetracycline to the chick's drinking water accelerates the process. Resistance to other antimicrobials is co-transferred along with tetracycline resistance (188).

Growing concern over antibiotic resistance, especially multidrug resistance, and the potential of bacterial strains affecting people acquiring transmissible resistance factors from bacteria in animals (see Public Health Significance), has led to changes in the way antimicrobials are used to treat colibacillosis in poultry (467). Additionally, new antimicrobials are not being developed for use in poultry and the ones that have been used previously have lost much of their efficacy because of acquired resistance. Fluoroquinolones became available in the United States and elsewhere for treatment of colibacillosis in poultry, which generally proved to be highly efficacious (151). However, resistance to fluoroquinolones has developed in parallel with their use in both chickens and turkeys (164), societal concerns about resistance in pathogenic bacteria (150, 226, 528), development of cross-resistance among different quinolones, and importance of this class of antibiotics for treating people have led to their withdrawal for use in poultry in many countries, including the United States. For a review of antimicrobial resistance of avian *E. coli* see (526).

When selecting an antimicrobial to use for treatment, it is important to determine the susceptibility of the isolate involved in the disease outbreak so that ineffective drugs can be avoided. APEC frequently are resistant to tetracyclines, sulfonamides, ampicillin, and streptomycin (25, 27, 335, 516, 526, 536). Multidrug resistance is common (516), may be linked to transmissible plasmids (233, 237) and may occur in conjunction with virulence factors (230, 235). Numerous recent studies on antimicrobial resistance of *E. coli* isolates from chickens (10, 47, 202, 279, 335, 346, 377, 396, 509, 536, 542), turkeys (8, 74, 509), ducks (341, 522), eggs (356), and poultry feed and ingredients (315) have been performed in different geographic areas. All showed some level of resistance in APEC and commensal strains, but there were regional variations. Most *E. coli* isolates from eggs were susceptible to all antibiotics (356). Occasionally resistance is higher among commensal strains compared with APEC, e.g., ampicillin resistance in turkeys (8) but, in general, resistance tends to be greatest in APEC strains. A high percentage of *E. coli* isolates from turkeys are resistant to gentamicin, which has been attributed to the widespread use of day-old gentamicin injection (8). Gentamicin resistance among chicken-origin *E. coli* was associated with significantly greater virulence in an embryo lethality assay (346).

Even a highly effective drug may not result in improvement of the flock if too little is used or it is incapable of reaching the site of infection. Therefore, underdosing may promote development of resistance. Chicks given feeds with increasingly lower concentrations of ampicillin (1.7 and 5 g/ton) developed resistance that was directly correlated to the amount of antibiotic in the feed (5). Paradoxically, certain antimicrobials and anticoccidials commonly used at subtherapeutic levels in poultry for growth promotion and coccidiosis control inhibited transfer of a plasmid that is responsible for multiresistance in *E. coli*. The basis for the inhibition was attributed to the ion-binding properties of the drugs and interference with DNA uptake channels in the organism (316). Selective pressure from exposure to an antibiotic is not always essential for resistance to develop (63). Although resistance generally occurs following prior contact with an antimicrobial, it can occur naturally in the absence of previous exposure. Resistance to florfenicol and chloramphenicol, which had never been used in poultry in the United States, was found in *E. coli* isolates from chickens in the southeastern United States (256).

Water administration of apramycin proved effective in reducing the numbers of organisms in the digestive tract and preventing bacteremia in chickens (286). Neomycin reduced mortality in turkey poults exposed naturally to litter from flocks with colibacillosis (314). Neomycin reduced mortality in turkey poults exposed naturally to litter from flocks with colibacillosis (314).

Anticoccidials also have antimicrobial activity that may be beneficial in the prevention and treatment of coliforms. Monensin reduced colonization of chickens with *E. coli* O157:H7 to undetectable levels 14 days postexposure compared with nonmedicated controls and chickens receiving other coccidiostats (479). In a recent study TAMUS 2032, a cationic amphipathic peptide antibiotic produced by *Brevibacillus texasporus*, improved performance and reduced mortality when

added to the feed of commercial broilers with or without monensin following natural environmental challenge. Improved livability and productivity also resulted from adding monensin with or without bacitracin. Bacitracin alone provided no protection against colibacillosis (224).

Antimicrobial resistance associated with colistin has recently emerged as a significant concern worldwide, threatening the use of one of the most important last resort antimicrobials for treating human disease. Of most concern is that colistin has been used as a growth promoter in certain regions. A broad screening of over 1200 APEC isolates from the United States and internationally identified the presence of the colistin associated *mcr-1* gene in 12 isolates recovered from diseased production birds in China and Egypt (26). Another study from South Africa identified *mcr-1* in APEC (412) and a second from China identified 2 *E. coli* isolates harboring *mcr-1* resistance in a Muscovy duck (538).

Other Treatments

The declining use of antibiotics for prevention and treatment of colibacillosis has stimulated interest in alternative methods including prebiotics, probiotics, enzymes, digestive acidifiers, vitamins, immune enhancers, anti-inflammatory drugs, and other antimicrobial products. Although prebiotics and probiotics are widely available for use in poultry, few studies on their effect on colibacillosis have been published. Colonization with *E. coli* begins immediately after hatching making early administration of probiotics essential (114). Administration of a bacteriocin-producing strain of *Lactobacillus plantarum* F1 or the purified bacteriocin provided chicks protection against challenge with an O2 APEC. Fermentation of coarsely ground wheat with a mixture of *L. plantarum* and *Pediococcus pentosaceus* completely eliminated *E. coli* when the pH of the mixture was lower than 4.0 for at least 24 hours (348). *L. johnsonii* significantly reduced colonization of the small intestine with *E. coli*, but had no effect on colonization of the ceca or large intestine (271).

In addition to lactobacilli, other microbes can inhibit colonization with *E. coli*. Specific strains of *Bacillus* spp. inhibit *E. coli* colonization in the digestive tract and have potential use as probiotics (30, 267). Formation of highly resistant spores simplifies administration of these bacteria to commercial flocks through feed. Extracts prepared from Bifidobacterium enhanced resistance to colibacillosis following oral administration. Cell-mediated immunity was enhanced in the treated chickens (259). Inoculating young birds with nonspecific competitive exclusion products derived from healthy adults reduced intestinal colonization by APEC (199).

Essential oils often have a substantial inhibitory effect on *E. coli in vitro* (180, 409) and in the lower intestinal tract of chickens (220). A commercial oregano oil product has been used in organic poultry production, but there are no studies on its effect on colibacillosis. Isopathic (immune enhancing) and homeopathic treatments with multiple products were unsuccessful in modifying the response of 8-day-old broilers challenged intratracheally with an O78 APEC strain (519). A Chinese herbal mixture ("XQT") significantly lowered plasma inflammatory mediators and reduced mortality when it was given orally prior to challenge (190).

Bacteriophage administration provides another possible alternative to antibiotic medication for controlling colibacillosis (33, 53). Two bacteriophages isolated from municipal wastewater that lysed an O2 APEC were effective in reducing mortality from experimental colibacillosis caused by the homologous APEC strain when high numbers of phage were mixed with the inoculum, given as a spray up to 3 days prior to challenge, or inoculated intramuscularly up to 48 hours postinfection (212, 213, 215, 216). Combination of bacteriophage treatment with enrofloxacin had a synergistic beneficial effect (214). Prior exposure to bacteriophage results in antibodies that interfere with subsequent exposure to bacteriophage and reduces their effectiveness (217). Whereas bacteriophage treatment has been shown to be efficacious, several challenges remain before a commercial product might be realized (212).

Several studies have shown vitamin E supplementation to have both prophylactic and therapeutic benefits for *E. coli* infections (207), but not all studies support this conclusion (see Management Procedures). Differences in results likely are because of differences in experimental designs, especially severity of challenge and the timing and manner in which vitamin E is administered. Use of aspirin or sodium salicylate reduced the impact of experimental colibacillosis in turkeys (207) and chickens (295) respectively. However, if used in high levels or in combination with other products that impair the inflammatory response, a reverse response can occur (207). Feeding a beta-glucan product obtained from yeast cell walls improved the response of chickens to *E. coli* challenge, but also depressed growth of unchallenged controls (210). A recently study has shown that administration of a combination of tetracycline and sertraline can have a synergic effect in isolates previously resistant to tetracycline (293).

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19

Pasteurellosis and Other Respiratory Bacterial Infections

Introduction

Martine Boulianne

Recent access to relatively cheap and rapid genome sequencing techniques is changing the face of microbiology. Determination of genetic relatedness among bacteria has clarified bacterial classification while introducing new taxonomy. As you will find out in the coming pages, sequencing of the complete genomes of several bacteria, and comparative genomic analyses of commensal and virulent strains, have facilitated the identification of a number of virulence-associated genes and are helping scientists to further understand pathogenesis.

The etiologic agents of the respiratory bacterial infections described in this chapter have seen their fair share of reclassification and name changes, which at times has made it difficult for the clinician to follow all the changes introduced by each edition of the microbiologists' bible: *Bergey's Manual of Systematic Bacteriology*. For example, many of the bacteria which used to be classified as members of the family Pasteurellaceae have been redesignated to new genera. Recent reclassifications included changing the name of *Pasteurella haemolytica* to *Gallibacterium anatis* biovar *haemolytica*, *Pasteurella gallinarum* to *Avibacterium gallinarum*, and *Haemophilus paragallinarum* to *Avibacterium paragallinarum*. The new taxonomy is used in this text.

Four distinct diseases are included in this chapter: fowl cholera caused by *Pasteurella multocida*, *Riemerella anatipestifer* infection, *Ornithobacterium rhinotracheale* infection, and bordetellosis caused by *Bordetella avium* or *Bordetella hinzii*. These diseases are grouped together because they are caused by small Gram-negative bacteria that are genotypically and phenotypically related and because they induce diseases in commercial poultry that may be clinically similar.

In diagnostic poultry medicine, the definitive diagnosis of these diseases depends upon the isolation and identification of the causative organism. This is essential in order to undertake the proper treatment and implement effective control measures. Several organisms, such as *Avibacterium gallinarum,* which are less important as disease agents, may be isolated and must be differentiated from the more important disease agents included in this chapter. A clinical diagnostic text will be helpful in this regard (1). There is no doubt, given the new tools now available, that whole genome sequencing will be not only used for epidemiological studies, but is likely to replace some traditional typing methods. Furthermore, our toolbox of control and preventive measures might be enhanced in a near future with the identification of new virulence genes and the development of new vaccines.

Fowl Cholera

Pat J. Blackall and Charles L. Hofacre

Summary

Agent, Infection, and Disease. Fowl cholera is a contagious disease affecting both domestic and wild birds and is caused by *Pasteurella multocida.* The organism can be classified into 16 somatic serovars by the Heddleston serotyping scheme. The disease occurs in all domestic poultry as well as a wide range of wild birds. In the acute form of the disease, clinical signs may only be present shortly before death. Mortalities range up to 20%, although even higher rates are reported. In the chronic form of the disease, clinical signs are typically related to localized infections with wattles, sinuses, leg or wing joints, foot pads, and sternal bursae often enlarged.

Diseases of Poultry, Fourteenth Edition. Editor-in-chief David E. Swayne.
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Diagnosis. The preferred diagnostic approach remains traditional culture, although a range of direct molecular detection assays, particularly PCR-based assays, are now available for laboratories with suitable resources. Culture allows antimicrobial sensitivity testing and serotyping to be performed. Serology, although not used as a diagnostic tool, has been used to monitor vaccination response.

Intervention. Both commercial and autogenous bacterins are widely used to reduce the economic losses associated with fowl cholera. In some areas, particularly North America, live vaccines are available. Sensitivity testing should be done to guide any antimicrobial use

Introduction

Definition and Synonyms

Fowl cholera (FC) (avian cholera, avian pasteurellosis, or avian hemorrhagic septicemia) is a contagious disease affecting domesticated and wild birds. It usually appears as a septicemic disease associated with high morbidity and mortality, but chronic or benign conditions often occur. This disease is historically important because of its role in the early development of bacteriology and because it was 1 of 4 diseases the Veterinary Division of the United States Department of Agriculture (USDA) was created to investigate.

Economic Significance

Fowl cholera is of major economic importance wherever poultry are raised. For intensively raised chickens, the disease has traditionally been regarded as a problem in broiler breeders. However, changes in production systems have resulted in the disease having been recognized as a problem in the free-range layers (113, 125) and organic broilers (114). The disease is also significant for backyard poultry (19) in the developed world as well as village chickens in the developing world (75). FC is of importance to both the turkey and the duck industries (18). In wild birds, FC has been associated with very large disease outbreaks, such as the death of over 80,000 wild birds in Chesapeake Bay, United States between February and April, 1994 (34).

Public Health Significance

Pasteurella multocida is a pathogen of humans, particularly in association with animal bites, and should be regarded as a zoonotic agent (9). Although there are no reports of a direct transmission of the organism from poultry to man, this possibility cannot be excluded (9).

History

A review of the history of epornitics among fowl that occurred in Europe during the latter half of the nineteenth century is provided in the previous edition of *Diseases of Poultry* (36). Pasteur isolated the causative organism in pure culture (89) and then used the FC organism to perform his classic experiments in attenuation of bacteria for use in producing immunity (88, 90). Salmon (108) appears to have been the first to study the disease in the United States. A good description of disease signs was reported, however, as early as 1867 in Iowa, where losses of chickens, turkeys, and geese had occurred (2).

Etiology

Classification

Pasteurella multocida is the causative agent of FC. In the past, the bacterium has been given many names and this history has been reviewed in the previous edition of *Diseases of Poultry* (36).

For a while, each isolate of *P. multocida* was named according to the animal from which it was isolated, such as *P. avicida* or *P. aviseptica*, *P. muricida* or *P. muriseptica*. *P. multocida*, proposed by Rosenbusch and Merchant (106), is now widely accepted and used throughout the world.

Based upon DNA homology studies *P. multocida* was divided into 3 subspecies, namely *P. multocida* subspecies *multocida*, *P. multocida* subspecies *septica*, and *P. multocida* subspecies *gallicida,* that could be recognized by physiological properties (77). However, subsequent studies using multilocus enzyme electrophoresis (11), multilocus sequence typing (116), and multilocus sequence analysis (10) have all identified only 2 lineages within *P. multocida*. It appears that the subspecies *gallicida* is an artificial unit. Although biotyping by fermentation patterns has provided useful information (32), it appears that identification at the subspecies level is not reliable and cannot be recommended.

Morphology and Staining

Pasteurella multocida is a Gram-negative, nonmotile, nonspore-forming rod occurring singly, in pairs, and occasionally as chains or filaments. It measures 0.2–0.4 × 0.6–2.5 μm but tends to become pleomorphic after repeated subculture. A capsule can be demonstrated in recently isolated cultures using indirect methods of staining (Figure 19.1). In tissues, blood, and recently isolated cultures, the organism stains bipolar (Figure 19.2). Pili have been reported (96).

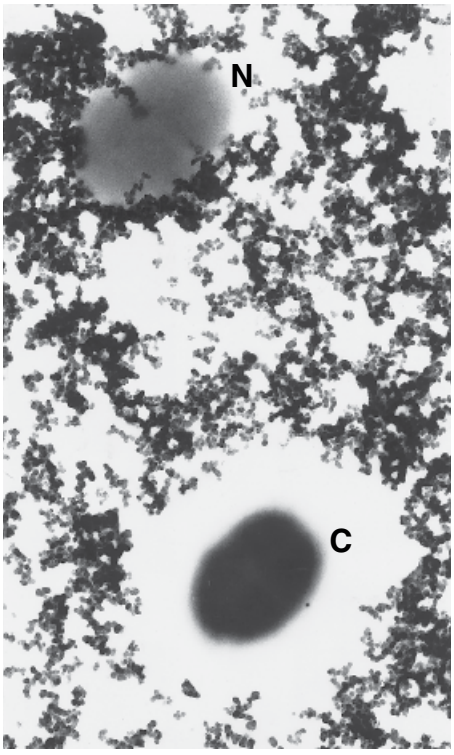


Figure 19.1 Electron photomicrograph of *Pasteurella multocida*-encapsulated cell (C) and nonencapsulated cell (N) suspended in India ink. ×19,000.

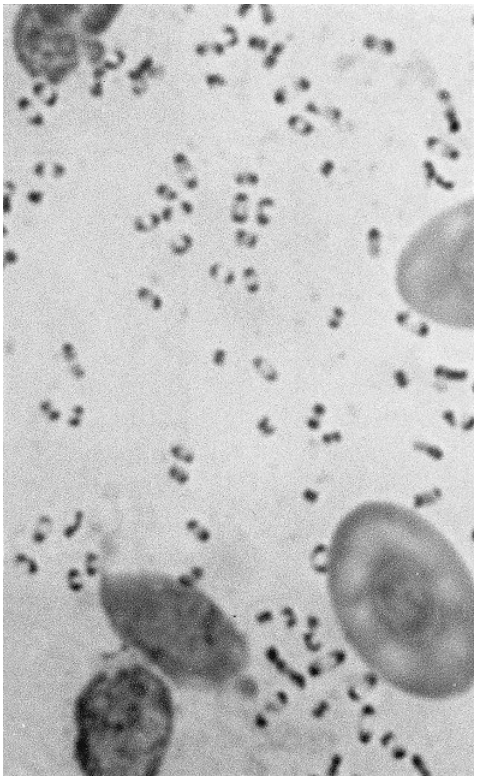


Figure 19.2 *Pasteurella multocida* in liver imprint from chicken with acute fowl cholera (note bipolarity). Wright's stain, ×2,500.

Growth Requirements

Pasteurella multocida grows aerobically or anaerobically. The optimal growth temperature is 37 °C. The optimal pH range is 7.2–7.8, but growth can occur in the range 6.2–9.0, depending upon composition of the medium. In liquid media, maximum growth is obtained in 16–24 hours. The broth becomes cloudy, and in a few days, a sticky sediment collects. With some isolates, a flocculent precipitate occurs.

The bacterium will grow on meat infusion media; growth is enhanced when the medium is enriched with peptone, casein hydrolysate, or avian serum. Blood or serum from some animals inhibits growth of *P. multocida*. Inhibition is greatest from blood of horses, cattle, sheep, and goats; blood of chickens, ducks, swine, and water buffalo has little or no inhibitory action (107). Chemically defined media have been reported and are listed in the previous edition of *Diseases of Poultry* (36). Berkman (5) found that pantothenic acid and nicotinamide are essential for growth. Dextrose starch agar with 5% avian serum is an excellent medium for growing *P. multocida*.

Colony Morphology

Colonial morphology observed with obliquely transmitted light can be a useful characteristic. On primary isolation from birds with FC, colonies may be iridescent, sectored with various intensities of iridescence, or blue with little or no iridescence. Full details of how to observe the iridescence and examples of colony appearance are provided in the previous edition of *Diseases of Poultry* (36).

Heddleston et al. (54) reported that a virulent isolate of *P. multocida* of avian origin produced iridescent colonies that dissociated *in vitro* and produced blue colonies. Organisms from blue colonies also mutated and produced gray colonies, which have not been reported in primary cultures from birds. Cells from iridescent colonies occurred singly or in pairs, did not agglutinate in immune serum, were encapsulated, and were virulent for chickens, turkeys, rabbits, and mice when administered on mucous membranes of the upper air passages. Cells from blue colonies occurred singly or in pairs, were agglutinated by immune serum, were unencapsulated, and were avirulent when applied to mucous membranes of chickens and mice but were virulent for rabbits and slightly virulent for turkeys. Cells from gray colonies occurred only as chains and were unencapsulated and avirulent. Killed organisms from all 3 colonial forms induced immunity in chickens. Antigens extracted with hot saline from highly virulent encapsulated cells of iridescent colonies by Yaw and Kakavas (123) actively immunized chickens and mice, whereas less virulent unencapsulated cells from blue colonies immunized chickens more effectively than mice.

Table 19.1 Differential tests for *Pasteurella multocida, Gallibacterium anatis* biovar *haemolytica*, and *Avibacterium gallinarum*.

Test	*P. multocida*	*G. anatis biovar haemolytica*	*Av. gallinarum*
Hemolysis	–	+	–
MacConkey agar	–	+U	–
Indole	+	–	–
Produce acid from			
Lactose	–U	+U	–
Maltose	–U	–U	+

Note: U = usually.
All three species are nonmotile, do not hydrolyse gelatin, are catalase and oxidase positive, fail to produce urease and ferment glucose and sucrose.

Biochemical Properties

Pasteurella multocida does not produce gas but produces indole, oxidase, and catalase, and has a characteristic odor. Unlike most Gram-negative bacteria, the organism is sensitive to penicillin. A listing of 29 other physiologic tests with 948 cultures of avian origin is provided in the previous edition of *Diseases of Poultry* (36). Significant differential characteristics are listed in Table 19.1.

Susceptibility to Chemical and Physical Agents

Pasteurella multocida is destroyed easily by ordinary disinfectants, sunlight, drying, or heat; it is killed within 15 minutes at 56 °C and 10 minutes at 60 °C. A 1% solution of formaldehyde, phenol, sodium hydroxide, beta-propiolactone, or glutaraldehyde and a 0.1% solution of benzalkonium chloride all killed, within 5 minutes, a suspension of 4.4×10^8 organisms of *P. multocida*/mL in 0.85% saline solution at 24 °C.

Skidmore (115) observed that the organism survived in dried turkey blood on glass for 8 days but not 30 days at room temperature. In studies of environmental influence on the incidence of FC, Van Es and Olney (119) found the infection hazard had apparently disappeared from a poultry yard 2 weeks after the last death and removal of birds.

The influence of temperature on the viability and virulence of *P. multocida* was studied by Nobrega and Bueno (80), who observed that broth cultures stored in sealed tubes at an average room temperature of 17.6 °C were still virulent after 2 years; at 2 °C–4 °C, while they were nonviable after 1 year. With controlled experiments, Dimov (27) observed that *P. multocida* died rapidly in soils with moisture content of less than 40%. At a moisture content of 50% and temperature of 20 °C, it survived for 5–6 days at pH 5.0, 15–100 days at pH 7.0, and 24–85 days at pH 8.0. A culture survived without loss of virulence for 113 days in soil with 50% moisture at 3 °C and pH 7.15.

Cultures may be maintained without dissociation or loss of virulence in the lyophilized state or sealed in glass tubes and stored at 4 °C or colder for up to 12 months (121).

Toxins

A dried culture filtrate of *P. multocida* was first demonstrated to produce signs of toxicity in chickens by Pasteur (89). A full review of work on the toxins of *P. multocida* is provided in the previous edition of *Diseases of Poultry* (36).

Endotoxins are produced by all *P. multocida*, both virulent and nonvirulent. They may contribute to virulence; however, invasion and multiplication of a strain are necessary for the production of sufficient quantities of endotoxin *in vivo* to contribute to pathologic processes. Heddleston and Rebers (50) demonstrated that a loosely bound endotoxin could be washed from *P. multocida* with cold formalinized saline solution. This endotoxin was a nitrogen-containing phosphorylated lipopolysaccharide, readily inactivated under mild acid conditions. Signs of acute FC were induced in chickens by injection of fractional amounts of endotoxin. The LD_{50} for chicken embryos was 5.2 mg via the chorioallantoic membrane and 1 dose of 1.9 mg injected intravenously killed 5 of 6 19-day-old turkeys (median death time was only 3 hours). The endotoxin was present in the vascular system of turkeys with FC and could be detected with the *Limulus* lysate test and antiserum in the gel diffusion precipitin test. The serologic specificity of the endotoxin was associated with the lipopolysaccharide. Free endotoxin induced active immunity (50). Week-old poults were relatively resistant to the lethal effects of purified lipopolysaccharides from 2 highly pathogenic FC strains of *P. multocida* (99).

Nielsen et al. (79) found 6 of 14 turkey strains produced a heat-labile protein toxic for embryonic bovine lung cells whereas none of the 58 tested chicken strains were positive for that protein. Four serogroup D strains isolated from turkeys were found to contain a heat-labile toxin (100).

Strain Classification

Antigenicity

Conventional serotyping of *P. multocida* has focused on capsular and somatic antigens. Specific capsule serogroup antigens are conventionally recognized using passive hemagglutination tests (16). Five capsular types (A, B, D, E, and F) are currently recognized (103). In a study of isolates representing a variety of avian hosts, Rhoades and Rimler (98) found organisms belonging to A, B, D, and F. Presumptive identification of capsular types A, D, and F can be determined by capsule depolymerization with specific mucopolysaccharidases (102). A multiplex capsular polymerase chain reaction (PCR) assay that recognizes all capsule types has been developed (117) and is now replacing the more difficult and laborious serological and depolymerization assays. It should be noted, however, that not all avian *P. multocida* can be capsule typed by this PCR, e.g. 9% of 100 isolates from England and Wales were nontypeable by this assay (23).

Somatic serotyping has been performed in the Namioka scheme by tube agglutination test (78) and in the Heddleston scheme by a gel diffusion precipitin test (47). The Heddleston scheme has become the main scheme used for somatic serotyping. To date, 16 Heddleston somatic serovars have been described (15). Somatic serovar specificity was found to be linked to the lipopolysaccharide (LPS) of the organism (101). A series of studies on the Heddleston type strains has identified the LPS structures and the genes responsible for LPS assembly, and has culminated in a multiplex PCR that recognizes 8 LPS types (43). The correlation of the LPS PCR with the classic Heddleston serovars was as follows: LPS PCR type 1 – serovars 1 and 14; LPS PCR type 2 – serovars 2 and 5; LPS PCR type 3 – serovars 3 and 4; LPS PCR type 4 – serovars 6 and 7; LPS PCR type 5 – serovar 9; LPS 6 – serovars 10, 11, 12 and 15; LPS PCR type 7 – serovars 8 and 13; LPS PCR type 8 – serovar 16. In the validation of the LPS PCR, the gold standard test was LPS compositional data as determined by mass spectrometry. This validation work highlighted that the traditional serological approach has significant deficiencies and problems – classic serotyping was only able to correctly and unambiguously type only 20 of the 58 isolates. In contrast, the LPS PCR correctly typed 57 of the 58 isolates (43). Overall, although the LPS PCR method is unable to recognize all 16 Heddleston serovars, the poor performance of the classic serotyping approach means that the PCR-based alternative is a significant improvement.

All the Heddleston somatic serovars have been isolated from avian hosts. Correlation between subspecies and serovars of *P. multocida* obtained by traditional serotyping systems has not been demonstrated (11). Over the years, somatic serotyping has provided useful information regarding the diversity of avian *P. multocida* strains and an overview of the use of the scheme has been presented in the previous edition of *Diseases of Poultry* (36).

Immunogenicity

The limited availability of Heddleston serotyping capacity has limited the number of definitive studies that have looked in detail at the correlation between immunogenicity and Heddleston serovars. This has changed in recent times with the development of molecular and chemical methods to define accurately the LPS structure. Harper et al. (42), in a study based on defined LPS mutants, have shown that bacterins (vaccines based on killed *P. multocida* cells) give poor protection across Heddleston serovars. Even more importantly, these bacterins only provide protection against strains with an identical or highly similar LPS structure – meaning that protection within a Heddleston serovar is only possible for nearly identical LPS structures (42). In contrast, a live vaccine (attenuated by an *aroA* mutation) provided protection that was not significantly influenced by LPS structure variation within a Heddleston serovar. Indeed, the live vaccines could provide significant cross-serovar protection (42).

Molecular

A number of nucleic-acid-based typing methods have been introduced for differentiation of avian strains of *P. multocida*. The advantages of these methods are that they do not depend on expression of phenotypic properties, all strains are typeable, and the discriminatory power is generally high (81). There are reviews of these methods and their applications to typing *P. multocida* and understanding outbreaks of FC (12, 36).

Restriction enzyme analysis (REA) with or without a hybridization step has been used extensively to obtain knowledge about routes of transmission and about strain diversity in outbreak situations as reviewed in the previous edition of *Diseases of Poultry* (36). The restriction enzymes *Hpa*II and *Hha*I are most frequently used (18). The problem with this approach is the difficulty in comparing results between laboratories and/or across time (12).

Multilocus sequence typing (MLST) is a widely used system for typing bacterial pathogens that involves the comparison of the sequences of highly conserved housekeeping genes, typically 7 genes with around 500 base pair sequences being used. MLST schemes allow easy comparisons across laboratories and across time as the information is simply DNA sequences (74). Two MLST schemes for *P. multocida* have been developed (24, 116). The RIRDC MLST developed by Subaaharan et al. (116) was originally based on avian *P. multocida* but now covers many different hosts. Both schemes are now available from a single combined database that contains sequences

from a total of over 300 avian isolates and a total of over 1,000 isolates from all hosts (https://pubmlst.org/pmultocida/). The RIRDC scheme has been used to examine the population structure of the species and suggest the existence of 2 lineages (116). Using the RIRDC MLST scheme, Hotchkiss et al. (58) have suggested that there may be a niche association, with *P. multocida* in different hosts being distinct and separate subpopulations. The RIRDC MLST scheme has been used to show that outbreaks of FC can be associated both with 1 or multiple genetic types. Further, repeated outbreaks on a property can be associated with a single genetic type or can change over time (113).

Pathogenicity

The pathogenicity of *P. multocida* is complex and variable, depending on the strain, host species, and variations within the strain or host and conditions of contact between the strain and the host. The variation in virulence across strains was shown in a study examining 5 strains administered via the intramuscular, intravenous, intratracheal or conjunctival routes to groups of 10 chickens (122). Although the route of the challenge had no significant impact, there were marked differences in virulence across the strains. The most virulent strain caused 100% mortality by all routes except the conjunctival route and the least virulent strain caused only a single mortality by the intravenous route (122).

Pasteurella multocida usually enters tissues of birds through mucous membranes of the pharynx or upper air passages, but it also may enter through the conjunctiva or cutaneous wounds. Hughes and Pritchett (59) were unable to infect chickens by placing a culture in a gelatin capsule and inserting it into the esophagus, but chickens were infected when the culture was dropped on the roof of the nasal cleft. Arsov (3) infected birds by mouth, using ^{35}P-labeled culture, and observed that the portal of infection was the mucous membrane of the mouth and pharynx, but not the esophagus, crop, or proventriculus. The Eustachian tube was suggested by Olson and McCune (83) as the most likely route of infection because the infection localizes in air spaces of the cranial bone, middle ear, and meninges.

Turkeys are much more susceptible than chickens to infection with *P. multocida*, and mature chickens are more susceptible than young ones (46). Hungerford (60) observed heavy losses in mature chickens, but no losses in birds up to 16 weeks of age in a case involving 90,000 birds. When testing infectivity of an isolate or susceptibility of a host, cohabitation is the most natural method of exposure. Unless the host is highly susceptible and the isolate highly invasive, however, results may be slow. Therefore, it is often advantageous to swab the nasal cleft with cotton saturated with the culture; if a more severe exposure is required, the culture can be injected parenterally.

Virulence Factors

Although no definitive and complete understanding of the virulence of *P. multocida* is currently available, there is a developing body of evidence on the roles of a number of virulence associated factors. In the following text, an overview of the current situation is provided. A review by Harper et al. (38) provides a detailed overview.

The capsule of *P. multocida* has long been regarded as an important virulence factor. Since the early 1960s, it has been observed that isolates that have a capsule are more virulent than the acapsular variants (54). Recent work has confirmed the key role of the capsule with a genetically defined, acapsular mutant of a serogroup A strain being shown to be markedly attenuated in both mice and chickens, and also unable to grow in chicken muscle (20).

There is clear evidence of the role of LPS in the virulence of *P. multocida*. The LPS of *P. multocida* consists of a highly conserved inner core that is linked to lipid A and an outer core that shows considerable variation with no polymeric O side chain (38). A series of studies have confirmed that the 16 Heddleston serovars do represent unique LPS structures (43). Studies using either the Heddleston serovar reference strains or VP161, a strain from a FC outbreak in Vietnam, have confirmed that apparently minor changes in the LPS structure can have major impacts on virulence: for example, decoration of the LPS with phosphocoline is important although not essential for virulence (40); truncating the LPS by inactivating a heptosyltransferase removes virulence (41); and that mutants expressing only inner glycoform B are avirulent whereas the parent with both forms (the wild type) or a mutant expressing only glycoform A were fully virulent (39). These and other studies have emphasized the critical importance of a full LPS structure in these reference strains and in experimental infections. In contrast, there are numerous variations in LPS structure, including truncations in the structure (far more than the 16 present in the Heddelston reference strains) of field isolates, particularly of serovars 3 and 4 (43). However, all of these field isolates – based on field evidence – appear to be fully virulent. This is an anomaly that is yet to be resolved.

The other potential virulence-linked genes are typically those associated with fimbrae/adhesins (e.g, *fimA*, *flp1*, *flp2*, *hsf_1, hsf_2, pfhA*, and *ptfA*), iron regulated and iron acquisition proteins (e.g., *tbpA, tonB* and *exbBD*), sialic acid metabolism (*nanB, nanH*), outer membrane proteins (e.g., *oma87*, *ompH*) and global regular genes (e.g., *crp* and *phoP*). As noted by Harper et al. (38), there has been little definitive evidence of the role of these genes in virulence. Indirect evidence of the role of these virulence genes is accumulating as the use of

assays such as the PCR-based virulence genotyping tool described by Ewers et al. (30) becomes more common. Also, comparative genomic studies such as those reported by Johnson et al. (67) provide novel insights into potential virulence-associated genes that can be followed up in more specific and detailed studies.

Pathobiology and Epizootiology

Incidence and Distribution

Fowl cholera is typically more prevalent in late summer, fall, and winter. This seasonal occurrence is one of circumstance, that is, exposure to vectors, rather than lowered resistance, except that chickens become more susceptible as they reach maturity.

Natural and Experimental Hosts

Most reported outbreaks of FC in commercial birds involve chickens, turkeys, ducks, or geese. However, this disease also affects other types of poultry, game birds raised in captivity, companion birds, birds in zoos, and wild birds. The wide range of avian hosts in which FC has been reported suggests that all types of birds are susceptible.

Among poultry, turkeys are most affected. The disease usually occurs in young mature turkeys, but all ages are highly susceptible. Death losses from FC in chickens usually occur in laying flocks, because birds of this age are more susceptible than younger chickens. Chickens younger than 16 weeks of age generally are quite resistant (60). However, the disease has been seen in broiler chickens (109, 114).

Domestic geese and ducks are also highly susceptible to FC. Van Es and Olney (119) recognized the marked susceptibility of geese to FC, in using them to test for persistence of viable organisms in lots after removal of infected chickens. FC in ducks has been a serious problem on Long Island, where it was diagnosed on 32 of 68 commercial duck farms (29).

Birds of prey, waterfowl, and other birds kept in zoologic gardens occasionally succumb to infection; *P. multocida* has been isolated from more than 50 species of feral birds (36). During a 2.5-year survey, Faddoul et al. (31) isolated *P. multocida* from 13 (7 species) of 248 feral birds submitted to the diagnostic laboratory.

Pasteurella multocida from birds with FC usually kills rabbits and mice, but other mammals are resistant to infection. According to Heddleston and Watko (53), rabbits, mice, pigeons, and sparrows died of acute septicemia when exposed intranasally to an isolate of *P. multocida* from an acute case of FC; rats, ferrets, guinea pigs, a sheep, a pig, and a calf did not show any clinical response to the same organism. One of 5 rats, 1 of 2 minks, and 11 of 19 mice fed viscera of infected chickens developed nasal infection, pneumonia, and fatal septicemia, respectively. A calf died of acute septicemia less than 18 hours after intramuscular exposure. Guinea pigs exposed by intramuscular inoculation developed necrosis at the inoculation site; those exposed intraperitoneally usually died.

Horses, cattle, sheep, pigs, dogs, and cats are refractory to oral inoculation, and subcutaneous inoculation results in localized abscesses. All of these animals, however, may succumb to intravenous inoculation (36).

Transmission, Carriers, and Vectors

How FC is introduced into a flock is often impossible to determine. Chronically infected birds are considered to be a major source of infection. The only limit to the duration of the chronic carrier state is the life span of the infected bird. Free-flying birds having contact with poultry may be a source of FC organisms. Transmission of the organism through the egg seldom, if ever, occurs. A study of more than 2,000 fresh and embryonating eggs from chickens infected with chronic FC yielded no evidence that *P. multocida* was transmitted through the egg (112).

Pritchett et al. (93, 94) and Pritchett and Hughes (95) examined 3 infected commercial flocks of white leghorns for *P. multocida* and found that many birds harbored the organism in nasal clefts. The presence of the bacterium was related to severity of upper respiratory infection in the flocks. They concluded that the enzootic focus of infection was healthy nasal carriers. Using an experimental infection model based on oral challenge, Lee et al. (72) found that some birds had viable *P. multocida* in the crop for at least 30 hours postchallenge. These studies, as well as those of Van Es and Olney (119) and Hall et al. (37), proved that survivors of an epornitic of FC may be reservoirs of infection. Carrier birds among the older flock, held over for a second year, provided a reservoir of infection for young susceptible pullets housed with them (28).

Most species of farm animals may be carriers of *P. multocida*. Generally, these organisms, except for those from swine and possibly those from cats, are avirulent for fowl. Iliev et al. (62) reported that isolates of *P. multocida* from the tonsils of cattle and sheep were not pathogenic for fowl, but all 18 isolates from pigs in areas where FC was common were highly pathogenic for fowl. Only 2 of 47 isolates from pigs in areas having low incidence of FC were pathogenic. Iliev et al. (63) also reported that healthy pigs that were carriers of *P. multocida* transmitted infection to fowl in the same enclosure. A serovar 5:A isolate, from a pneumonic pig

lung, was highly virulent for chickens, whereas a serovar 1:A isolate from pneumonic pig lung was avirulent in chickens (76). There was no cross-immunity in chickens between the 2 serovars.

Contaminated crates, feed bags, or any equipment used previously for poultry may serve in introducing FC into a flock. Organisms are disseminated throughout the carcasses of birds that die of acute FC and may serve as an infection source, especially because fowl tend to consume such carcasses. Hendrickson and Hilbert (56) were able to isolate *P. multocida* from the blood of a naturally infected chicken for 49 days preceding death. They noticed a rapid increase in the number of organisms immediately preceding and following death and that the organisms remained viable for 2 months at 5°C–10°C. Serdyuk and Tsimokh (111) demonstrated experimentally that sparrows, pigeons, and rats could become infected with *P. multocida* when exposed to chickens with FC and that they in turn could infect susceptible chickens. Sparrows and pigeons carried organisms without showing clinical signs, but 10% of infected rats developed acute pasteurellosis. Cats on a free-range organic broiler farm suffering repeated outbreaks of FC have been shown to have the same genotype as that present in the chickens (114). However, the direction of transmission (cat to chicken or chicken to cat) could not be resolved (114).

The possibility that insects may serve as vectors of FC has been investigated. Skidmore (115) experimentally transmitted FC to turkeys by feeding them flies that had previously fed on infected blood. He pointed out that under natural conditions, ingestion of flies might be a means of introducing the disease into a flock. Transmission by flies, however, is probably not common, because FC that was maintained in 2 lots of chickens during the height of the fly season did not spread to adjoining lots separated only by poultry netting (119). Iovcev (64) observed that larvae, nymphs, and adult ticks (*Argas persicus*) contained *P. multocida* after feeding on infected hens. Petrov (91) demonstrated that the red mite (*Dermanyssus gallinae*) became infected with *P. multocida* after feeding on infected birds, but the mite did not transmit the organism.

Heddleston and Wessman (55) showed that 27 cultures of *P. multocida* from the upper respiratory tract of humans were not pathogenic for turkeys. Humans can become infected, however, and may infect poultry via excretion from the nose or mouth.

Dissemination of *P. multocida* within a flock is primarily by excretions from the mouth (Figure 19.3), nose, and conjunctiva of diseased birds that contaminate their environment, particularly feed and water. Feces very seldom contain viable *P. multocida*. Turkeys drinking from the same water trough with those experimentally infected with *P. multocida* developed FC (86).



Figure 19.3 Acute fowl cholera; mucous excretion from the mouth contains large numbers of *Pasteurella multocida* that can contaminate feed and water.

Clinical Signs

Acute Disease

Signs of infection in acute FC are often present for only a few hours before death (Figure 19.3). Unless infected birds are observed during this period, death may be the first indication of disease. Signs that often occur are fever, anorexia, ruffled feathers, mucous discharge from the mouth, diarrhea, and increased respiratory rate. Cyanosis often occurs immediately prior to death and is most evident in unfeathered areas of the head, such as comb and wattles. Fecal material associated with the diarrhea is initially watery and whitish in color but later becomes greenish and contains mucus. Birds that survive the initial acute septicemic stage may later succumb to the debilitating effects of emaciation and dehydration, may become chronically infected, or may recover.

Chronic Disease

Chronic FC may follow an acute stage of the disease or result from infection with organisms of low virulence. Signs generally are related to localized infections. Wattles (Figure 19.4), sinuses, leg or wing joints, foot pads, and sternal bursae often become swollen. Exudative conjunctival (Figure 19.5) and pharyngeal lesions may be observed, and torticollis (Figure 19.6) sometimes occurs. Tracheal rales and dyspnea may result from respiratory tract infections. In the past, the term roup was used to



Figure 19.4 Chronic fowl cholera. Swollen wattle resulting from localized infection.

indicate a condition in which signs were associated with chronic infections of cephalic mucous membranes. The term was not limited to FC, but included other diseases as well. Chronically infected birds may succumb, remain infected for long periods, or recover.

Morbidity and Mortality

In naturally infected chickens, mortality usually ranges from 0% to 20%, but greater losses have been reported. Reduced egg production and persistent localized infection often occur. Chickens are more susceptible to FC after feed and water withdrawal or after an abrupt change of diet (14). Heat or rough treatment on a shaking machine increased the incidence in chickens exposed experimentally (69). The move to organic and free-range production systems has resulted in some changes in disease expression. In a free-range organic broiler flock, an ongoing FC outbreak caused mortalities of 55% through to processing (114).



Figure 19.5 Chronic fowl cholera. Serous inflammation of conjunctiva.



Figure 19.6 Chronic fowl cholera. Torticollis resulting from meningeal infection.

Under experimental conditions, 90%–100% of mature chickens exposed by swabbing the palatine cleft may die within 24–48 hours, depending on the strain of *P. multocida* used. In contrast, only 10%–20% usually die within a 2-week period when exposed by contact with infected birds. Pritchett et al. (93) observed mortality of 35%–45% in 3 houses of pullets. In South Carolina and adjoining areas, FC existed mainly as a persistent, subacute chronic disease that clinically resembles avian monocytosis (7).

Most or all turkeys in an infected flock may die within a few days. Under experimental conditions, 90%–100% of mature turkeys may die within 48 hours when exposed to a highly virulent strain of *P. multocida* by swabbing the palatine cleft or by contact with infected birds.

An FC outbreak in geese in Rhode Island resulted in about 3,200 of a flock of 4,000 dying in a short period (22). Losses usually occur in ducks greater than 4 weeks of age, with mortality reaching 50% (29).

Fowl cholera outbreaks in wild birds are often associated with large mortalities. Jaksic et al. (65) described an acute epornitic among pheasants, in which 1,700 died. An FC outbreak in the San Francisco Bay area was reported to have been responsible for an estimated loss of 40,000 waterfowl (105). Gershman et al. (35) observed a serious outbreak among eider ducks (*Somateria mollissima*) in a nesting area 6 miles off the coast of Maine, where more than 200 birds died and more than 100 nests were lost. More than 60,000 waterfowl died of FC during the winter of 1956–1957 at the Muleshoe National Wildlife Refuge in Texas (66). Rosen (104) reported that there are 2 areas in the United States where FC is enzootic in waterfowl: the Muleshoe National Wildlife Refuge and the north central area of California. Both locations have had periodic outbreaks since 1944.



Figure 19.7 Acute fowl cholera. Hyperemia of chicken duodenum.

Pathology

Lesions of FC are not constant but vary in type and severity. The greatest variation is related to the course of the disease, whether acute or chronic. Although it is convenient for descriptive purposes to refer to either acute or chronic FC, it is sometimes difficult to categorize the disease in this manner. Signs of infection and lesions that occur may be intermediate to those described for acute and chronic forms.

Gross Pathology: Acute Disease

When the course of the disease is acute, most of the postmortem lesions are associated with vascular disturbances. General hyperemia usually occurs, being most evident in veins of the abdominal viscera, and may be quite pronounced in small vessels of the duodenal mucosa (Figure 19.7). Petechial and ecchymotic hemorrhages are frequently found and may be widely distributed on the serosal surfaces of various organs. Subepicardial (Figure 19.8A) and subserosal hemorrhages are common, as are hemorrhages in the lung, abdominal fat, and intestinal mucosa. Increased amounts of pericardial and peritoneal fluid frequently occur. Disseminated intravascular clotting or fibrinous thrombosis has been observed in chickens and ducks that died from acute experimentally induced FC (61, 87).

Livers of acutely affected birds may be swollen and usually contain multiple small focal areas of coagulative necrosis (Figure 19.8B). Lungs of turkeys are affected more severely than those of chickens, with pneumonia being a common sequela. Large amounts of viscid mucus may be observed in the digestive tract, particularly in the pharynx, crop, and intestine.

Ovaries of laying hens are commonly affected. Mature follicles may appear flaccid; thecal blood vessels, which are usually easily observed, are less evident (Figure 19.8E). In some hens, the blood vessels of the ovaries will be very prominent. Yolk material from ruptured follicles may be found in the peritoneal cavity. Immature follicles and ovarian stroma are often hyperemic.

Gross Pathology: Chronic Disease

Chronic FC is characterized by localized infections, in contrast to the septicemic nature of the acute disease. These generally become suppurative and may be widely distributed anatomically. They often occur in the respiratory tract and may involve any part, including sinuses and pneumatic bones (Figure 19.9). Pneumonia (Figure 19.8C,D) is an especially common lesion in turkeys. Infections of the conjunctiva and adjacent tissues occur (see Figure 19.5), and facial edema may be observed. Localized infections also may involve the hock joints (Figure 19.8F), foot pads, peritoneal cavity, and oviduct.

Chronic localized infections can involve the middle ear and cranial bones and have been reported to result in torticollis. In turkeys, torticollis and eventual death can

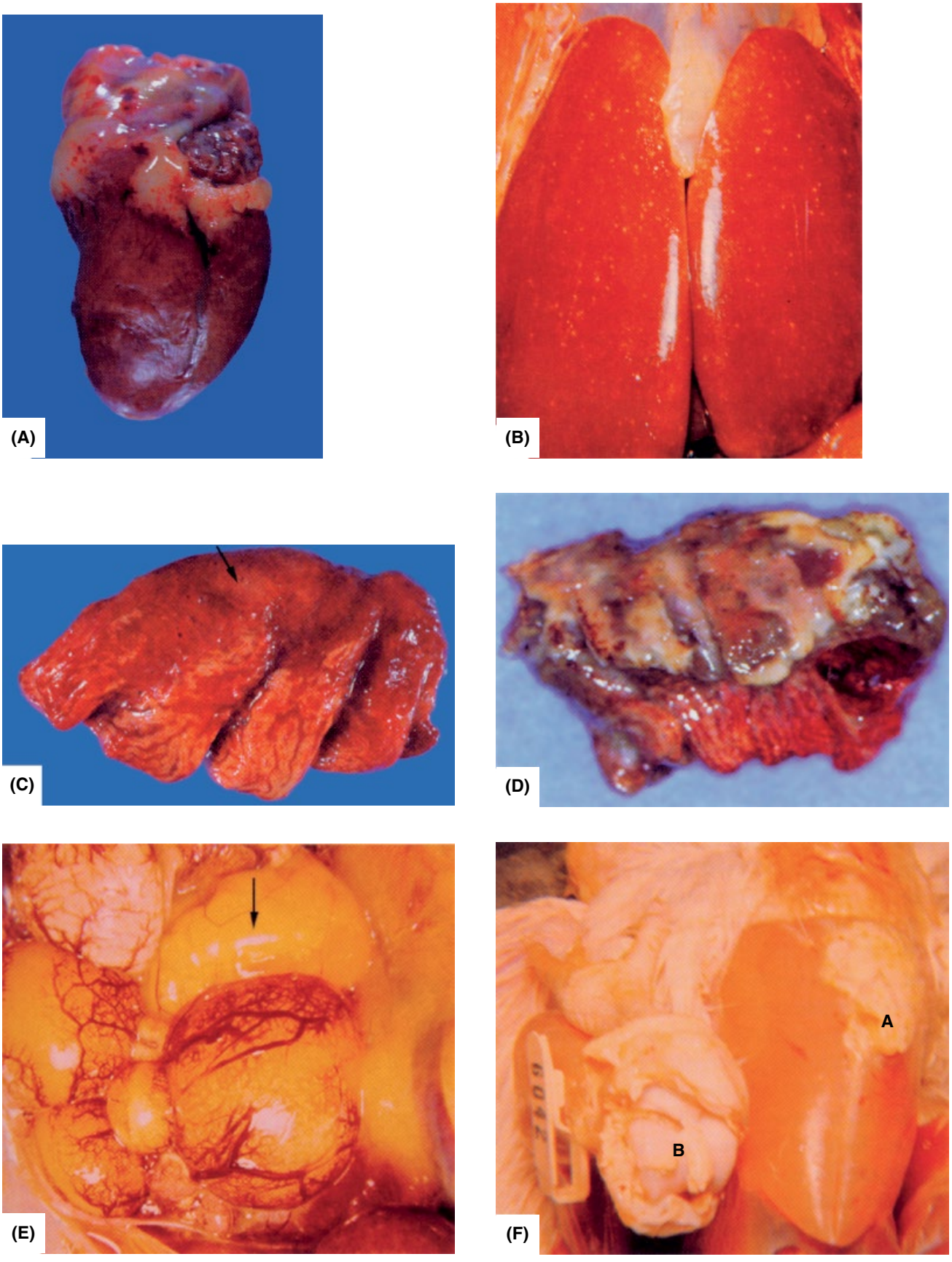


Figure 19.8 (A) Acute fowl cholera (FC). Subepicardial hemorrhages in a turkey. (B) Acute FC. Multiple necrotic foci in turkey liver. (C) Acute FC. Turkey lung with extensive hemorrhage and patchy areas of necrosis (arrow) and emphysema. (D) Submassive necrosis with fibrous exudate on pleural surface. (E) Acute FC. Flaccid ovarian follicle (arrow) with thecal blood vessels less evident than normal. (F) Chronic FC. Caseous exudate in sternal bursa (A) and hock joint (B) of a turkey. (*For color detail, please see the color section.*)

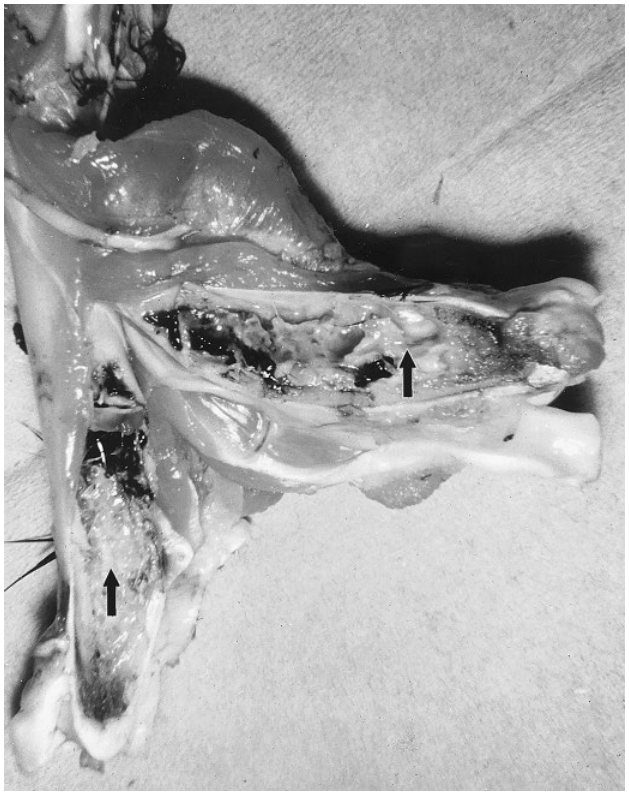


Figure 19.9 Chronic fowl cholera. Caseous exudate (arrows) in turkey humerus.

be associated with infections of the cranial bones, middle ear, and meninges.

Microscopic Pathology: Acute Disease

Experimental infection of mature chickens resulted in an acute general passive hyperemia with heterophilic infiltration of the lung, liver, and other organs (97) (see Figure 19.10). In experimental infections of ducks, the acute disease presents as a hemorrhagic septicemia with widespread vascular damage and focal necrosis in liver, spleen, and other organs (61).

Microscopic Pathology: Chronic Disease

In a study of naturally infected turkeys exhibiting torticollis, Olson (82) described lesions in cranial bones, middle ear, and meninges. The outstanding gross lesion was yellowish caseous exudate in air spaces of the calvarial bones. Heterophilic infiltration and fibrin were consistently observed in the air spaces, middle ear, and meninges. Multinuclear giant cells often were associated with necrotic masses of heterophils in air spaces. Similar lesions were found in experimentally exposed turkeys (83). Localized meningeal infections (Figure 19.11), without involvement of cranial bones or the middle ear, have been observed in turkeys exhibiting torticollis, as have cerebellar infections (33).

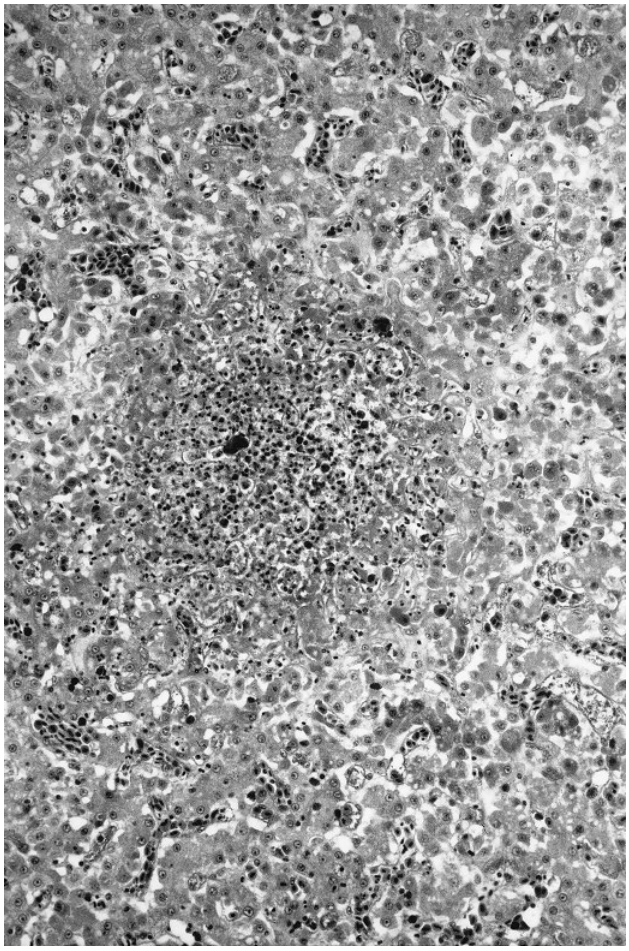


Figure 19.10 Acute fowl cholera. Coagulative necrosis and heterophilic infiltration in turkey liver. H&E, ×600.

Immunity

Pasteur (90) used an avirulent culture attenuated by prolonged growth on artificial medium and produced immunity that protected fowl against subsequent exposure. In field use, his method did not prove practical because uniform attenuation could not be obtained, and heavy losses sometimes occurred in vaccinated flocks.

Since Pasteur's classic work, numerous attempts have been made to produce efficient vaccines against FC, but results have not been consistent. There can be little doubt, however, that a substantial, but not absolute, immunity can be induced in fowl by using killed *P. multocida* vaccines under controlled conditions (4, 44). Killed *P. multocida* vaccines usually are prepared by growing selected immunogenic strains on a suitable medium and suspending them in formalinized saline solution. The killed organisms usually are incorporated with an adjuvant and injected subcutaneously or intramuscularly.

Heddleston et al. (48) showed that bacterins only protected against the homologous strain and not a heterologous serovar. This finding has long been assumed to

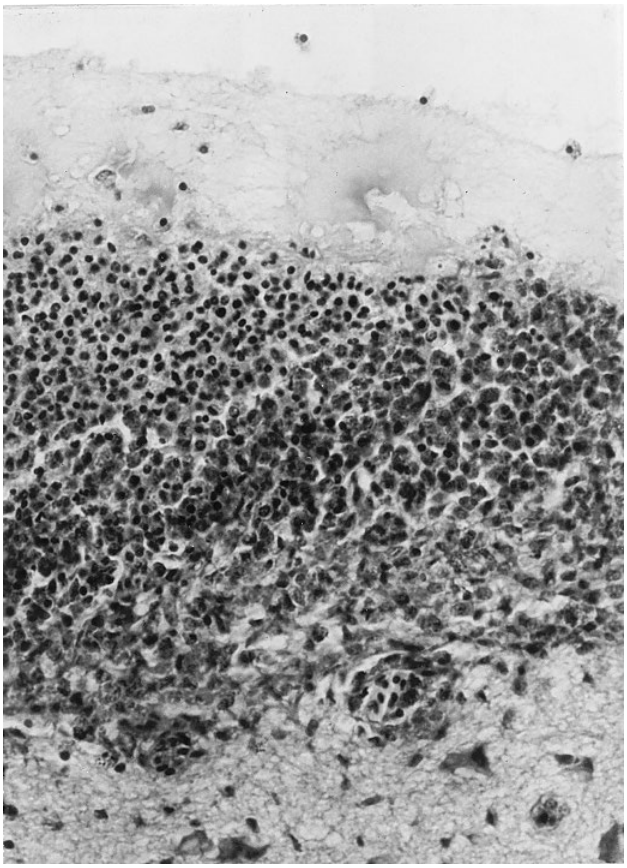


Figure 19.11 Chronic fowl cholera. Fibrinoheterophilic meningitis in turkey. H&E, ×400.

be evidence that bacterins provide serovar specific protection. However, this is now known not to be correct. The improved molecular knowledge of the LPS biosynthesis genes has allowed a far better understanding of the importance of the LPS outer core structure in the protection provided by killed cells of *P. multocida* (42). Essentially, a bacterin will provide protection only against challenge by strains with a highly similar or identical LPS structure (42). Hence, cross-protection for bacterins is not at the serovar level, but rather, at the LPS outer core structure level (42).

Under field conditions, losses from FC sometimes occur in vaccinated flocks for reasons other than mismatch with the challenge strain. This failure may be because of improperly prepared or administered vaccine or immune-impaired birds. Heddleston and Reisinger (52) demonstrated that stress caused by changing the social or peck order of vaccinated males, as well as fowlpox infection in chickens at time of vaccination and exposure, significantly reduced the efficacy of vaccination. In experimental studies (92), the manifestation of acquired resistance was impaired in turkeys vaccinated against *P. multocida* while receiving aflatoxin in their feed. It also was observed that an isolate of *P. multocida* recovered from an FC outbreak in previously vaccinated turkeys differed serologically from the culture used in preparing vaccine (48).

There has been interest in live vaccines since the work of Heddleston and Rebers (49) showed that bacterins prepared with tissues from infected turkeys or live *P. multocida* administered in drinking water will induce immunity in turkeys against a different immunogenic type. This early work is reviewed in the previous edition of *Diseases of Poultry* (36). In later work Bierer and Derieux (8) demonstrated good immunity in 14-week-old turkeys that were given a live culture of *P. multocida* (CU strain, previously CS-148) in drinking water 2 weeks before challenge exposure. The vaccine, however, killed 4.2% of 120 turkeys. The best results were obtained by inoculating 8-week-old turkeys with a killed bacterin and then administering the live vaccine 2 weeks later; the live vaccine killed only 2.5% of 120 turkeys. Derieux and Bierer (26) stated that good immunity may be obtained in 6-week-old turkeys by administering 2 doses of vaccine in drinking water on the same day and repeating the vaccination 4 weeks later. No data were given, however, as to duration of immunity or number of turkeys killed by vaccination. The CU strain is more effective in chickens by wing-web or subcutaneous inoculation than in drinking water (25).

Maheswaran et al. (73), using a high temperature mutant that was avirulent, induced immunity in turkeys with live vaccines via drinking water. Scott et al. (110) produced 2 auxotrophic mutants (one Heddleston serovar 1 and the other serovar 3) that were avirulent in chickens and which provided both homologous protection and heterologous protection, the latter against Heddleston serovar 4 for both candidate vaccine strains.

Passive Immunity

Bolin and Eveleth (14) reported that *P. multocida* antiserum prepared in chickens gave maximum protection 16–24 hours after injection; protection began to decline after 48 hours and had disappeared after 192 hours. Heddleston et al. (51) showed that serum from birds vaccinated via drinking water would induce passive immunity in chicks and turkeys. Although passive immunity was used in the past, the short duration of the protection means that the approach is no longer used.

Diagnosis

A presumptive diagnosis of FC may be made from clinical observations, necropsy findings, or isolation of *P. multocida*. A conclusive diagnosis should be based on all 3. Signs and lesions of the disease are described above.

Isolation and Identification

Pasteurella multocida can be isolated readily from viscera of birds that die of acute FC and usually from lesions of chronic cases; it is less likely to be isolated from dehydrated, emaciated survivors of an acute outbreak. A tentative diagnosis of acute FC can be made by demonstrating bipolar organisms in liver imprints (Figure 19.2) using Wright's stain.

Bone marrow, heart blood, liver, meninges, or localized lesions are preferred for culturing. To isolate *P. multocida*, sear the tissue or exudate with a spatula and obtain a specimen by inserting a sterile cotton swab or wire loop through the seared surface. For live birds, squeeze mucus from the nostril or insert a cotton swab into the nasal cleft. The specimen should be inoculated onto blood agar and MacConkey agar and incubated aerobically at 37 °C.

Colonies characteristic of *P. multocida* (described under Etiology) are transferred to a fresh blood agar plate incubated for 18–24 hours. For traditional phenotypic identification, tubes of phenol red broth base containing 1% glucose, lactose, sucrose, mannitol, and maltose, respectively, are inoculated. Fermentation of glucose, sucrose, and mannitol without gas is characteristic of *P. multocida*. Lactose usually is not fermented. Indole and oxidase activity can be tested directly from the blood agar using commercial strips. For the indole test, the classic method, a broth of 2% tryptose in 0.85% saline solution that is incubated for 24 hours at 37 °C and then tested (Kovac's indole reagent), can be performed. The oxidase reaction should be positive and typically indole is almost always produced by *P. multocida*. There should be no hemolysis of blood and no growth on MacConkey agar (Table 19.1).

Selective media (both broths and agar plates) have been developed. Reviews of the various options available have been published (13, 36). There has been no formal comparison of the various options. However, when sampling the upper respiratory tract, the simple approach of Catry et al. (17), the addition of bacitracin (16 mg/L) to the isolation medium, appears the most effective. When sampling sites with a high normal flora, for example the alimentary tract, the medium of Lee et al. (72), which contains polymyxin B, crystal violet, thallous acetate, bacitracin and cycloheximide, appears suitable.

Animal inoculation is useful although there are increasing ethical concerns associated with the use of this technique. Details are available in the previous edition of *Diseases of Poultry* (36). Serologic diagnosis of FC by rapid whole-blood agglutination, serum plate agglutination, agar diffusion tests, or enzyme-linked immunosorbent assay (ELISA) has limited value in chronic cholera and no value with the acute form of the disease.

A range of PCR assays for the detection or identification for *P. multocida* have been described (1). In a direct comparison of 4 of these assays (1), the PCR originally reported by Townsend et al. (118) was shown to have the required specificity and sensitivity.

Use of commercial MALDI-TOF instruments for the identification of bacteria in diagnostic laboratories is now common. The available evidence is that MALDI-TOF-based identification is rapid and generally of acceptable specificity (71), although both molecular and phenotypic methodologies performed better in 1 study (124). The depth and extent of the spectral reference library may explain why the capacity of MALDI-TOF to identify *P. multocida* and other veterinary pathogens may vary from laboratory to laboratory.

Serology

Commercial ELISA kits to detect antibodies are available. These kits are typically used to monitor vaccination responses. Serology has, in general, not been used as a diagnostic tool.

Differential Diagnosis

Avibacterium gallinarum and *Gallibacterium anatis* biovar *haemolytica* are 2 closely related bacteria that may be isolated from diseased poultry and incorrectly identified as *P. multocida* (45). Full details of the disease conditions associated with these 2 agents are provided in Chapters 20 and 23, respectively. Differential characteristics of *P. multocida*, *Avibacterium gallinarum*, and *Gallibacterium anatis* biovar *haemolytica* that may be isolated from poultry are listed in Table 19.2. The large and sudden mortalities associated with acute FC outbreaks means that avian influenza is often a part of the differential diagnosis list.

Intervention Strategies

Management Procedures

Prevention of FC can be effected by eliminating reservoirs of *P. multocida* or by preventing their access to poultry flocks. Good management practices, with emphasis on sanitation, are the best means of preventing FC. Unlike many bacterial diseases, FC is not a disease of the hatchery. Therefore, infection occurs after birds are in the hands of the producer, and consideration must be given to the many ways that infection might be introduced into a flock. Genotyping studies have shown that FC outbreaks on a free-range layer farm were associated with a single genetic type that persisted for over 10 years (125).

Table 19.2 Physical and genetic properties of *Bordetella avium* and *Bordetella hinzii*.

	***B. avium* (References)**	***B. hinzii* (References)**
Capsule	Positive (67, 71, 114)	Positive[1] (71)
Fimbriae	Positive (63, 108)	Positive[1] (71)
Motility	Positive (67, 135)	Positive (67, 135)
Dermonecrotic (heat labile) toxin	Positive (33)	Negative (71)
Osteotoxin	Positive (35)	Unknown[2] (96, 137)
Tracheal cytotoxin	Positive (33)	Unknown
Hemagglutination of guinea pig erythrocytes	Positive (6, 64)	Negative (135)
Genome size (Mb)	~3.7 (108)	~4.9 (137)
Mol% genome guanine + cytosine content	61.5-62.6 (67, 86, 108)	66.0-67.1 (96, 135, 137)

[1] Inferred, based on the presence of orthologous genes or operons predicted to encode the indicated factor.
[2] An open reading frame with ~81% nucleotide identity and ~85% predicted amino acid identity to the gene encoding the *B. avium* osteotoxin (*metC*) is present in the genome of several isolates but there has been no demonstration of related activity.

The primary source of infection is usually sick birds or those that have recovered and still carry the causative organism, or other carriers, such as rodents or cats (114, 120). Address any holes, cracks, or other physical breaches in the barn structure that would allow pests to enter the barn and barn entryway. Only young birds should be introduced as new stock; they should be raised in a clean environment completely isolated from other birds. Isolation should be extended to housing. Unless separate houses can be provided for first- and second-year layer flocks, the older flock should be marketed in its entirety. Different species of birds should not be raised on the same premises. The danger of mixing birds from different flocks cannot be overemphasized. Farm animals (particularly pigs, dogs, and cats) should not have access to the poultry area. Birds raised outside should have drinkers that are self-cleaning, and feeders should be covered to prevent contamination as much as possible.

Pasteurella multocida has been recovered from many species of free-flying birds and these birds warrant consideration as a potential source of bacteria to poultry. Measures should be taken to prevent association of wild birds with the flock. Raising turkeys in areas where FC is a serious problem may warrant their confinement in houses from which free-flying birds, rodents, and other animals are excluded. If an outbreak of FC occurs, the flock should be quarantined and disposed of as soon as economically feasible. All housing and equipment should be cleaned and disinfected before repopulation.

Vaccination

Vaccination should be considered in areas where FC is prevalent, but it should not be substituted for good sanitary practice.

Types of Vaccines

Inactivated. Commercially produced bacterins are available and usually contain whole cells of Heddleston serovars 1, 3, 4 and 3X4 emulsified in an oil adjuvant. The limits of bacterins are that the protection is limited to strains that share a highly similar or identical LPS structure (42). Because the determination of LPS structures is highly specialized, it is not possible to confirm that a field isolate will be covered by a bacterin, even if the field isolate is identified and shown to be a serovar that matches the bacterin content. Hence, autogenous bacterins containing a locally isolated strain other than serotypes 1, 3, or 4 may be used, although the results are often mixed (70, 84). The mixed results with autogenous bacterins may be cause by the presence of multiple genotypes and serovars as has been recently shown in ducks, turkeys and layers (113). Also, the need for a close match in LPS structure (42) may explain the mixed results.

The choice of adjuvant for a bacterin (commercial or autogenous) can be water-in-oil emulsion or aluminum hydroxide (6). Bacterins using aluminum hydroxide as the adjuvant are useful for the vaccination of turkey breeder or broiler breeder flocks that are in lay because the water-in-oil emulsion, in combination with the whole bacterial cell, results in a significant tissue response by the bird. This response can result in significant declines in egg production. The negative effect on egg production is less with aluminum hydroxide adjuvant whole-cell FC bacterins. It has been well documented that aluminum hydroxide bacterins do not stimulate the immune response as well as water-in-oil bacterins (52). Therefore, if an aluminum hydroxide bacterin is used, revaccination may be required to afford immunity to a flock for an entire laying cycle.

Live. Three live vaccines available for use in the United States are CU, a strain of low virulence; M-9, a mutant of

CU with very low virulence; and PM-1, a mutant of CU intermediate in virulence between CU and M-9. A rationally attenuated strain (PMP1) (110) is used in Australia. Vaccination of chickens and turkeys with these live *P. multocida* vaccines induces protection against heterologous serovar challenge. Importantly, the live vaccine strain has no need to be matched to the LPS structure of the challenge strain (42). The use of live FC vaccines stimulates an effective immune response but has the disadvantage of potentially resulting in mortality in the vaccinated birds (8). If postvaccination mortality becomes excessive, it can be reduced by the administration of an antibiotic. This should be avoided, if possible, until at least 4 days postvaccination when there will be at least partial immunity induced by the vaccine (85).

Field Vaccination Protocols and Regimes

When considering the most appropriate vaccination program for FC, the following should be taken into consideration: prevalence of FC in the area, most prevalent serovars (and LPS structure types if possible) of *P. multocida* in area, age of birds to be vaccinated, and the value of the birds to be vaccinated (i.e., breeder turkeys vs. commercial turkeys or parent chicken breeders vs. grandparent chicken breeders). There have been many successful vaccination protocols for chicken breeders against FC. Bacterins, live vaccines, or both are used, and usually 2 doses are given: the first at 8–10 weeks of age and the second at 18–20 weeks of age. The protection provided by bacterins appears to be limited to challenge strains that match the LPS structures of the bacterin strains and does not give solid immunity for an entire laying cycle. Some of the more commonly used vaccination programs consist of administering a live vaccine in the wing web at 10–12 weeks of age followed by either another live vaccine in the wing web or a bacterin at 18–20 weeks. Vaccination with live vaccine provides protection against multiple serovars, but some vaccines can cause chronic FC. The use of a bacterin at 10–12 weeks and a live vaccine at 18–20 weeks, just prior to movement to the laying house, gives protection against multiple serotypes and minimizes live vaccine-induced chronic FC (57).

One of the most successful programs for vaccination of both breeder turkeys and commercial meat turkeys is the use of a live vaccine in the drinking water every 4 weeks, beginning at 6–8 weeks of age and continuing for the life of the flock. Bacterins also can be used in breeder turkeys. They are vaccinated 2–5 times before the onset of egg production, with the first vaccination beginning at 6–8 weeks.

Treatment

Antibacterial chemotherapy has been used extensively in the treatment of FC with varying success, depending to a large extent on the promptness of treatment and drug used. Sensitivity testing should be performed because *P. multocida* isolates vary in susceptibility to chemotherapeutic agents (68) and resistance to treatment may develop, especially during prolonged use of these agents. Wherever possible, the sensitivity testing should be performed using validated and standardized methodologies accepted by the relevant national authorities such as the Clinical and Laboratory Standards Institute (21).

The use of antimicrobial agents is a highly regulated area and can vary from nation to nation. Hence, any use of antimicrobial agents to treat an FC outbreak has to be undertaken with full recognition of the relevant national regulations and guidelines. The previous edition of *Diseases of Poultry* (36) contained a full review of the literature on the historical use of antimicrobial agents. A general observation is that many *P. multocida* isolates from poultry remain sensitive to traditional agents such as amoxicillin, penicillin, and tetracyclines as shown in a 2013 study in Mississippi (68).

Riemerella anatipestifer Infection

Jaime A. Ruiz and Tirath S. Sandhu

Summary

Agent, Infection, and Disease. *Riemerella anatipestifer* (RA) infection is a highly contagious disease of domestic ducks, geese, turkeys, and various other domestic and wild birds, occurring worldwide. It causes acute to chronic septicemia with polyserositis.

Diagnosis. RA can be isolated in trypticase soy agar containing 0.05% yeast extract. Molecular diagnostic tests available include: (1) polymerase chain reaction amplifying 16S rDNA and, (2) a rapid assay based on the *groEL* and *ompA* genes sequence using loop-mediated isothermal amplification (LAMP).

Intervention. The use of autogenous bacterins alone or in combination with avirulent live vaccines prevents infection and reduces mortality in duck breeders and susceptible progeny. Specific antibiotic therapy can be effective to control and reduce mortality in the first stages of the disease.

Introduction

Definition and Synonyms

Riemerella anatipestifer (RA) infection is also known as new duck disease, duck septicemia, anatipestifer syndrome, anatipestifer septicemia, and infectious serositis. In geese, RA infection has been called goose influenza or septicemia anserum exsudativa (54). It affects both ducks and geese, and occurs as an acute or chronic septicemia characterized by fibrinous pericarditis, perihepatitis, airsacculitis, caseous salpingitis, and meningitis. *Riemerella columbina* (RC), a similar organism to RA, has been isolated from clinically diseased pigeons (76).

Economic Significance

Riemerella anatipestifer infection is a major disease confronting the duck industry worldwide. It accounts for significant economic losses because of high mortality, weight loss, condemnations, downgrading, and salvage. Prevention and control programs consist of diagnosing the infection, vaccinating at-risk flocks, and treating the disease, which all add to the production cost.

Public Health Significance

The disease has no public health significance.

History

Riemerella anatipestifer infection was first described in 1932 in Pekin ducks from 3 farms on Long Island, New York (37). The report referred to a new disease which became known in the area as the "new duck disease." The disease was first observed in 7- to 10-week-old ducks with about 10% mortality and later spread to younger ducklings of about 3 weeks of age. For additional historical information please refer to this subchapter in previous editions of *Diseases of Poultry* and to the following references: 21, 27, 39, 53, 73 and 88.

Etiology

Classification

Originally called *Pfeifferella anatipestifer* (37) then *Moraxella anatipestifer* (10), it was finally listed in the 7th edition of *Bergey's Manual of Determinative Bacteriology* as *Pasteurella anatipestifer* (88). However, because of its uncertain taxonomic status, RA was placed as species *incertae sedis* in the 8th (88) and 9th (59) editions of *Bergey's Manual of Systematic Bacteriology*.

Comparison of DNA-base composition, DNA-DNA homology, and cellular fatty-acid profile indicated it should be excluded from the genus *Moraxella* as well as *Pasteurella* (5, 59). Suggestions to transfer RA to the *Flavobacterium/Cytophaga* group was made based on its low but significant DNA binding and ability to produce menaquinones and branched-chain fatty acids (70). However, given significant differences between RA and its close genotypic relatives *Flavobacterium* and *Weeksella* (87), it was suggested that a separate genus should be created, *Riemerella*, in honor of Riemer (73), who first described the disease "septicemia anserum exsudativa" in geese in 1904. It was ultimately named *Riemerella anatipestifer* on the basis of DNA-ribosomal RNA hybridization analysis, protein and fatty acid methylester (FAME) profiles, and phenotypic characteristics such as lack of pigment production and presence of the respiratory quinone, menaquinone 7.

Riemerella anatipestifer-like organisms of taxon 1502 (40) isolated from ducks and geese were assigned to the genus *Coenonia* and named *Coenonia anatina* gen. nov., sp. nov. on the basis of its phenotypic and genotypic characteristics and FAME profiles (93). *C. anatina* differs from RA by the absence of arginine dihydrolase and gelatinase and by the presence of hyaluronidase, chondroitin sulfatase activity, aesculin hydrolysis, and b-glucosaminidase activity. Genome sequences of RA strains ATCC 11845, RA-GD, and RA-YM, have been published (61, 101, 105).

Morphology and Staining

Riemerella anatipestifer is a Gram-negative, nonmotile, nonspore-forming rod that occurs singly, in pairs, and occasionally in chains. The cells vary from 0.2 to 0.4 mm in width and 1 to 5 mm in length. Many cells stain bipolar with Wright's stain, and a capsule can be demonstrated in preparations with India ink.

Growth Requirements

The organism grows well on chocolate agar, blood agar, and trypticase soy agar. Growth of fastidious strains can be enhanced by adding 0.05% yeast extract and 5% newborn calf serum. Growth is more abundant with increased carbon dioxide (27). Based on results obtained with the pyrogallic acid and sodium hydroxide procedure for removing oxygen, RA appears to be a strict aerobe (37). However, because carbon dioxide also would be depleted by reacting with the sodium hydroxide, neither oxygen nor carbon dioxide was available to the organism. Although some strains of RA grow at an incubation temperature of 45 °C, no growth is observed at 4 °C or 55 °C (4); indeed, maximum growth usually occurs in 48–72 hours when

incubated at 37 °C in a candle jar that provides increased carbon dioxide and moisture, both of which favor growth.

Colony Morphology

Colonies on blood agar, when grown for 24–48 hours at 37 °C in a candle jar, are 1–2 mm in diameter, convex, entire, transparent, glistening, and butyrous. Some strains produce slimy growth. Colonies on clear media are iridescent when observed with obliquely transmitted light.

Biochemical Properties

Riemerella anatipestifer does not ferment sugars in routine media, but has been reported to produce acid in dextrin, glucose, maltose, inositol, trehalose, mannose, and fructose by growing it in buffered single substrate medium (2, 4, 39). The organism liquefies gelatin and produces a slight alkaline reaction in litmus milk. Usually, indol and hydrogen sulfide are not produced; however, some strains are indol positive (40). Nitrate is not reduced to nitrite, and starch is not hydrolyzed. No growth occurs on MacConkey's agar and no hemolysis takes place on blood agar. RA produces oxidase, catalase, and phosphatase (31). It is negative for aesculin hydrolysis, hyaluronidase, and chondroitin sulfatase (40). Some strains produce urease and arginine dihydrolase. RA requires iron for growth; genes involved in iron uptake have been identified (55).

Riemerella anatipestifer is positive for: acid and alkaline phosphatase; ester lipase C8 (APIZYME system); leucine-, valine- and cystine-arylamidases; phosphoamidase; α-glucosidase; and esterase C4. It is negative for the following enzyme activities: α- and β-galactosidases, β-glucuronidase, β-glucosidase, α-mannosidase, β-glucosaminidase, lipase C14, fucosidase, and ornithine and lysine decarboxylases (70, 87).

Susceptibility to Chemical and Physical Agents

Most RA strains do not survive on solid media for more than 3–4 days at 37 °C or room temperature; in contrast, cultures in broth may be viable for 2–3 weeks when stored at 4 °C. Incubation at 55 °C for 12–16 hours resulted in nonviability of the organism (4). RA has been reported to survive in tap water and turkey litter for 13 and 27 days, respectively (6). It is sensitive to penicillin, novobiocin, chloramphenicol, lincomycin, enrofloxacin, ceftiofur, streptomycin, erythromycin, ampicillin, bacitracin, neomycin, and tetracycline, but is resistant to kanamycin and polymyxin B (4, 14). RA is also relatively resistant to gentamicin.

Strain Classification

Riemerella anatipestifer isolates have been serotyped using agglutination and agar-gel precipitin (AGP) reactions. Both of these tests involve surface antigens that are presumed to be polysaccharides (9). Plate agglutination is rapid and convenient; tube agglutination is favored over AGP because it is quantitative in terms of antibody titers.

To date, 21 serotypes have been reported. Based on agglutination reactions, Harry (30) identified 16 serotypes (A through P), 4 of which (E, F, J, and K) were lost when they were stored. He also found serotypes G and N to be identical to serotypes I and O, respectively (7, 30). Seven serotypes (1–7) were differentiated using AGP reaction (9). Subsequently, serotypes 1, 2, 3, 4, 5, and 6 were reported to be serologically identical to Harry's types A, I/G, L, H, M, and B, respectively (7). Bisgaard (7) also suggested designating serotypes numerically to avoid confusion and to standardize serotype nomenclature to recognize new serotypes such as serotypes 12 and 13. Serotype 7 was reported to be identical to serotype O/N, and a new serotype, 8, was isolated (84).

Revision of this typing scheme led to the redesignation of Harry's serotypes C and D as types 9 and 10, exclusion of serotype 4, which was not RA, and the identification of 5 new serotypes: 11, 14, 15, 16, and 17 (85). Loh et al. (58) reported serotypes 13 and 17 were identical. They redesignated Harry's type P as serotype 4 and added 3 new serotypes 17, 18, and 19, which were isolated from ducks in Singapore. Two new serotypes, 20 and 21, were isolated from ducks in Thailand (68); one of these, serotype 20, was later excluded because it was determined it was not RA (78). A new serotype isolated from ducks in Thailand replaced serotype 20 (78). All serotypes reacted specifically with homologous-type antisera with the exception of serotype 5, which gave minor cross reactions with serotypes 2 and 9 (58, 84).

Higgins et al. (38) demonstrated that cell lysates of various serotypes showed many bands when subjected to polyacrylamide gel electrophoresis. Most of the bands were common to all serotypes, but some were specific to individual serotypes. An outer membrane protein gene (*OmpA*) that encoded for a 42-kDa major antigenic outer membrane protein (OmpA) was found in all RA reference strains, although some minor genetic differences were observed in different strains (90). Hu et al. (44) demonstrated that *OmpA* is a virulent factor for RA. Tsai et al. (91) reported that all of the RA strains fall into a single cluster based on the phylogenetic analysis of the 16S rRNA gene and that the 16S rRNA gene-based polymerase polymerase chain reaction (PCR) may be a suitable test for screening RA infections.

Recently, most of the RA strains were shown to contain plasmids (13). A 3.9b plasmid carried protein genes

similar to the virulence-associated genes of other bacteria. An insertion sequence element found on a second plasmid may be important in epidemiological studies (96). Yu et al. (99) studied the genomic diversity of pathogenic RA isolates using profiles of plasmid patterns. DNA fingerprinting has been useful in investigating disease outbreaks (26).

Virulence Factors

Several RA virulence factors have been identified that associate with disease severity, including VapD (13), CAMP cohemolysin (19), outer membrane protein A (OmpA) and *P45* (44, 47), nicotinamidase PncA (94), and putative genes associated with lipopolysaccharide (LPS) synthesis (100). In addition, Wang et al. (95) studied the fact that two-component signaling systems (TCS), a basic stimulus-response coupling mechanism for some bacteria, regulates gene expression and virulence in RA.

Pathobiology and Epidemiology

Incidence and Distribution

Riemerella anatipestifer infection occurs worldwide and has been recognized in countries that have intensive duck production (80). RA pathogenic infections in domestic ducks (*Anas platyrhynchos*) have been recently reported in Japan (16).

Wide variation has been observed in the severity of the disease depending on the strain of the organism, age of the host, and route of exposure (35, 86). Often, more than one serotype is responsible for the disease at a single farm or in the same hatch of birds.

Natural and Experimental Hosts

Riemerella anatipestifer infection is a disease that primarily affects domestic ducks and geese. Naturally occurring outbreaks, however, have been reported in turkeys (36, 102). Serious outbreaks in turkeys in the United States and other countries showed that RA is a potential pathogen of domestic turkeys (25, 64, 65, 89). RA also has been isolated from pheasants (11), chickens (75), guinea fowl and quail (65), partridge (98), and other waterfowl (22, 51, 63, 71, 97). It has also been isolated from gulls, budgerigars, guillemots, and pigs (40). RA has been also identified by PCR and rRNA gene sequence in wild bird populations in South Korea (12).

Chickens, geese, pigeons, rabbits, and mice were reported to be refractory to infection with RA; guinea pigs succumbed to inoculation of large doses intraperitoneally (27, 37). However, Heddleston (35) observed that 8×10^6 organisms inoculated into the foot pad killed 5 of 7 1-day-old chicks; 4×10^6 organisms in 2-week-old white Chinese goslings produced signs and lesions similar to those seen in Pekin ducklings.

Ducklings 1–8 weeks of age are highly susceptible. Ducklings younger than 5 weeks of age usually die within 1–2 days after clinical signs appear; older birds may survive longer. The disease is rare in breeder ducks.

Transmission, Carriers, and Vectors

Infection takes place via the birds' respiratory tract (52) or through skin wounds, particularly on the feet (2). RA and RA-like bacteria have been isolated from pharyngeal mucosa of clinically normal ducklings (77). Cooper (18) suggested that in turkeys, the disease may be transmitted via arthropod vectors based on its seasonal occurrence and the apparent affinity of RA for host erythrocytes. The disease can be reproduced most consistently by injecting the organism intravenously, subcutaneously, intraperitoneally, intramuscularly, in the foot pad, or in the infraorbital sinus. Experimental infection by subcutaneous and intravenous routes caused high mortality, whereas no or low mortality was observed in ducklings infected by the oral or nasal route (3, 33, 86).

Incubation Period

The incubation period is usually 2–5 days. Artificial infection of ducklings by the subcutaneous, intravenous, or infraorbital sinus routes resulted in clinical signs and deaths as early as 24 hours postinfection.

Clinical Signs

Signs most often observed are listlessness, ocular and nasal discharge, mild coughing and sneezing, greenish diarrhea, ataxia, torticollis, tremor of head and neck, and coma (Figure 19.12).



Figure 19.12 *Riemerella anatipestifer*. Commercial Pekin duck. Torticollis resulting from meningeal infection. (Jaime Ruiz) (*For color detail, please see the color section*.)

Affected ducklings lie on their backs paddling with their legs and are unable to move. Surviving ducks may be stunted (69). Adverse environmental conditions or concomitant disease often predispose birds to outbreaks of RA infection. Mortality may vary from 5% to 75%; morbidity is usually higher.

Pathology

Gross

The most obvious gross lesion in ducks is fibrinous exudate, which involves serosal surfaces in general, but is most evident on the pericardium, and air sacs (Figure 19.13). Similar lesions have been reported in turkeys and other birds. Fibrinous airsacculitis is common; both abdominal and thoracic air sacs may be involved. The spleen may be enlarged and mottled. Mucopurulent exudate in nasal sinuses and caseous exudate in oviducts also have been observed (21).

Chronic localized infections may occur under the skin and occasionally in the joints. Skin lesions usually take the form of necrotic dermatitis on the lower back or around the vent. Yellowish exudate has been observed between layers of the skin and fat.

Microscopic

Fibrinous exudate on the heart contains a few inflammatory cells, primarily mononuclear cells, and heterophils. In acute cases, severe focal necrosis of the heart muscle is present (Figures 19.14 and 19.15). Liver lesions observed in the acute stage of the disease are mild periportal mononuclear leukocytic infiltration, cloudy swelling, and hydropic degeneration of parenchymal cells. In less acute cases, moderate periportal lymphocytic infiltration may be observed (69). In air sacs, mononuclear cells are the predominant cell type in the exudate. Multinuclear giant cells and fibroblasts may be observed in chronic cases (21). The respiratory tract also may be infected without showing clinical signs. The lungs of infected ducks may be unaffected; there may be interstitial cellular infiltration and proliferation of lymphoid nodules adjacent to parabronchi



Figure 19.13 *Riemerella anatipestifer* infection. Fibrinous epicarditis in a commercial Pekin duck. (Jaime Ruiz) (*For color detail, please see the color section*.)

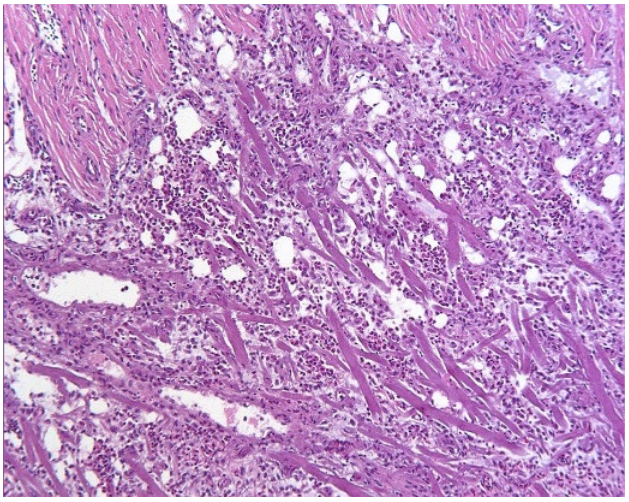


Figure 19.14 *Riemerella anatipestifer* infection. Necrosis of the heart muscle. H&E, ×100. (Susan Williams) (*For color detail, please see the color section*.)

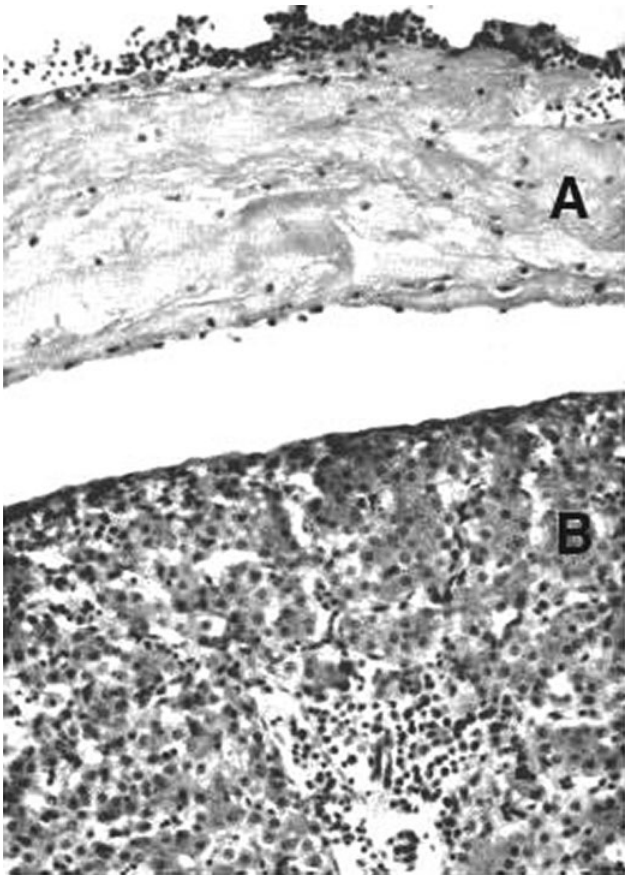


Figure 19.15 *Riemerella anatipestifer* infection. Fibrinous exudate (A) over surface of liver (B). H&E, ×300.

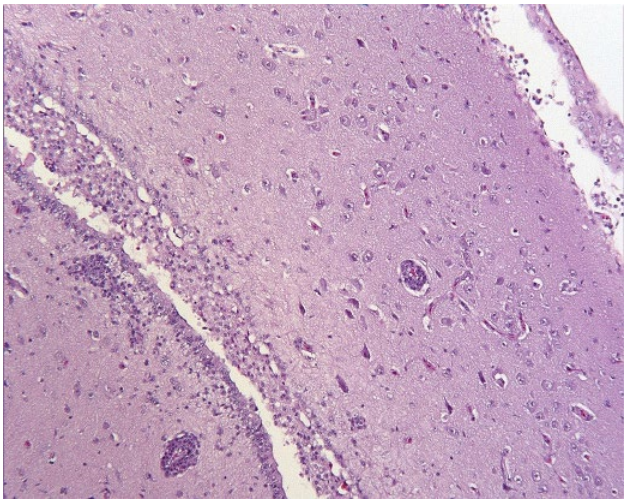


Figure 19.16 *Riemerella anatipestifer* infection. Lymphocytic meningitis. H&E, ×100. (Susan Williams) (*For color detail, please see the color section.*)

(69), or there may be an acute fibrinopurulent pneumonia (27). Infections of the central nervous system can produce fibrinous and lymphocytic meningitis (Figure 19.16). Jortner et al. (49) studied lesions in the central nervous system of naturally infected ducklings and described diffuse fibrinous meningitis with leukocytic infiltration in and around the walls of meningeal blood vessels. Extensive exudate was observed in the ventricular system. Slight to moderate leukocytic and microglial infiltrates were observed in subpial and periventricular brain tissue. Lymphoid necrosis and depletion of lymphocytes have been observed in the spleen and cloacal bursa (86). Biofilm formation has been described as a contributing factor in persistent RA infections (43).

Immunity

Ducklings that recover from the disease are resistant to subsequent infection (2, 27, 37). Inactivated bacterins have been used in ducks to prevent RA infection. Ducks vaccinated with formalin-inactivated bacterins and subsequently challenged with strains representing serotypes 1, 2, and 5 developed homologous, but not heterologous, protection. A trivalent bacterin containing these strains protected against challenge with each serotype, but the protection lasted only a short time (79). Harry and Deb (32) evaluated the effectiveness of several types of bacterins and conducted a field trial with a formalin inactivated bacterin. A single dose of oil-emulsion bacterin provided longer lasting immunity in ducklings (24, 79). Cell-free culture filtrate also has been reported to provide significant protection against homologous challenge (67). Outer membrane proteins *OmpA* and *P45* failed to protect against a virulent challenge, but produced RA-specific antibodies (47). One-day-old ducklings exposed to live avirulent strains by aerosol or through the drinking water were resistant when challenged at 3–6 weeks of age with virulent homologous strains (81). Passive protection of progeny can be achieved by immunizing the female breeder ducks; maternal immunity lasts for about 2–3 weeks (82). RA-specific antibodies were detected in the egg yolk and sera of vaccinated breeder ducks (57); maternal antibodies in the progeny lasted up to 10 days of age. Cell-mediated immunity to RA antigens was transient (similar to vaccination with the bacterin), and live vaccine induced longer lasting protection (38, 81).

Han et al. (29) identified and described an immunogenic protein, chaperonin GroEL, from the outer membrane of RA strain WJ4 using an immunoproteomic assay based on matrix-assisted laser desorption/ionization time of flight mass spectrometry. They found that the *groEL* gene is highly conserved among RA strains; indeed, the DNA sequence identity was more than 97.5% between WJ4 and the 9 additional RA strains.

Fernandez et al. (23) described the importance of interleukin-17A (IL-17A) in the pathogenesis of RA infections in ducks.

Diagnosis

Isolation and Identification of the Causative Agent

Although a presumptive diagnosis may be made from clinical signs and necropsy findings, a definite diagnosis should be based on isolating and identifying RA. The bacterium can be isolated most readily when birds are in the acute stage of the disease. Suitable tissues for culture are the brain, blood from the heart, air sacs, bone marrow, lungs, liver, and exudates from lesions. Samples should be taken aseptically, streaked on blood agar or trypticase soy agar containing 0.05% yeast extract, and incubated in a candle jar at 37 °C for 24–72 hours. Adding newborn calf serum (5%) and gentamicin (5 mg/1,000 mL) to plate media is helpful for isolating RA from contaminated specimens. Isolated colonies should be selected for inoculation of the differential media and identified on the basis of characteristics described in "Etiology." Serotype identification can be established by agglutination and/or AGP reactions with specific antisera. Molecular fingerprinting by restriction endonuclease analysis and repetitive sequence PCR are useful to differentiate RA strains and may be helpful in epidemiological studies (17, 48, 50, 74, 99). Using PCR amplifying 16S rDNA has been reviewed by Qu et al. (72). A rapid assay for detecting RA has been developed based on the *groEL* and *ompA* genes sequence of RA using LAMP (28, 103). In addition, Hu et al. developed a multiplex polymerase chain reaction (m-PCR) that discriminates RA, *Escherichia coli*, and *Salmonella enterica* in clinical samples from diseased

ducks (45). Colloidal gold immunochromatographic strips have been used for detection of RA (41).

Serology

Immunofluorescent procedures can be used to identify RA in tissue or exudate from infected birds (60). Agglutination tests and ELISA can be used to detect serum antibodies. ELISA is more sensitive than agglutination tests but is not serotype-specific (34, 46, 57).

Differential Diagnosis

Riemerella anatipestifer infection should be differentiated from other septicemic diseases caused by *Pasteurella multocida*, *C. anatina*, *Escherichia coli*, *Streptococcus faecium*, and salmonellae. Because these diseases produce gross lesions that cannot be distinguished from those caused by RA, diagnosis must include isolating and identifying the causal organism.

Differential diagnosis also should include chlamydiosis and *O. rhinotracheale*, especially in turkeys.

Intervention Strategies

Management Procedures

The most important aspects of preventing RA include biosecurity, management, and sanitation practices. This includes proper ventilation, especially in houses where ducks are raised in total confinement. Predisposing factors such as stress caused by overcrowding or being exposed to hot or cold weather should be avoided. Strict measures should be taken to prevent the infection from spreading from diseased to healthy flocks. If ducks are raised on wire, the floors should be washed and sanitized periodically to avoid accumulating manure and to reduce exposing healthy birds to infection

Vaccination

Types of Vaccine

Inactivated Bacterins. Inactivated bacterins prevent or reduce mortality caused by RA (32, 52, 79). Because immunity induced by bacterins is serotype-specific, an ideal bacterin should contain cells of the predominant serotypes to provide effective protection. A bacterin containing serotypes 1, 2, and 5 has been used in the United States and Canada. Ducklings are vaccinated at 2 and 3 weeks of age to provide adequate protection up to market age (52). A single inoculation of oil-emulsified bacterin has been reported to produce longer lasting protection, but it may cause unfavorable lesions at the inoculation site (24, 79). Using autogenous bacterins containing virulent field strains provided good protection against homologous virulent challenge.

Live Vaccines. A live RA vaccine developed against serotypes 1, 2, and 5 provided significant protection against experimental or field infections with virulent organisms when administered to day-old ducklings by aerosol or in the drinking water (80). A single vaccination protected ducklings up to at least 42 days of age. The vaccine strains grew in the upper respiratory tract and produced a humoral antibody response. The vaccine was demonstrated to be avirulent to day-old ducklings when administered by aerosol or injected into the infraorbital sinus. The vaccine strains were safe in ducks up to 10 back-passages using the contact-exposure method.

Breeder ducks can be vaccinated with the bacterin or live vaccine to provide protection in progeny through maternal immunity, which may last up to 2–3 weeks of age. Maternally immune ducklings respond successfully to active immunization with a live or inactivated vaccine (82).

Treatment

Riemerella anatipestifer antibiotic resistance profiles change over time (104). Antibiotics and sulfa drugs have been tested to treat RA with varying degrees of success. Sulfamethazine, 0.2%–0.25%, delivered via drinking water or feed, was reported to prevent the onset of clinical signs in ducks experimentally exposed to RA (2). Sulfaquinoxaline at levels of 0.025% or 0.05% in feed was effective in reducing mortality in both field and experimental infections (20, 83). Medicated feeds containing novobiocin (0.0303%–0.0368%) or lincomycin (0.011%–0.022%) were reported to be highly effective in reducing mortality when started 3 days prior to experimental infection. A combination of sulfadimethoxine and ormetoprim, when administered at 0.02%–0.12% levels in feed, prevented or reduced mortality and gross lesions in experimentally exposed ducks (62, 83).

RA resistance to chloramphenicol has been also reported in Taiwan and China (15, 42). Tetracyclines were of little value for treating RA infection (1, 76). Subcutaneous injection of lincomycin-spectinomycin, penicillin, or a combination of penicillin and dihydrostreptomycin were reported to be effective in reducing mortality in artificially infected ducklings (83). Enrofloxacin has been shown to be highly effective in preventing mortality in ducklings when given in drinking water at levels of 50 ppm for the first day followed by 25 ppm for the next 4 days (92). Ceftiofur, a broad-spectrum cephalosporin, reduced mortality in experimentally infected ducklings given a single dose of 2 mg/kg body weight subcutaneously 5 hours after infection (14). Kirby-Bauer tests of 224 RA isolates revealed higher resistance levels for aztreonam, cefepime, oxacillin, penicillin G, ceftazidime, and trimethoprim/sulfamethoxazole (87.8%, 64.3%, 88.6%,

86.9%, 75.9%, and 79.2% resistance, respectively) (104). The lowest resistance rates were observed for amikacin (9.5%), cefoperazone (7.2%), imipenem (3.2%), and neomycin (9.5%). Li et al. (56) studied RA *in vitro* susceptibility to cefquinome, ceftiofur, tilmicosin and florfenicol. They found that cefquinome had the highest risk of selecting resistant mutants among these 4 antimicrobial agents.

Ornithobacterium rhinotracheale Infection

Hafez M. Hafez and Richard P. Chin

Summary

Agent, Infection, and Disease. *Ornithobacterium rhinotracheale* is a Gram-negative bacterium which causes respiratory disease in numerous bird species. Additionally, it is associated with heavy economic losses caused by increased mortality and condemnation rates, drop in egg production, and reduced growth. *O. rhinotracheale* is worldwide in distribution.

Diagnosis. The preferred diagnostic test is isolation and identification of *O. rhinotracheale*. Additionally, antigen detection by polymerase chain reaction and detection of antibodies in the blood is also used.

Intervention. Increased biosecurity practices are primarily used to prevent the introduction and spread of *O. rhinotracheale*. Vaccination using commercial or autogenous inactivated vaccines reduces mortality and condemnation rates.

Introduction

Definition and Synonyms

Ornithobacterium rhinotracheale infection is a contagious disease of birds that causes respiratory distress, mortality, and decreased growth. The severity of clinical signs, duration of the disease and mortality are extremely variable and are influenced by several factors such as concurrent infections, poor management, inadequate ventilation, high stocking density, poor litter conditions, and poor hygiene.

Economic Significance

Ornithobacterium rhinotracheale can be associated with high economic losses in poultry caused by increased mortality and condemnation rates, drop in egg production, or decreased growth.

Public Health Significance

Currently, *O. rhinotracheale* has not been found to be of any public health significance.

History

Ornithobacterium rhinotracheale was first characterized in 1993 by Charlton et al. (9) and subsequently named in 1994 by Vandamme et al. (80) the following year. Since its identification, *O. rhinotracheale* has been isolated from birds in numerous countries throughout the world. For additional earlier references on *O. rhinotracheale*, see (11).

Etiology

Classification

Name and Synonyms

The genus *Ornithobacterium* is a member of the Flavobacteriaceae within the Cytophaga-Flavobacterium-Bacteroides phylum and represents a rather distinct line of descent within this family. Related bacteria are the bird pathogens *Riemerella anatipestifer* and *Coenonia anatina* (79, 80). The family includes Flavobacterium, the type genus, and the genera *Bergeyella*, *Capnocytophaga, Chryseobacterium, Ornithobacterium, Riemerella* and *Weeksella* (6).

Morphology and Staining

Ornithobacterium rhinotracheale is a Gram-negative, nonmotile, highly pleomorphic, rod-shaped, nonsporulating bacterium. From agars, it appears as short, plump rods measuring 0.2–0.9 μm in width and 0.6–5 μm in length (Figure 19.17). Very long rods measuring up to 15 μm can be observed from fluid media.

Growth Requirements

Ornithobacterium rhinotracheale grows aerobically, microaerobically, and anaerobically. The optimal growth temperature is 37°C; however, growth can occur at 30–42°C. The bacteria grows best on 5%–10% sheep blood agar, but readily grows on tryptose soy agar and chocolate agar. No growth occurs on MacConkey agar, Endo agar, Gassner agar, Drigalski agar, or Simmons citrate media. The growth in fluid media can be strain-dependent, and

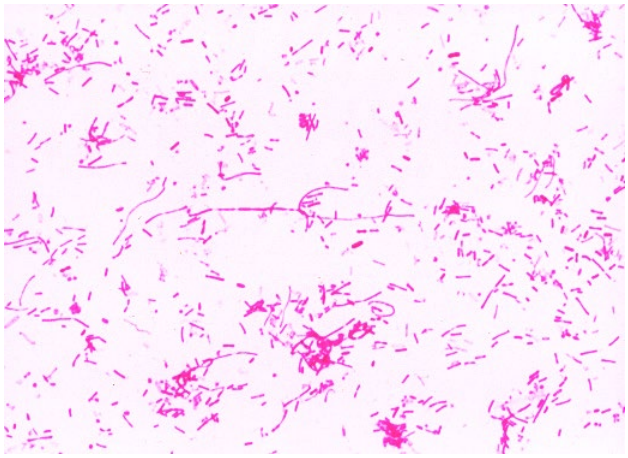


Figure 19.17 *Ornithobacterium rhinotracheale* showing highly pleomorphic nature. Gram stain of bacteria from a 48-hour culture. Gram stain, ×375. (*For color detail, please see the color section.*)

media such as brain heart infusion broth, Pasteurella broth, or Todd Hewitt broth are needed.

Colony Morphology

Ornithobacterium rhinotracheale develop very small, nonhemolytic colonies that are circular, gray to gray-white, sometimes with a reddish glow, and convex with an entire edge. On primary isolation, the colonies of most *O. rhinotracheale* cultures show great differences in size (1–3 mm after 48 hour incubation) but when subcultured, the colony size will become more uniform. Prolonged incubation can give hemolytic activity on sheep blood agar. β-hemolytic activity has also been found in field isolates (61). Zahra et al. (84) characterized 27 small-colony variants (SCVs) of *O. rhinotracheale* isolated from tracheal samples collected from different avian species. Of the 27 *O. rhinotracheale* isolates, 21 (77.8%) showed SCVs in their primary cultures. Five of them showed high levels of stability and were chosen for further characterization with their wild type isolates. SCVs were oxidase negative, whereas their wild type isolates were positive. Growth curves for stable *O. rhinotracheale* SCVs indicated lower growth rates and longer lag phases than for their wild type isolates. In addition, Mirzaie et al. (40) isolated 5 *O. rhinotracheale* from pigeon and all 4 isolates from turkey, which showed smaller colony size, whereas other isolates had larger colonies, when cultured in blood agar. Fifty percent of the isolates with larger colony but none of the isolates with small colony size could agglutinate red blood cells. The relationship between colony morphology and hemagglutination abilities of *O. rhinotracheale* and their virulence is yet to be determined.

Biochemical Properties

Conventional biochemical tests can be inconsistent. Phenotypic characteristics include the production of oxidase, lack of catalase production, lack of motility, no reaction on triple sugar iron agar, production of beta-galactosidase, the inability to reduce nitrate to nitrite, and the inability to grow on MacConkey agar. There some reports of a cytochrome oxidase-negative strain of *O. rhinotracheale* isolated from turkeys in Germany (50, 82).

Susceptibility to Chemical and Physical Agents

Ornithobacterium rhinotracheale strains were completely inactivated by a 0.5% solution containing formic and glyoxyl acid, and a 0.5% solution of an aldehyde-based (20% glutaraldehyde) product after 15 minutes exposure time (24). These preparations were able to inactivate *O. rhinotracheale in vitro* at concentrations of 0.5% within 15 minutes.

Antigenic Structure and Toxins

Currently, no special structures or properties such as pili, fimbriae, plasmids, or specific toxic activities have been reported.

Strain Classification

Antigenicity

Using boiled extract antigens (BEAs) and monovalent antisera in the agar gel precipitation (AGP) and enzyme-linked immunosorbent assay (ELISA) tests, 18 serotypes (A to R) of *O. rhinotracheale* have been determined (71). Serotype A was the most prevalent serotype among chicken isolates (97%) and turkey isolates (61%). There appears to be a correlation between the geographic origin of the *O. rhinotracheale* isolates and their serotype. Serotype C could be isolated only from chickens and turkeys in South Africa and the United States (71). There is no indication of host specificity of the serotypes.

Hafez and Sting (25) compared the efficacy of using different antigen extractions (heat-stable, proteinase K-stable and sodium dodecyl sulfate) for serotyping *O. rhinotracheale* in the AGP and ELISA tests. Results indicate that the AGP test with heat-stable or proteinase K-stable antigen extractions is a suitable method for serotyping. Numerous cross-reactions were seen with the ELISA making it unreliable for serotyping.

Immunogenicity or Protective Characteristics

Using a novel experimental method of combining immune depletion and passive transfer of immunity within the same host, Schuijffel et al. (52), found that the antibody-mediated immunity in chickens was a key component in the protection against *O. rhinotracheale* infection.

Molecular

Amonsin et al. (2) using multilocus enzyme electrophoresis, repetitive sequence based-polymerae chain reacation (PCR), and 16S rRNA gene sequencing demonstrated that the majority of 55 *O. rhinotracheale* isolates recovered from domesticated poultry throughout the world had limited heterogeneity and were represented by a small group of closely related clones. They propose that the bacterium was recently introduced to domesticated poultry from wild bird populations.

Twenty-three isolates of *O. rhinotracheale* from France were tested using the random amplified polymorphic DNA (RAPD) analysis (32). Results showed that this method gave reproducible DNA fingerprints and a good level of discrimination, thus appearing to be another method for typing.

Investigation of several *O. rhinotracheale* isolates from turkeys and chickens originated from Germany, Hungary, and Spain by pulsed-field gel electrophoresis (PFGE) of genomic macro-restriction fragments using the enzyme *SalI* (27). In general, most isolates showed differences in DNA fingerprints although the overall profiles were very similar and a correlation between geographic origin, serotype and DNA fingerprint pattern was observed. In contrast, Koga and Zavaleta (31) investigated 25 *O. rhinotracheale* isolates from broilers, breeders, and layers from several geographic zones of Peru using PCR and repetitive extragenic palindromic PCR (rep-PCR) techniques. All isolates tested had a genetic profile similar to that of the *O. rhinotracheale* type strain (American Type Culture Collection 51463) isolated from a turkey in the United Kingdom. Molecular typing of *O. rhinotracheale* isolates has also been performed using the primers M13 (5′-TAT GTA AAA CGA CGG CCA GT- 3) and ERIC 1R (5′- ATG TAA GCT CCT GGG GAT TCA C -3′) and variations were found between all tested serotypes (29).

Recently, Thieme et al. (63) established an multilocus sequence typing (MLST) scheme for *O. rhinotracheale,* which allows for worldwide comparison of sequence data. The overall identified low genetic diversity among strains isolated from turkeys and chickens, independent of host and geographical origins, suggests that *O. rhinotracheale* has only recently been introduced into domestic poultry and dispersed worldwide. In addition, results clearly showed that *O. rhinotracheale* strains from birds of prey had close genetic relationships to pathogenic strains circulating among turkeys and chickens. On the other hand, the results further indicate that strains isolated from pigeons are genetically distant from all other *O. rhinotracheale* strains and may taxonomically represent their own *O. rhinotracheale*-like species (64).

Pathogenicity

Pathogenicity differences appear to exist between isolates of *O. rhinotracheale*. Three South African *O. rhinotracheale* field isolates inoculated into the caudal abdominal air sacs of 28-day-old broiler chickens showed significant differences in the production of airsacculitis and arthritis (66). In addition, van Veen et al. (78) found that Dutch and South African isolates were more pathogenic than an American isolate in broiler chickens when aerosol challenged.

The pathogenicity of 88 *O. rhinotracheale* isolates, collected in Germany from turkeys and chickens between 2003 and 2006, was examined using the embryo lethality test. In total, 54 isolates (61.4%) were mildly pathogenic, whereas 34 isolates (38.6%) were classified as moderately pathogenic. There was no correlation between serotype and pathogenicity. No highly pathogenic isolates were detected, and no increase in pathogenicity over the years was observed (82).

Soriano et al. found *in vitro* adherence of *O. rhinotracheale* isolates to chicken tracheal epithelial cells (55).

Pathobiology and Epidemiology

Incidence and Distribution

After a first recognition, *O. rhinotracheale* has been diagnosed throughout the world (11).

Natural and Experimental Hosts

Ornithobacterium rhinotracheale has been isolated worldwide from numerous bird species, including chicken, chukar partridge, duck, falcons, goose, guinea fowl, gull, ostrich, partridge, pheasant, pigeon, quail, rook and turkey (11, 83).

In commercial poultry, all ages appear to be susceptible. Many case reports of *O. rhinotracheale* infection describe a concomitant infection with other respiratory pathogens, such as *Escherichia coli* (16, 17, 51), *Bordetella avium* (17), Newcastle disease virus (65), infectious bronchitis virus (18), avian metapneumovirus (30, 38), Avian Influenza H9N2 (5), *Streptococcus zooepidemicus* (45), *Avibacterium paragallinarum* (41), *Mycoplasma synoviae* (85), and *Chlamydia psittaci* (12, 73). Most experimental studies have concluded that, when experimentally inoculated by itself, *O. rhinotracheale* causes minimal pathologic lesions in chickens and turkeys and that the severity of lesions are enhanced when there is a concurrent infection with respiratory viruses or bacteria.

However, some studies report production of pathologic lesions similar to those seen in field cases in chickens and turkeys using *O. rhinotracheale* alone (11, 48, 81).

Transmission, Carriers, and Vectors

Ornithobacterium rhinotracheale infection appears to have become endemic and can affect every new restocking

even in previously cleaned and disinfected poultry houses, especially in areas with intensive poultry production as well as in multiple age farms (26). The infection appears to spread horizontally by direct and indirect contact through aerosols or drinking water. *O. rhinotracheale* was found to survive 1 day at 37 °C, 6 days at 22 °C, 40 days at 4 °C, and at least 150 days at −12 °C (35). The survival *of O. rhinotracheale* at lower temperatures may be associated with the higher incidence of reported infections during winter months. It did not survive 24 hours at 42 °C.

Vertical transmission is suspected based on some reports of the isolation of *O. rhinotracheale* at a very low incidence from reproductive organs and hatching eggs, infertile eggs and dead embryos (15, 62). In addition, *O. rhinotracheale* has been isolated from the ovaries, oviduct, hatching eggs, infertile eggs. However, when *O. rhinotracheale* was inoculated into embryonated chicken eggs, the embryos were killed by the ninth day and *O. rhinotracheale* was not isolated from the eggs suggesting it is not transmitted via eggs during hatching (81). However, van Veen et al. (77), observed that specific pathogen-free broiler chickens placed in a hatcher at a commercial turkey hatchery during hatch showed respiratory tract lesions at postmortem examination that were positive for *O. rhinotracheale* by bacteriological and immunohistological examination.

Incubation Period

Experimental inoculation of 22-week-old turkeys with *O. rhinotracheale* resulted in depression, coughing, and decreased feed intake within 24 hours (57). In 48 hours, turkeys were coughing bloody mucus. Five days postinoculation, the coughing had decreased and surviving turkeys were less depressed.

In experimental infection, broiler chickens were challenged at 14 days or 21 days with an aerosol and observed for 2 weeks postinfection. Pathologic lesions at postmortem investigation in general were mild, with only significant airsacculitis at first week postinfection and severe exudate in the trachea at second week postchallenge (70).

Clinical Signs

The severity of clinical signs, duration of the disease and mortality of *O. rhinotracheale* outbreaks are extremely variable. They can be influenced by many factors such as poor management, inadequate ventilation, high stocking density, poor litter conditions, poor hygiene, high ammonia levels, concurrent diseases and the type of secondary infection.

Clinical signs in broiler chickens generally appear at 3–6 weeks of age with a mortality rate of 2%–10%. Affected birds show listlessness, decreased food intake, reduced weight gains, and transient nasal discharge and sneezing, followed by facial edema (44, 68). *O. rhinotracheale* can also cause sudden death (up to 20% in a couple of days) in young birds with infections of the brains and skull with or without respiratory symptoms (4).

In broiler breeders the disease affects the birds in the laying period, primarily at the peak of production or soon before entering production. There is a slight increase in mortality, a decrease in feed intake, and some mild respiratory symptoms. Mortality is variable and relatively low in uncomplicated cases. There can be a drop in egg production, decrease in egg size, and poor eggshell quality. Fertility and hatchability are unaffected in many cases (21).

In commercial laying-type chickens, decreased egg production, increased misshapen eggs, and increased mortality have been associated with *O. rhinotracheale* infection (59).

Roepke (47) found a higher severity of clinical signs and mortality in older turkeys, and the majority of young infected flocks appeared clinically normal. In many cases young poults are affected between 2 and 8 weeks of age. Mortality ranges between 1% and 15% during the acute phase (8 days), but infections can be accompanied with mortality rates of up to 50% (13). Initial symptoms are coughing, sneezing, and nasal discharge followed, in some cases, by severe respiratory distress, dyspnea, prostration, and sinusitis. These symptoms are accompanied with a reduction in feed consumption and water intake. In turkey breeder flocks, there can also be a decrease in egg production and an increase in the number of unsuitable hatching eggs (13, 68).

Ornithobacterium rhinotracheale has been reported to cause neurological signs or paralysis through arthritis, meningitis, osteitis, and osteomyelitis in chickens and turkeys (17, 60, 68).

Pathology

Gross

In broiler chickens, the common gross lesions include pneumonia, pleuritis, and airsacculitis. At slaughter or postmortem examination, foamy, white, yogurt-like exudate can be seen in the air sacs (predominantly abdominal) (Figure 19.18), most of the time accompanied by unilateral pneumonia (70). Lesions caused by *O. rhinotracheale* can lead to condemnation rates of 50% or more (74, 76). In addition, subcutaneous edema over the cranium with adjacent osteitis, osteomyelitis and encephalitis has been reported in chickens.

In turkeys, there is edema and unilateral or bilateral consolidation of the lungs (26, 27) with fibrinous exudate on the pleura (Figure 19.19). In addition, there could be fibrinosuppurative airsacculitis, pericarditis, peritonitis,

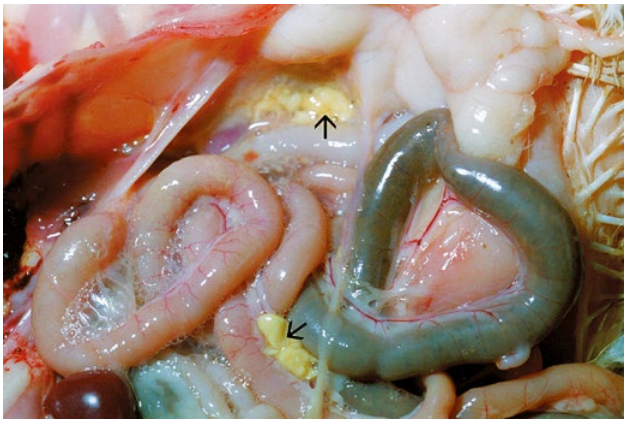


Figure 19.18 Thickened, opaque air sacs with white to yellow exudate (arrows) associated with infection of *Ornithobacterium rhinotracheale* in 9-week-old turkeys. (*For color detail, please see the color section*.)



Figure 19.19 Consolidation of the lungs with fibrinous exudate on the pleura (pleuropneumonia) associated with *Ornithobacterium rhinotracheale* infection in a 16-week-old turkey. (*For color detail, please see the color section*.)

and mild tracheitis. In some cases, swelling of the liver and spleen, as well as degeneration of heart muscles, has been observed. Infections of the joints and vertebrae can be seen in older birds.

Microscopic

Most histologic lesions can be seen in the lungs, pleura, and air sacs. In field cases, the lungs (Figure 19.20) are congested, and throughout the parenchyma, there are

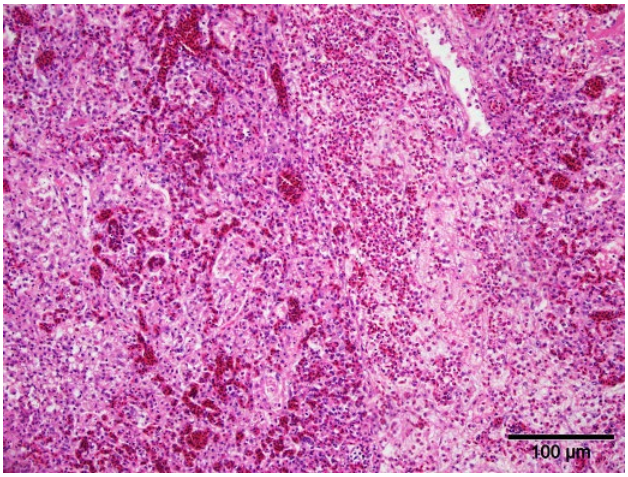


Figure 19.20 Severe fibrinoheterophilic inflammation of lung associated with *Ornithobacterium rhinotracheale* infection in a turkey. H&E, ×20. (S. Stoute) (*For color detail, please see the color section*.)

large collections of fibrin admixed with macrophages and heterophils lying free within the lumen of air capillaries and parabronchi. There are pronounced and diffuse interstitial infiltrates of macrophages with smaller numbers of heterophils. Widespread coalescing foci of necrosis often centered within the lumen of parabronchi with extension of the necrosis into the adjacent parenchyma are present. These necrotic foci usually are filled with dense aggregates of necrotic heterophilic infiltrate or exudate, and there can be scattered small clusters of bacteria seen within the necrotic foci. Numerous blood capillaries can be distended and filled with fibrin thrombi. The pleura and air sacs can be severely thickened and edematous with interstitial fibrin deposits, diffuse heterophilic infiltrate, scattered small foci of necrotic heterophilic infiltrate, and fibrosis.

Immunity

Minimal information is available regarding immunity to *O. rhinotracheale*. The active immunity induced by inactivated vaccines was found to be serotype specific, but live vaccination can induce a degree of cross-protection between some serotypes (54). Passive immunity can be induced for up to 3–4 weeks by maternally derived antibodies.

Diagnosis

A presumptive diagnosis may be made based on clinical signs and necropsy findings. However, a definitive diagnosis must be based on the isolation and identification of *O. rhinotracheale* and/or detection of antibodies (10, 22).

Isolation and Identification of Causative Agent

Bacterial Isolation and Identification

The trachea, lungs, and air sacs are the best tissues from which to isolate *O. rhinotracheale*, though it has been isolated from numerous tissues, including joint, brain, and oviduct. The infraorbital sinus and nasal cavity are also suitable sites for culture, but *O. rhinotracheale* can be masked easily by other bacteria overgrowth.

Fresh tissues and/or swabs should be collected within the first week of infection, and shipped cooled in a transport medium, such as Amies gel medium or Stuart gel medium (43).

Ornithobacterium rhinotracheale can be isolated on common, nonselective blood or chocolate agar. Colonies grow in 24 hours, but it is best to hold inoculated plates for 48–72 hours in air enriched with 7.5%–10% CO_2. Colonies will appear pinpoint to small (approximately 1–2 mm diameter), gray to gray-white, circular, and convex with an entire edge. Gram stain will reveal characteristic pleomorphic Gram-negative bacteria. Colonies are catalase-negative and oxidase-positive. Pure *O. rhinotracheale* cultures have a distinct odor, similar to that of butyric acid.

In contaminated samples with fast growing bacteria, such as *E. coli*, *Proteus* sp. or *Pseudomonas* sp., *O. rhinotracheale* colonies may be overgrown and are difficult to detect in routine investigation. Since most *O. rhinotracheale* isolates are resistant to gentamicin the use of 10 μg of gentamicin per milliliter of blood agar medium is recommended in an effort to isolate *O. rhinotracheale* from contaminated samples. Blood agar containing 5 μg/mL of gentamicin and polymyxin B was also effective (22).

The API-20NE system (bioMérieux, France) was found useful for the identification of *O. rhinotracheale* (71). Ninety-nine percent of isolates were found to have biocodes 0-2-2-0-0-0-4 (β-galactosidase, urease and oxidase positive) (65%) or 0-0-2-0-0-0-4 (β-galactosidase and oxidase positive) (34%). For those isolates that were positive for the arginine dihydrolase test, biocodes 0-3-2-0-0-0-4 or 0-1-2-0-0-0-4 were found. Also isolates with the code 0-0-2-0-0-0-0 (β-galactosidase positive and urease as well as oxidase negative) are highly suspected (50).

The rapid slide agglutination test also has been used for diagnostic purposes. However, auto-agglutinable strains were regularly found (3).

The AGP test, using known positive antisera, is currently used to identify and serotype *O. rhinotracheale* isolates. Conventional and real-time PCR tests have been developed and are used for the identification of suspect isolates (1, 29).

Antigen Detection

As mentioned above, PCRs was used for the detection of *O. rhinotracheale* in tracheal swabs of heavily infected birds (1, 29). In addition, the immunofluorescence antibody test and immunohistochemical staining were used to detect *O. rhinotracheale* in chickens (72). Subsequently, van Veen et al. (76) found that the immunofluorescence assay and the peroxidase-antiperoxidase test were equally sensitive. Using these tests, they were able to identify a higher percentage of *O. rhinotracheale*-infected chicken broiler flocks at slaughter, when compared with conventional diagnostic methods (i.e., serology and/or bacteriology).

Serology

Serology is useful for flock monitoring or as an aid in the diagnosis of *O. rhinotracheale* infection.

The serum plate agglutination test (SPAT) has been used as a rapid test for the detection of antibodies against *O. rhinotracheale* (19, 28). One SPAT was developed using a nonserotyped Minnesota isolate of *O. rhinotracheale* and was reported to have good sensitivity and specificity (3). However, in another study (36) the SPAT detected only 65% of infected birds during the first 2 weeks of infection and declined significantly thereafter. This suggests that the SPAT detects IgM antibodies, which are efficient in agglutination with specific antigens. Erganis et al. (19) developed a dot immunobinding assay (DIA) which appeared to be less sensitive than other agglutination tests.

ELISAs have been developed using different serotypes and extracted antigens of *O. rhinotracheale*. Boiled extract antigens, which are used for serotyping, tend to give the best results for serotype-specific tests (68). Conversely, sodium dodecyl sulfate (SDS)-antigen extraction (25) and extracted outer membrane proteins of *O. rhinotracheale* (36) will result in more cross-reactions allowing detection of antibodies against different serotypes with 1 test. Field surveys using these ELISAs or commercial ELISA kits have been useful for monitoring flocks and the diagnosis of *O. rhinotracheale* infections (23, 46, 49, 67). Commercial ELISA kits are currently available and able to detect antibodies against all tested *O. rhinotracheale* serotypes.

The effect of amoxicillin treatment on the antibody kinetics after experimental infection showed that immediate treatment did not influence the antibody response, whereas treatment that started 7 days postinoculation resulted in a lower antibody response (11).

Differential Diagnosis

Respiratory lesions associated with *O. rhinotracheale* are similar to those caused by numerous bacteria, such as *E. coli, Pasteurella multocida*, *Riemerella anatipestifer*, *Avibacterium paragallinarum*, *Coenonia anatine*, and *Chlamydia psittaci*.

Intervention Strategies

Management Procedures

Ornithobacterium rhinotracheale appears to be highly contagious and strict biosecurity measures should be followed to prevent its introduction into a flock. However, after a ranch is infected, *O. rhinotracheale* becomes endemic, especially in multiple-age farms and in areas with intensive poultry production.

Vaccination

Types of Vaccines

Several attempts to combat infection by using several types of vaccines such as bacterins, live vaccines, and subunit recombinant vaccines under experimental and field conditions have been carried out with various results (11, 20).

Inactivated Vaccines. Vaccination of broiler chickens with inactivated vaccines was found to be effective (69), but is probably impractical in most, because the efficacy of the vaccine is negatively influenced by the presence of maternal antibodies. On the other hand, vaccination of broiler breeders with mineral oil adjuvant inactivated vaccines stimulated the development of high maternal antibodies (7, 8), which were sufficient to protect progeny against experimental challenge for up to 4 weeks of age (69) and produced lower mortality and condemnation rates in the progeny from vaccinated breeders (8). Using inactivated vaccines with mineral oil adjuvant, layers vaccinated at 8 weeks of age with a booster dose at 12 weeks of age, produced an early immune response and reduced the incidence of airsacculitis and pneumonia (42).

Vaccination of meat turkey flocks using monovalent or trivalent bacterins in field trials resulted in production of antibodies for a short duration. Nonetheless, the mortality and condemnation rates were lower in the vaccinated group when compared with the unvaccinated group. Vaccination of young turkeys with autogenous bacterins successfully reduced the number of outbreaks (11).

Because of the possibility of infection by several serotypes, it may be necessary to use different serotypes in the vaccines.

Live Attenuated Vaccines. A temperature-sensitive mutant of *O. rhinotracheale* was developed and used as a live vaccine in turkeys (33, 34). Turkeys were vaccinated at 5 days of age via the drinking water, and challenged 7 weeks postvaccination. Vaccinated birds had a significantly lower mean score for gross lesions when compared with unvaccinated birds, as well as a lower rate of reisolation and number of colony forming units of *O. rhinotracheale* per gram of lung tissue.

Sprenger et al. (58) vaccinated 6-week-old turkeys either intranasally with a live vaccine or subcutaneously with a killed *O. rhinotracheale* vaccine, and challenged them intratracheally with live *O. rhinotracheale* at 14 or 21 weeks of age. Airsacculitis and pneumonia occurred less frequently in vaccinated birds than in unvaccinated birds after challenge, and *O. rhinotracheale* was recovered from unvaccinated, challenged birds, but not from vaccinated, challenged or unchallenged birds.

Administration of an autogenous live vaccine (oral route) in 6-week-old turkeys resulted in a decrease in pathologic lesions and mortality when the birds were older. It is interesting to note that the birds were simultaneously spray vaccinated with a live avian paramyxovirus-1 vaccine without any problems (11).

Recombinant Vaccines. Schuijffel et al. (52) demonstrated that cross-protective immunity against different *O. rhinotracheale* serotypes can be induced by live vaccination in chickens. The genes encoding 8 cross-reactive antigens (Or01, Or02, Or03, Or04, Or11, Or77, Or98A, and Or98B) were amplified, cloned in an expression vector, and expressed in *E. coli*. Purified recombinant proteins with a molecular mass ranging from 35.9 to 62.9 kDa were mixed and tested as a subunit vaccine for protection against challenge with homologous and heterologous *O. rhinotracheale* serotypes. Subunit vaccination resulted in the production of antibodies reactive to the recombinant proteins on Western blot, and this eight-valent vaccine provided both homologous and heterologous protection against *O. rhinotracheale* challenge in chickens. In a subsequent study (53), they found that these 8 antigens are highly conserved among different *O. rhinotracheale* serotypes, but the different antigens were not expressed by all serotypes. In addition, their 4 component subunit vaccine was able to protect chickens against challenge with a heterologous *O. rhinotracheale* serotype.

Treatment

The treatment of *O. rhinotracheale* infections with antibiotics is very difficult because of the variable susceptibility of strains. *O. rhinotracheale* can acquire reduced susceptibility or resistance against antibiotics such as amoxicillin, ampicillin, doxycycline, enrofloxacin, flumequine, gentamicin, lincomycin, trimethoprim-sulfonamide, tetracycline and tylosin (14, 37, 39, 56, 75). Susceptibility can be dependent on the antibiotic regime used by the poultry industry in various geographical locations.

In 1996, Hafez reported that water medication using amoxicillin at a dose of 250 ppm for 3–7 days gave satisfactory results in most cases, and application of chlortetracycline at a dose of 500 ppm in drinking

water for 4–5 days appeared to be effective (21). However, further studies have shown that treatment with amoxicillin is no longer efficacious (39). In some cases, injections with various tetracyclines and penicillins were found to be effective.

Sixty-eight *O. rhinotracheale* isolates from the United States were found susceptible to ampicillin, erythromycin, penicillin, spectinomycin, and tylosin, and 54 of the 68 isolates were susceptible to neomycin, sarafloxacin, and tetracycline. It was also found that German isolates had a significantly lower susceptibility to erythromycin and sarafloxacin when compared with isolates from the United States. Furthermore, Zahra et al. (84) found that antibiotic sensitivity of SCVs *O. rhinotracheale* isolates had higher minimum inhibitory concentration (MIC) values in comparison with their wild type isolates. They suggested that successful antibiotic treatment of respiratory diseases associated with *O. rhinotracheale* must take into consideration the resistance patterns of *O. rhinotracheale* SCVs.

Bordetellosis (Turkey Coryza)

Karen B. Register and Mark W. Jackwood

Summary

Agent, Infection, and Disease. Bordetellosis is an acute, highly contagious respiratory disease of young turkeys. *Bordetella avium* was once considered the sole etiologic agent but *B. hinzii* is now also known to be a potential cause. Mortality in uncomplicated outbreaks is low but 80%–100% morbidity is typical and the economic impact can be substantial. Mortality may be at least 50% under poor management conditions or when additional pathogens are present. Bordetellosis occurs in nearly all areas of the world where turkeys are intensively raised.

Diagnosis. Diagnosis is based on clinical signs and lesions and isolation of *B. avium* or *B. hinzii* from the respiratory tract.

Intervention. *B. avium* vaccines may reduce disease severity or delay onset but they fail to prevent infection and are not widely used.

Introduction

Definition and Synonyms

Bordetellosis in poultry is a highly contagious disease affecting primarily the upper respiratory tract. Once thought to be caused by only *Bordetella avium*, it is now apparent that a closely related bacterium with which it has frequently been confused, *B. hinzii*, may also cause bordetellosis in turkeys (97). The disease is still at times referred to as turkey coryza. Other synonyms that have been largely abandoned are alcaligenes rhinotracheitis (ART), adenovirus-associated respiratory disease, acute respiratory disease syndrome, *B. avium* rhinotracheitis (BART), and turkey rhinotracheitis. The variety of names used for this disease reflects the initial confusion that surrounded its etiology.

Economic Significance

There are few data available addressing the current prevalence of bordetellosis in poultry and no detailed analysis of its economic impact. The number and severity of outbreaks appears to have lessened in recent years but the disease remains a major concern for turkey producers. Impaired growth and high mortality resulting from secondary colisepticemia probably cause significant losses to the turkey industry. *B. avium* or *B. hinzii* alone is not known to cause disease in chickens, but related losses may occur when *B. avium* infections are complicated by prior or concurrent infection with other pathogens. It is not known whether *B. hinzii* may also contribute to losses in cases of complicated infections.

Public Health Significance

For many years it was thought that *B. avium* infected only avian hosts but it is now apparent that the bacterium is also a rare, opportunistic human pathogen (41, 121). *B. hinzii* is likewise an opportunist in humans, occasionally reported as a cause of respiratory disease, bacteremia or infections of the digestive system (29, 121). Some evidence suggests that contaminated poultry or other avian reservoirs may be among the sources from which *B. hinzii* can be transmitted to humans.

History

Turkey rhinotracheitis (coryza) attributable to a bacterium of the genus *Bordetella* was first reported in Canada in 1967 (32) and then in Germany, nearly a decade later (45). Outbreaks with a similar presentation occurring in the United States and elsewhere were frequently associated with adenovirus, infectious bursal disease virus (IBDV) or a variety of other bacterial or viral agents but none were consistently capable of reproducing the

disease in experimentally infected turkeys (see the 12th edition of *Diseases of Poultry* for more detail). In the early 1980s, several investigators reported *Alcaligenes faecalis* as the etiologic agent (57, 114). Additional characterization of isolates revealed dimorphic colony morphologies and 2 distinct biochemical and physiological profiles, which served as the basis for classification as either *A. faecalis* Type I or *A. faecalis* Type II (64, 103). Type I isolates were noted to be uniformly pathogenic in turkey poults. A subsequent, more exhaustive study of the phenotypic and genetic features of 28 disease-causing isolates led to their classification as a novel taxon, *B. avium* (67). It was further recognized that *B. avium* was the proper classification of pathogenic isolates previously identified as *A. faecalis* Type I (64). Type II isolates, only a few of which were reported to be disease-causing in turkeys (103), were informally designated *B. avium*-like so as to differentiate them from *B. avium* (62, 65). In 1995, *B. avium*-like isolates were formally re-classified as a unique *Bordetella* species, *B. hinzii* (135).

In 1985, a disease of turkeys also referred to as turkey rhinotracheitis appeared in England and Wales (2). While initially adding to confusion surrounding the etiology of what is now recognized as bordetellosis, the cause of that disease was later shown to be a pneumovirus (21) (see Pneumovirus Infection in Chapter 3).

Etiology

Classification

The major etiologic agent of bordetellosis in turkeys is *B. avium*. Disease is exacerbated by environmental stressors or when birds are simultaneously infected with other respiratory pathogens. Experimental transmission of the disease to susceptible poults clearly established the causative agent as a small Gram-negative bacillus (113). The bacterium, tentatively identified at that time as *A. faecalis*, closely resembled *B. bronchiseptica*, except for its failure to split urea. A systematic investigation by Kersters et al. (67) that evaluated an extensive array of phenotypic and genetic characteristics determined that the bacterial cause of turkey rhinotracheitis was a previously unrecognized species of *Bordetella*, given the name *B. avium*. Further molecular characterization of *B. avium* confirmed its unique taxonomic position among species of the *Bordetella* and *Alcaligenes* genera (14, 46, 65, 84, 108).

More recently, it has been shown that some isolates of *B. hinzii* also cause clinical signs consistent with bordetellosis in experimentally infected turkeys (97), but there are no data regarding its prevalence in the field and, thus far, only anecdotal accounts of *B. hinzii* outbreaks. For nearly 15 years after its recognition as a distinct taxon there was no readily available technique for reliably distinguishing between *B. hinzii* and *B. avium*. Re-evaluation of disease-causing isolates initially classified as *B. avium*, *B. avium*-like, *B. bronchiseptica* or *A. faecalis* identified several as *B. hinzii* (98), suggesting that *B. hinzii* infections may have been underdiagnosed for a considerable period of time.

Morphology and Staining

Bordetella avium and *B. hinzii* are both Gram-negative, nonfermentative, motile, aerobic bacilli (64, 67, 135). Filamentous forms of *B. avium* have been observed following growth in broth media high in nutrients (24). Some additional characteristics of these bacteria are detailed in Table 19.2.

Growth Requirements

Bordetella avium and *B. hinzii* grow readily on MacConkey, Bordet-Gengou, veal infusion, trypticase soy blood agar, brain heart infusion (BHI), and many other solid media; *B. hinzii*, but not *B. avium*, grows on minimal essential medium (64, 67, 106, 135). Trypticase soy or BHI broth is generally used when a liquid medium is required. Since *B. avium* is a strict aerobe, agitation of cultures to provide aeration is recommended (7). Leyh et al. (69) have developed a defined minimal medium for growth of *B. avium* and detection of auxotrophic mutants. For both *B. avium* and *B. hinzii* the optimal growth temperature is 35 °C– 37 °C (106), but incubation at 45 °C is lethal to *B. avium* (7).

Colony Morphology

Most strains of *B. avium* produce small, compact, translucent, pearl-like colonies (type I) with entire edges and glistening surfaces (67). Type 1 colonies are typically 0.2–1 mm in diameter after 24 hours of incubation and 1–2 mm in diameter after 48 hours of incubation. Many isolates develop a slightly raised, brown-tinged center when grown for 48 hours on MacConkey agar (Figure 19.21). A small percentage of strains dissociate into a rough colony type with a dry appearance and a serrated irregular edge (58). Rough colonies were found to be nonpathogenic (58). A third colony type has also been reported (46), characterized as circular and convex, with an entire edge, smooth surface, and larger size than type I colonies.

Two colony types have been described for *B. hinzii* (135). Following 48 hours of incubation at 37 °C in 5% CO_2, some isolates form colonies described as round, raised, glistening and grayish, and about 2 mm in diameter. Under these same conditions colonies of other

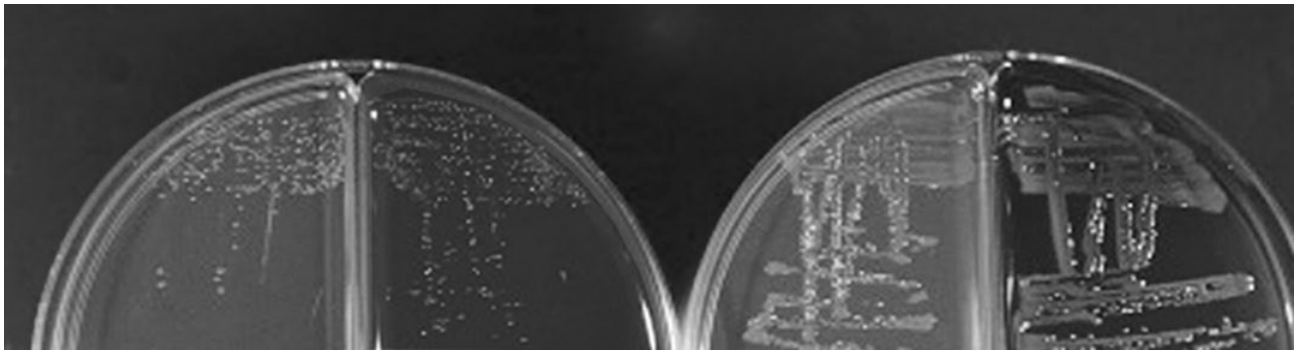


Figure 19.21 Colonies of *Bordetella avium* strain 002 (left) and *B. hinzii* strain 128 (right) grown on MacConkey and blood agar, for 48 hours at 37 °C.

Table 19.3 Biochemical properties of *Bordetella avium* and *Bordetella hinzii*.

Biochemical Test	*B. avium*	*B. hinzii*
Oxidase (Kovac's reagent)	Positive	Positive
Catalase	Positive	Positive
Urease	Negative	Variable[1]
Nitrate reduced to nitrite	Negative	Negative
Growth on MacConkey agar (lactose not fermented)	Positive	Positive
Triple sugar iron agar	Alkaline slant, no change in butt	Alkaline slant, alkaline butt
Alkali production from:		
Malonamide	Negative	Positive
Malonate	Negative	Positive
Valerate	Negative	Positive

[1] Most isolates are negative but some may test positively, depending on the method used.

isolates appear flat, dry, and crinkled and may grow up to 5 mm in diameter.

Biochemical Properties

Bordetella avium and *B. hinzii* are nonfermenting bacteria and are generally unreactive in biochemical tests. Biochemical properties of these organisms are listed in Table 19.3 (67, 106, 135).

Susceptibility to Chemical and Physical Agents

Most commonly used disinfectants appear to kill *B. avium* when applied according to manufacturers' recommendations. Survival of *B. avium* is prolonged by low temperatures, low humidity, and neutral pH (19). On simulated carrier materials, such as dust and feces from turkey houses, the organism survived 25–33 days at 10 °C with a relative humidity of 32%–58%, whereas at 40 °C with similar humidity the organism survived less than 2 days (19). Survival for at least 6 months in undisturbed damp litter has been reported (9). In BHI broth culture, the bacterium is killed within 24 hours at 45 °C (7). Survival may be greatly prolonged at 10 °C on smooth surfaces such as glass or aluminum (19). Fumigation of an unclean room with methyl bromide effectively stopped transmission of acute bacterial rhinotracheitis to day-old poults, but the investigators did not specifically identify the causative agent (112). *B. hinzii* has been shown to remain viable and grow in soil at 25 °C (40). There are otherwise no studies addressing the susceptibility of *B. hinzii* to most chemical disinfectants and no information as to its ability to survive within or on the surface of various materials or in different environments. A recent report detailing the ability of various bacteria of laboratory animal origin to survive in cage bedding and following treatment with hydrogen peroxide vapor is stated to include a mouse isolate of *B. hinzii* (12). Subsequent analysis revealed the true identity of the isolate to be *Bordetella pseudohinzii*, a novel species only recently recognized as distinct from *B. hinzii* (55).

Several studies have evaluated antibiotic resistance patterns in *B. avium* but it is difficult to compare results because there is no standard methodology. Most isolates appear to be sensitive *in vitro* to a variety of antibacterials but regional differences may exist (10, 39, 126). Resistance to streptomycin, sulfonamides, and tetracycline by some strains of *B. avium* is encoded on up to 6 plasmids ranging in size from 12 to 51.5 kb (10, 22, 87). The absence of penicillin binding protein 3 in some isolates has been postulated to underlie their resistance to aztreonam (10). Treatment of *B. avium*-infected turkeys with oxytetracycline administered parenterally or by aerosol resulted in either no effect or only a transient reduction in bacterial numbers, even though the strain of *B. avium* used was sensitive to oxytetracycline *in vitro* (30, 115, 134). Human isolates of *B. hinzii* have been reported as resistant to a number of antibiotics, including most β-lactams, fluoroquinolones, and many cephalosporins (54). The few

turkey isolates so far evaluated have resistance profiles similar to those found in *B. avium* (10)

Antigenic Structure and Toxins

The antigenic structure of *B. avium* and related bacteria has been studied by agar gel precipitation, cross agglutination, and Western immunoblotting (7, 10, 34, 42, 63, 64, 67). All evidence indicates that *B. avium* isolates from various sources are closely related antigenically. Using antisera produced in rabbits, Kersters et al. (67) identified 6 different surface antigens, 3 of which were cross-reactive among 3 strains of *B. avium*. They additionally demonstrated a limited degree of antigenic relatedness with *B. bronchiseptica, Alcaligenes denitrificans* and *Achromobacter xylosoxidans* but no cross-reactivity with *A. faecalis*. Using convalescent serum and tracheal washings in immunoblotting procedures, Hellwig and Arp (42) found that antibodies in infected turkeys recognize at least 8 outer-membrane proteins of *B. avium*, ranging in size from 14 to 116 kDa. A 21 kDa protein appeared to be the most immunogenic, eliciting increasing levels of both local and systemic antibody for up to 4 weeks after infection. Gentry-Weeks et al. (34) identified 6 outer-membrane proteins, from 21–56 kDa, that were reactive with convalescent sera from *B. avium*-infected turkeys and/or sera from a rabbit immunized with *B. avium*. The 21 kDa protein, perhaps analogous to that reported by Hellwig and Arp (42), was shown to be a member of the surface-exposed, highly immunogenic OmpA family of proteins, which often play important roles in various aspects of pathogenicity. Little information exists regarding the antigenic structure of *B. hinzii* but sera from turkeys infected or immunized with *B. avium* cross-react extensively with protein preparations from *B. hinzii* (10, 64). Similar cross-reactivity was demonstrated with sera from rats immunized with *B. avium* when tested by enzyme-linked immunosorbent assay (ELISA) using *B. hinzii* as the source of antigen (16). Cross-reactivity appears to be unidirectional, because ELISA values were low when antigen preparations from *B. avium* were tested with sera from rats immunized with *B. hinzii*.

Other studies have used sodium dodecyl sulfate-polyacrylamide gel electrophoresis to compare protein patterns among various isolates of *B. avium* and between *B. avium* and related bacteria (43, 67, 68, 135). Leyh and Griffith (68) examined the outer-membrane protein profiles of 50 *B. avium* isolates and found 2 major sarkosyl-insoluble proteins of 21 and 37 kDa, and at least 13 other lesser proteins. *B. avium* was found to have an electrophoresis profile distinctly different from those of both *B. hinzii* and *B. bronchiseptica*. A comparison of whole-cell proteins from 13 isolates each of *B. avium* and *B. hinzii* similarly revealed species-specific patterns, despite many similarities in the overall profiles (135). Varley and Carter (136) compared whole-cell protein profiles from 7 *Bordetella* turkey isolates obtained in the United Kingdom during the early 1980s with profiles from reference strains of *B. avium*, *B. bronchiseptica*, and *A. faecalis*. Only the *A. faecalis* profile was distinct from all others. It was concluded that *B. avium* was the proper classification of the 7 turkey isolates, although only minor differences could be discerned in the profile of *B. bronchiseptica*.

Three toxins have been definitively identified in *B. avium*; none have so far been documented in *B. hinzii* but additional study is needed (Table 19.2). These toxins are considered to be virulence factors and their activities are described in that section.

Strain Classification

Phenotypic Characteristics

Serotyping based on fimbrial antigens has been used to classify some species of *Bordetella*, but *B. avium* is reactive with sera representing only 1 of the fimbrial types (83) and no similar analysis has been reported for *B. hinzii*. A classification scheme for *B. avium* based on cross-agglutination absorption testing has been described (67) but it has not been adopted for general use and there is currently no method for discriminating among isolates of *B. avium* or those of *B. hinzii* on the basis of phenotypic properties.

Several reports from the 1980s suggested there may be isolate-specific differences in pathogenicity for the etiologic agent of bordetellosis in turkeys, which was classified at that time as *A. faecalis* (14, 44, 64, 103, 105). In hindsight, it seems likely that initial confusion as to the true etiology of the disease largely explains these observations. Uniformly pathogenic isolates, formerly referred to as Type 1 or Group 1 *A. faecalis* or as *Bordetella*-like, were subsequently recognized as the novel taxon *B. avium* (67). Isolates originally classified as Type II *A. faecalis* or as *B. avium*-like, only a portion of which appear to be pathogenic in turkey poults, were later assigned to a new species designated *B. hinzii* (135). Herzog et al. (44) noted that the proportion of poults developing tracheal lesions after infection with "*A. faecalis*" ranged from 40% to 100% depending on which of 3 isolates was used. However, the criteria used for identification of isolates are not given and sera from turkeys infected with the least pathogenic isolate were minimally cross-reactive with antigen preparations from the 2 more pathogenic isolates. Thus, it cannot be known with certainly whether all the isolates used in this study are *B. avium*.

Genetic and Molecular Characteristics

Restriction endonuclease analysis (REA) of chromosomal DNA has been used to characterize isolates of both *B. avium* and *B. hinzii* (104). Species-specific restriction fragment patterns were obtained using the

enzyme *Hin*fI and the method also clearly distinguished these *Bordetella* species from *A. faecalis*. Digestion of DNA with *Dde*I provided additional discriminatory power. Overall, 16 *Hin*fI/*Dde*I fingerprint profiles were identified among 42 isolates of *B. avium* whereas 7 fingerprint profiles were found among 15 isolates of *B. hinzii*. Ribotyping with the enzyme *Pvu*II also reliably discriminates between *B. avium* and *B. hinzii* (98). Although the results from ribotyping are more easily interpreted than those from REA, only REA appears to be sufficiently discriminatory for routine use as an epidemiologic tool.

A multilocus sequence typing method (MLST) has been developed for the "classic" *Bordetella*, i.e., *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* (23), and a related database is publically available (https://pubmlst.org/bordetella/). BLAST queries of the GenBank nucleotide and genome databases identify orthologs of the 7 genes used in the MLST scheme in both *B. avium* and *B. hinzii*. Nonetheless, because their sequences are relatively divergent compared with those from the classic *Bordetella*, the polymerase chain reaction (PCR) primers specified for amplification and sequencing of the MLST gene targets are not optimal for use with *B. avium* and *B. hinzii*. Thus far, only a few isolates of *B. avium* have been fully typed (https://pubmlst.org/bordetella/).

Only 1 complete genome sequence is available for *B. avium*, from the extensively studied isolate 197N (108). Almost one-third of the predicted genes have no identifiable orthologs in *B. bronchiseptica* and many of the unique genes appear to encode secreted or cell surface proteins that may be involved in interactions defining avian host specificity. A draft genome sequence from a *B. avium* isolate causing temporomandibular rigidity in a cockatiel was recently reported (86). A thorough comparative analysis has not been undertaken but genetic features of this isolate are largely congruent with those of 197N and other partially characterized isolates.

Two complete genome sequences are currently available for *B. hinzii*, both from human isolates (137). Also available are draft genome sequences from 3 additional human isolates, 3 from turkeys, and 1 each from a chicken and a rabbit (96, 109). No comprehensive comparisons among these isolates have been reported, nor have any detailed comparisons been made with genome sequences from *B. avium*.

Virulence Factors

As for several other *Bordetella* species, *B. avium* has a strong tropism for cilia of the respiratory epithelium (3, 64). This characteristic has also been observed for disease-causing isolates of *B. hinzii* (97). Although fimbriae have been implicated in attachment of *B. avium* to turkey tracheal explants *in vitro* (43, 63, 73), morphologically similar fimbriae were also noted on adhesion-defective mutants and on nonadherent, "*B. avium*-like" isolates (43, 63). Orthologs of genes encoding pili in *B. avium* are present in *B. hinzii* (71) but there has been no direct demonstration of related structures on the bacterium nor is anything known regarding their possible contribution to attachment. It seems likely that multiple adhesins of *B. avium*, and perhaps *B. hinzii*, contribute to attachment *in vivo*, as has been demonstrated for other species of *Bordetella* (94). Additional attachment factors of *B. avium* may include antigenically distinct fimbriae encoded by genes within a second fimbrial locus (73) as well as the autotransporter protein Baa1 (122). Filamentous hemagglutinin (FHA), a 220 kDa cell surface and secreted protein, is an important adhesin and virulence factor for the classic *Bordetella*. A similar but highly divergent FHA-like protein produced by *B. avium* is essential for virulence but appears not to contribute to tracheal cell adherence (119). Genes predicted to encode similar FHA-like proteins are found in the genomes of several *B. hinzii* isolates (71, 109, 137). Biofilm formation, which may contribute to adhesion and/or persistence, has been demonstrated for *B. avium* although the components that comprise the biofilm matrix appear to be distinct from those of biofilms produced by other *Bordetella* species (80).

Hemagglutination (HA) of guinea pig erythrocytes by *B. avium* correlates closely with virulence (33, 64, 128). HA-negative mutants are also impaired in tracheal attachment, both *in vitro* and *in vivo* (6, 127, 128), and reversion or complementation of the mutations restores their original phenotypes (6, 127). Although it seems clear that HA is not mediated by fimbriae (63) nor by the FHA-like protein (119), various investigators have reached different conclusions as to the identity of the hemagglutinin. Moore and Jackwood (85) identified a 41 kDa protein that binds directly to the surface of guinea pig erythrocytes and found that monoclonal antibodies reactive with the protein prevented HA. Treatment of intact cells of *B. avium* with either proteinase K or periodic acid also blocked HA, leading these investigators to conclude that the hemagglutinin is a carbohydrate closely associated with the 41 kDa surface protein. More recently, Temple et al. (127) demonstrated that the products of 2 adjacent genes, *hagA* and *hagB* (predicted to be ~59 kDa and ~141 kDa, respectively), are required for HA and that antibodies specific for HagB block both HA and attachment to turkey tracheal rings. Bioinformatic analysis predicts that HagA may facilitate export of HagB or otherwise serve to properly localize the protein. These authors note their results do not rule out the possibility that additional factors may participate in HA, including the 41 kDa protein characterized by Moore and Jackwood (85). *B. hinzii* does not agglutinate guinea pig erythrocytes and neither *hagA* nor *hagB* orthologs are apparent in any *B. hinzii* genome sequence. Thus, it appears that

adhesins of importance for *B. hinzii* are likely to be distinct from those of *B. avium*.

Lipopolysaccharide (LPS) is a known virulence factor for several *Bordetella* species, including *B. avium*. Spears et al. (120) showed that mutations in the *B. avium* genes *wlbA* and *wlbL*, part of an LPS biosynthesis locus, alter the LPS profile and dramatically decrease colonization of turkey poults, binding to turkey tracheal rings *in vitro* and survival in 50% turkey serum. LPS has also been implicated in the induction of apoptosis and nitric oxide synthase in ciliated cells from turkey tracheal explants following exposure to *B. avium* (81). The structure of lipid A, the LPS component that mediates toxic activities, differs in *B. avium* as compared with *B. hinzii* (90). Loci encoding the O–antigen, the outermost domain of LPS, are also distinct in these 2 species (71). Whether or how these differences may affect virulence traits is not understood.

Several relatively well-characterized toxins also contribute to the virulence of *B. avium*. A search for toxins was prompted by the observation that inoculation of turkey tracheal organ cultures with disease-causing isolates resulted in acute cytotoxicity and ciliostasis (75). The first toxin to be described was a heat-labile protein lethal for turkey poults (101) that was later shown to produce necrotic and hemorrhagic lesions after intradermal or intraperitoneal injection (100, 102). Further characterization revealed the toxin to be a cell-associated, 155 kDa protein functionally analogous to the dermonecrotic toxin (DNT) produced by the classic *Bordetella* (33). A role for the *B. avium* DNT *in vivo* was established by Temple et al. (128), who reported that a DNT-negative mutant was nonpathogenic in turkey poults. However, the mechanism through which DNT contributes to virulence is not known and the toxin appears not to be responsible for either ciliostasis (102) or local epithelial damage (131). Instead, those activities may be mediated by the tracheal cytotoxin (TCT) isolated by Gentry-Weeks et al. (33), which was found to be chemically identical to that produced by *B. pertussis*. This 921 kDa disaccharide-tetrapeptide fragment of peptidoglycan, a by-product of growth-related cell wall remodelling, specifically damages ciliated epithelial cells leading to a loss of epithelium and poor clearance of mucus (37). These toxic effects require bacterial attachment so that TCT is concentrated at the epithelial cell surface, mirroring an earlier observation that the cytotoxic and ciliostatic effect of *B. avium* on turkey tracheal organ cultures requires adherence (75). An additional toxin produced by *B. avium*, identified as beta-cystathionase, was shown to be lethal for osteogenic, osteosarcoma, and tracheal cells (35). Beta-cystathionase may be at least partially responsible for the cartilage lesions that lead to tracheal softening and collapse.

There is currently no evidence to suggest that *B. hinzii* produces DNT and no orthologous gene has been identified in the genome of any isolate (71). Whether TCT and/or beta-cystathionase are produced by *B. hinzii* is unknown, although a gene that shares ~81% nucleotide identity and ~85% predicted amino acid identity with the *B. avium* beta-cystathionase gene is present in the genomes of all *B. hinzii* isolates so far sequenced (71, 109, 137). Other toxins contributing to the virulence of the classic *Bordetella*, including adenylate cyclase toxin and pertussis toxin, have not been detected in either *B. avium* or *B. hinzii* and the corresponding genes are absent from their genomes (33, 71, 96, 102, 108, 109, 137).

Many virulence factors of the classic *Bordetella* are coordinately regulated at the genetic level by a 2-component system (BvgA and BvgS) encoded by the *bvg* (*Bordetella* virulence genes) locus, a phenomenon termed phenotypic modulation (11). Expression of BvgAS-controlled virulence genes can be reversibly up- and downregulated *in vitro* by changes in environmental conditions, including temperature and the concentration of sulfate ions or nicotinic acid. The cues that trigger modulation *in vivo* are not well defined but there is ample evidence that precise control of virulence gene expression in response to changes in the local environment is crucial for optimal growth and survival of these bacteria. The *bvgAS* genes also undergo phase variation, in which spontaneously occurring deletions or frameshift mutations irreversibly abolish expression of BvgAS-activated genes, regardless of the environmental conditions. A *bvgAS* locus has been identified in both *B. avium* and *B. hinzii* (71, 119) though the predicted amino acid sequences of the related proteins are somewhat divergent from those of the classic *Bordetella*. Phase variation has been reported for *B. avium* (36) and BvgS appears to be required for *B. avium* virulence (119) but there is otherwise little known regarding the function of the BvgAS system in either *B. avium* or *B. hinzii*. The data available suggest regulation of virulence gene expression in these *Bordetella* species may be somewhat unique as compared with the classic *Bordetella* (71, 108).

Pathobiology and Epizootiology

Incidence and Distribution

Bordetellosis is an important disease in major turkey-producing regions around the world (60). A survey in Poland, carried out from 2012 to 2014 and representing 29 commercial flocks, detected *B. avium* antibodies in all but 1 of 612 sera from turkeys 23 weeks of age or older (118). A study of North Carolina broilers revealed 63% of the 27 flocks tested were infected with *B. avium*; in flocks with respiratory disease the infection rate was 75% (13).

A 2016 report from the Turkey Health subcommittee of the US Animal Health Association indicates bordetellosis continues to significantly impact turkey production in several geographic regions and is among the top 5 infectious disease problems facing the industry (20). Wild turkeys may also be infected by *B. avium* (51, 93). Whether bordetellosis is a significant disease problem in wild turkeys is unknown, but they should be considered a possible reservoir of infection.

Natural and Experimental Hosts

The natural host of *B. avium* is the turkey. Naturally occurring infection is typically recognized at 2–6 weeks of age (17, 45, 91), although older turkeys and breeder flocks may also develop disease (66). Experimental inoculation of poults older than 1–2 weeks frequently results in colonization but only mild disease. *B. avium* has also been isolated from chickens and other avian species, including mallards, wild turkeys, and Canada geese (46, 93, 110). Serological surveys suggest *B. avium* may infect a wide variety of wild or domesticated birds (50, 93). Strains isolated from avian species other than turkeys are pathogenic for day-old turkeys (46).

B. avium is an opportunistic pathogen in chickens (59) and disease tends to be less severe than in turkeys (82, 110). Chickens challenged experimentally with *B. avium* displayed clinical signs of disease only when there was prior exposure to vaccine strains of infectious bronchitis virus or Newcastle disease virus (59). Turkey and chicken isolates are indistinguishable based on physical and biochemical characteristics (67, 110). A strain of *B. avium* pathogenic for turkeys and Japanese quail failed to produce disease in guinea pigs, hamsters, and mice (74).

B. hinzii has been isolated primarily from poultry and humans but a single instance of rabbit infection has been reported (67, 98). Mice were also once thought to be natural hosts for *B. hinzii* but further characterization of mouse isolates led to their reclassification as a new species, *B. pseudohinzii* (55).

Transmission and Carriers

Bordetellosis is highly contagious and is readily transmitted through close contact with infected poults or exposure to contaminated litter or water (112). Infection is not transmitted between adjacent cages, thus providing evidence against aerosol transmission (112). Litter contaminated with *B. avium* likely remains infective for 1–6 months (19). Although a carrier state has not been demonstrated in turkeys that recover from bordetellosis, the possibility remains. Transmission from turkeys to other avian species can occur (112).

Incubation Period

The incubation period for *B. avium* is 7–10 days when exposure occurs through direct contact (112). Intranasal or intraocular inoculation of poults results in clinical signs of bordetellosis in 4–6 days (3, 38, 105). Poults experimentally infected with *B. hinzii* displayed clinical signs as early as 24 hours after exposure (97).

Clinical Signs

An abrupt onset of sneezing (snick) in a high percentage of 2- to 6-week-old turkeys over the course of a week is suggestive of bordetellosis. Older turkeys may also develop a dry cough (66). A clear nasal discharge can be expressed by placing gentle pressure over the bridge of the beak between the nostrils. During the first 2 weeks of disease, the nares and feathers of the head and wings become crusted with wet, tenacious, brownish exudate (Figure 19.22), and some birds develop submaxillary edema. Mouth breathing, dyspnea, and altered vocalization result when the nasal cavity and upper trachea become partially occluded with mucoid exudate, typically in the second week. Tracheal softening can be palpated through the skin of the neck in some birds beginning in the second week of disease. Behavioral changes include reduced activity, huddling, and decreased consumption of feed and water. Concurrent infections and poor weight gains contribute to poor flock performance and stunted growth (60). Signs of disease begin to subside after 2–4 weeks (38, 91, 105, 132).



Figure 19.22 Clinical appearance of a poult with bordetellosis: open-mouth breathing, dark stains around eye and nostril, and foamy exudate at the medial canthus of the eye.

Morbidity and Mortality

Bordetellosis is typically characterized by high morbidity and low mortality. In turkeys 2–6 weeks of age, morbidity reaches 80%–100% (105), whereas mortality is generally less than 10%. Infection of a breeder flock with *B. avium* resulted in only 20% morbidity with no mortality (66). Instances of high mortality (greater than 40%) are frequently associated with concurrent *Escherichia coli* infection (17, 105). Experimental studies of concurrent *B. avium* and *E. coli* infections in 2- to 4-week-old turkeys revealed defective clearance of *E. coli* from tracheas (31, 133) and increased severity of *E. coli* airsacculitis (130). Adverse environmental temperatures (7), high humidity (116), poor air quality, and concurrent respiratory pathogens may increase mortality rates (105).

Pathology

Gross

Gross lesions are confined to the upper respiratory tract and vary with the duration of infection. Nasal and tracheal exudates are initially serous in character but become tenacious and mucoid as the disease progresses. Tracheal lesions consisting of generalized softening and distortion of the cartilaginous rings, dorsal-ventral compression, and fibrinomucoid luminal exudate are highly suggestive of bordetellosis (3, 132). In isolated cases, there is severe infolding of the dorsal tracheal wall into the lumen immediately below the larynx (Figure 19.23) (3, 134). In cross-section, tracheal rings appear to have thick walls and a diminished lumen. Distortion of tracheal cartilage persists for at least 53 days postinfection (3). Accumulation of mucoid exudate in an area of tracheal infolding can lead to death by suffocation (3). Hyperemia of the nasal and tracheal mucosae and edema of interstitial tissues of the head and neck are apparent during the first 2 weeks of infection.

Microscopic

Distinctive microscopic lesions include cilia-associated bacteria, depletion of mucus from goblet cells, cytoplasmic inclusions, cystic mucosal glands, and generalized loss of ciliated epithelium (3). Colonization of ciliated epithelium begins on the nasal mucosa, progresses down the trachea, and moves into primary bronchi within 7–10 days. Bacteria adhere specifically to cilia and are not found attached to nonciliated cells (4). As seen by scanning electron microscopy, surfaces of adherent bacteria are covered with numerous knob-like surface projections (Figure 19.24) (4). Tracheal cells with adherent bacteria may have increased eosinophilia of the apical cytoplasm and protrude slightly from the mucosa (134). Bacterial colonies (Figure 19.25) are most apparent on the tracheal mucosa 1–2 weeks after onset of clinical signs, before loss of ciliated cells is extensive (3, 4).

During the first 2 weeks of disease, ciliated tracheal epithelium is gradually lost and replaced by nonciliated cuboidal epithelium (Figure 19.26). These immature hyperplastic cells have basophilic cytoplasm with variable numbers of small mucous granules (3, 134). Late in the disease, squamous metaplasia of tracheal epithelium may occur (Figure 19.27). Linear, eosinophilic inclusions occur in the cytoplasm of the tracheal epithelium during the first 3 weeks of disease (3, 4). Ultrastructurally, these inclusions

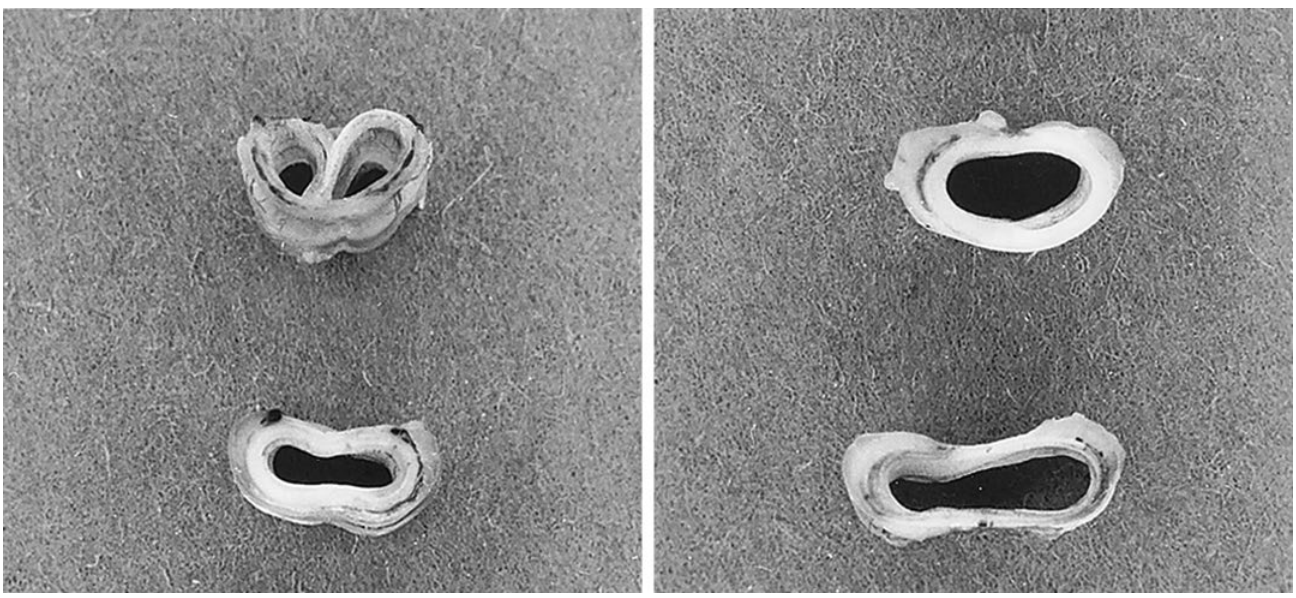


Figure 19.23 Cross-sections of a collapsed trachea from a poult with bordetellosis. The section on the top left, taken immediately below the larynx, has extreme dorsal-ventral infolding. Other sections were taken at 5 cm intervals along the trachea. *Source:* Arp and Cheville, 1984 (3). Reproduced with permission of American Veterinary Medical Association.

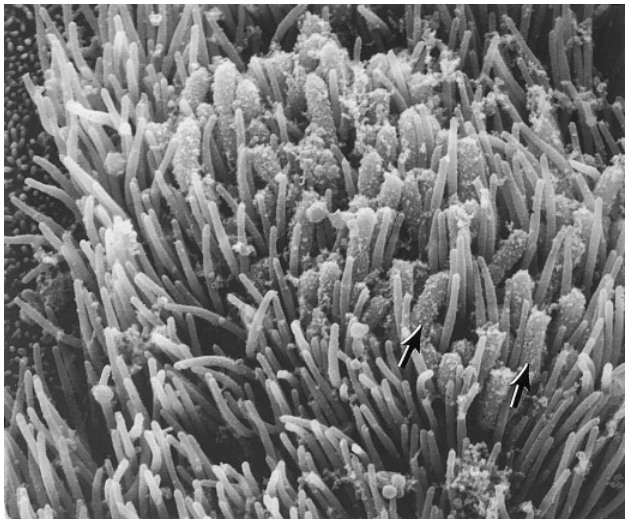


Figure 19.24 Numerous *Bordetella avium* bacteria (arrows) intimately associated with cilia of tracheal epithelial cells. The bacterial surfaces are covered with irregularly shaped, knob-like projections, hypothesized to contribute to adhesion.

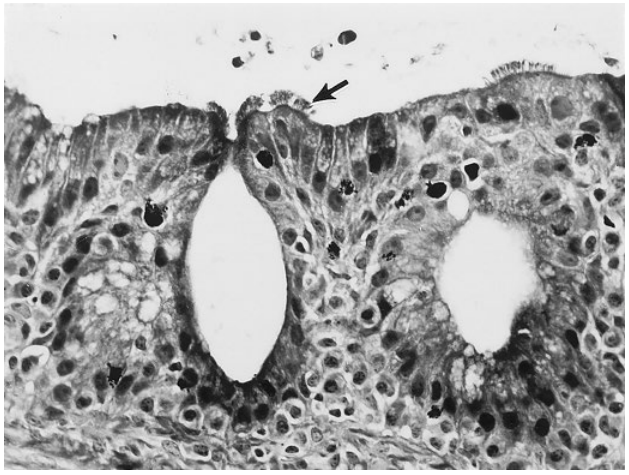


Figure 19.25 Trachea from a poult infected 3 weeks previously with *Bordetella avium*. Characteristic lesions of bordetellosis include cilia-associated bacterial colonies (arrow), loss of ciliated epithelium, dilated mucous glands depleted of mucus, and interstitial infiltration of plasma cells and lymphocytes.

are proteinaceous crystals composed of parallel filaments surrounded by a membrane (4). During the third and fourth week of disease, the tracheal mucosa becomes distorted by numerous folds and mounds of dysplastic epithelium. Depending on the severity of the disease, the tracheal epithelium returns to normal 4–6 weeks after the onset of signs (3, 38), when *B. avium* can no longer be isolated.

Copious mucoid exudate from the upper respiratory tract is accompanied by depletion of mucus from isolated goblet cells and mucous glands along the mucosa (3, 134). Alveolar glands become cystic and lined by immature epithelium with small mucous granules (Figure 19.25). Goblet

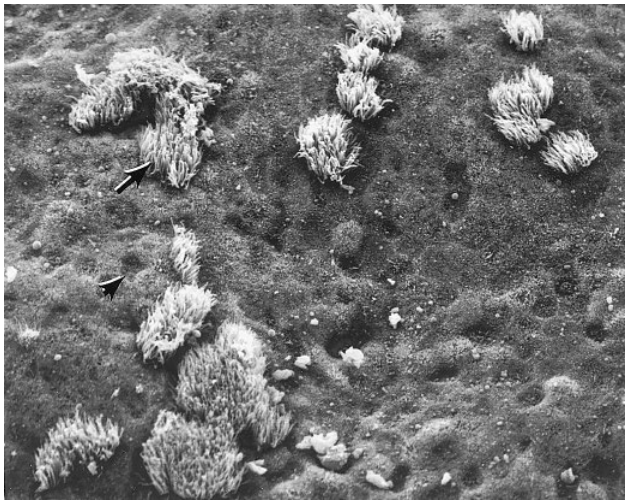


Figure 19.26 Loss of ciliated epithelium from the tracheal mucosal surface. Isolated clumps of ciliated cells (arrow) and dark pits left where ciliated cells have sloughed (arrowhead).

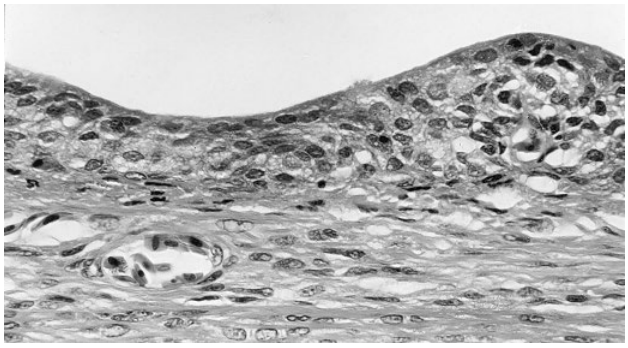


Figure 19.27 Squamous metaplasia of tracheal epithelium occurs in some poults late in the course of bordetellosis.

cells remain largely depleted of mucous granules from the first to the third week of clinical disease.

Cellular exudates in the tracheal lamina propria begin with multifocal infiltrates of heterophils and change to predominantly lymphocytes and plasma cells as clinical signs subside (3, 38). In the third to fifth weeks of disease, a diffuse increase in mucosal plasma cells is accompanied by multifocal lymphoid nodules in the submucosa. Mucosal surface exudates change from mucopurulent to fibrinopurulent after the first week of disease (4).

Pulmonary lesions are restricted to primary bronchi and bronchus-associated lymphoid tissue (130, 132). In contrast to the tracheal mucosa, the bronchial mucosa maintains a near normal appearance including ciliated columnar epithelium and goblet cells (132). Ciliated cells with adherent *B. avium* may occasionally be evident, accompanied by a mild infiltrate of heterophils. Bronchus-associated lymphoid tissue, normally found at the junction of primary and secondary bronchi, becomes grossly apparent, and lymphoid nodules protrude into

the bronchial lumen (132). Other changes in lymphoid tissues include the depletion of cortical lymphocytes from the thymus during early stages of disease (111).

Pathogenesis of the Infectious Process

Initial adhesion of bacteria to ciliated cells of the oronasal mucosa leads to progressive colonization from the upper trachea to the primary bronchi over the first week postinfection. Expansion of the bacterial population along the respiratory mucosa stimulates acute inflammation and the release of mucus from goblet cells, leading to sneezing, coughing, and nasal obstruction. Spread of infection against the flow of mucociliary clearance occurs as motile "swarmer" bacteria break free from microcolonies and move within the layer of mucin to other ciliated cells. During the next week, many of the cells colonized by *B. avium* slough into the tracheal lumen, leaving large surfaces devoid of cilia.

The mechanism by which *B. avium* damages the tracheal mucosa and cartilage remains unknown, but TCT and/or beta-cystathionase may be involved. The formation of cytoplasmic protein crystals and delayed restitution of normal mucosa are suggestive of a toxin that alters cell growth and differentiation. The molecular basis for softening and collapse of tracheal rings may be related to abnormal connective tissue metabolism leading to qualitative and quantitative changes in collagen and elastin (138).

As ciliated cells are progressively lost, the flow of mucus and exudates becomes sluggish, particularly in the upper trachea and nasal cavity. Obstruction of nasolacrimal ducts causes foamy ocular exudate to accumulate at the medial canthus of the eye. Signs of bordetellosis result from local and systemic products of the inflammatory response, the actions of bacterial toxins, and physical obstruction of large air passages.

Within a week of the onset of clinical signs, local and systemic immune responses develop to *B. avium* antigens. Antibody transported from serum and antibody produced by submucosal plasma cells accumulates in respiratory secretions. Local antibody interacts with free "swarmer" *B. avium* cells to inhibit their motility and to prevent adhesion to other ciliated cells. Colonies of bacteria among the cilia are largely protected from host defenses; however, numerous bacteria are shed along with colonized epithelial cells. The bacterial population diminishes over the next several weeks as colonized cells are lost, and newly formed ciliated cells are protected from colonization by antibody.

Some convalescent birds are probably slow to clear all *B. avium* from their respiratory tissues and may serve as a source of infection for susceptible flocks. As mucosal immunity wanes over the next 4–8 weeks, any residual population of *B. avium* in the nasal cavity or sinuses can again expand to produce clinically apparent infection or be transmitted to susceptible birds.

It is not clear whether *B. avium* is immunosuppressive. Infection has been reported to cause a decrease in lymphocyte blastogenesis response to concanavalin A and a depletion of thymic lymphocytes (111). However, subsequent studies of cell-mediated immunity in *B. avium*-infected poults showed the reverse effect with enhanced graft-versus-host and delayed hypersensitivity responses (78, 79). A number of systemic pathophysiologic effects have been attributed to *B. avium* infection including elevation of serum corticosterone (76), enhanced leukocyte migration (77), altered electrocardiograms (139), reduced body temperature (26), reduced levels of monoamines in brain and lymphoid tissues (27, 28), reduced levels of liver tryptophan 2,3-dioxygenase (141), and reduced thyroid hormones in conjunction with fasting (25).

Immunity

Active

Most turkeys develop a humoral immune response to infection with *B. avium* (3, 56, 125). Serum antibodies appear within 2 weeks and reach peak levels by 3–4 weeks postexposure (3, 56). The period of peak antibody titer is followed within 1 week by resolution of clinical disease and a decline in bacterial numbers in the trachea (3). This, combined with evidence for maternal immunity, suggests an important role for humoral immunity in the prevention and recovery from infection (9, 48).

Passive

Resistance to clinical disease and gross lesions was observed in poults with maternal antibody (89). Convalescent serum and tracheal secretions from turkeys infected with *B. avium* inhibit adherence of the bacteria to the tracheal mucosa in turkeys (5). Moreover, adherence of *B. avium* is inhibited whether convalescent serum is administered locally or parenterally. The passive administration of convalescent serum is believed to mimic many aspects of maternal immunity. Suresh et al. (125) evaluated antibody levels in serum, tracheal washings, and lacrimal secretions and found that maternal antibody was undetectable by 3 weeks of age.

Diagnosis

The diagnosis of bordetellosis is based on clinical signs and lesions, isolation of *B. avium* or *B. hinzii* from the respiratory tract, a positive serologic test, or some combination of these.

Isolation and Identification of Causative Agent

Bacterial isolation is accomplished on MacConkey agar inoculated with a swab used to sample the tracheal mucosa.

Samples collected from the choanal opening and nostril, or by passing a swab into the trachea through the larynx, are not recommended as they commonly yield large numbers of nonpathogenic bacteria (113, 117). When turkeys are available for necropsy examination, swab samples should be collected aseptically through an opening in the midcervical trachea. After 24–36 hours of incubation at 37°C on MacConkey agar, colonies of *B. avium* are 1 mm or less in diameter whereas those of *B. hinzii* are larger, perhaps up to 2 mm. Most contaminating bacteria are recognizable as large, mucoid, lactose-fermenting colonies but their overgrowth in heavily inoculated areas of the plate can mask smaller colonies of *B. avium*. *B. avium* and *B. hinzii* may be more easily recognized in less heavily inoculated portions of the plate. Extending the incubation time to 48 hours may also more readily reveal colonies of *B. avium*, which sometimes develop a brownish, raised center (Figure 19.21). Early in the course of infection, pure cultures can be obtained from the trachea but in later stages *E. coli* and other opportunistic bacteria may be isolated (105). Physical, genetic, and biochemical characteristics of *B. avium* and *B. hinzii* that distinguish these bacteria from one another and from closely related bacteria are presented in Tables 19.2 and 19.3.

It is difficult to differentiate between *B. avium* and *B. hinzii* on the basis of standard biochemical tests, although isolation of either bacterium from birds with appropriate clinical signs is sufficient for a diagnosis of bordetellosis. Nonetheless, accurate identification of these bacteria is essential as it will provide important information about their relative prevalence and contribute to an understanding of the basis for strain-specific variation in the virulence of *B. hinzii*. In addition to hemagglutination of guinea pig erythrocytes (a property of *B. avium* but not *B. hinzii*, as noted previously), several other methods may be useful. A monoclonal antibody-based latex bead agglutination test for *B. avium* was reported to be nonreactive with 24 "*B. avium*-like" isolates (124). The same monoclonal antibody has been used in a fluorescent staining technique for detection of *B. avium* in tracheal sections (123). Although these studies suggest the monoclonal antibody may be specific for *B. avium*, further evaluation of both avirulent and disease-causing isolates definitively identified as *B. hinzii* is needed. Cellular carbohydrate patterns have been proposed as a basis for identification of *B. avium* (88), but the method has not been tested with isolates of *B. hinzii* or "*B. avium*-like" bacteria. Highly sensitive and specific PCRs for *B. avium* (99, 107) and *B. hinzii* (95) have been reported. It has recently become clear that isolates of *B. pseudohinzii*, so far found only in mice, may test falsely positively with the *B. hinzii* PCR (55, 95). Given the apparent host-restriction of *B. pseudohinzii*, it seems unlikely that false positives would arise from poultry isolates. Newly available genome sequences for several isolates each of *B. hinzii* and *B. pseudohinzii* may permit the design of PCR assays capable of accurately distinguishing between these species.

Serology

Serologic testing is useful for detection of serum antibodies to *B. avium* in both experimentally and naturally infected birds. Jackwood and Saif (56) developed a microagglutination test (MAT) using killed, neotetrazolium-chloride-stained *B. avium* as the antigen that correlates well with bacterial isolation. Serologic tests may sometimes remain positive beyond the time during which *B. avium* can be cultured. In a field study by Slavik et al. (117), 4 of 10 flocks with a history of respiratory disease were positive for antibodies reactive with *B. avium*, even though the bacterium was not isolated. In experimentally infected poults, antibody is detectable by the MAT from 2 weeks postinfection until at least 5–7 weeks postinfection, with peak titers occurring at about 3–4 weeks postinfection (3, 7, 56). Use of heterologous *B. avium* antigen has little effect on agglutination titers (7). Because the basis for a positive MAT is the presence of *B. avium*-specific immunoglobulin M (IgM), the test will not reliably detect maternal antibody in poults, which is primarily immunoglobulin G (IgG).

Hopkins et al. (52) developed an ELISA for detection of serum IgG reactive with a whole-cell bacterial antigen prepared from *B. avium*. ELISA results correlated well with those from the MAT, except for sera from day-old poults for which only the ELISA could detect maternal antibody. A commercially available ELISA kit for detection of *B. avium*-specific IgG has also proven to be useful (70). A dot-immunobinding assay (129), a particle concentration fluorescence immunoassay (15), and several additional variations of ELISA procedures for detection of *B. avium*-specific antibodies have been developed (8, 89, 125). All detect maternal antibody and are reproducible and sensitive. It appears that *B. avium* and *B. hinzii* share some degree of antigenic relatedness, because antibodies elicited by *B. avium* cross-react with proteins of *B. hinzii* (10, 16, 64). The limited data so far available suggests antibodies elicited by *B. hinzii* may not be cross-reactive with *B. avium* (16) but whether birds infected with *B. hinzii* could test positively in 1 or more of the assays described above is unknown. There is currently no serologic test suitable for identification of *B. hinzii*-infected birds.

Differential Diagnosis

Bordetellosis must be differentiated from other primary and secondary causes of rhinotracheitis. Mycoplasmosis, chlamydiosis, and respiratory cryptosporidiosis may mimic or contribute to many of the clinical signs of bordetellosis (1, 47, 66, 72). Of the viral agents, Newcastle disease virus, avian paramyxovirus type 2 (Yucaipa virus),

adenovirus, influenza virus, and pneumovirus should be considered (21, 72). Although *B. avium* alone can produce all of the clinical signs and lesions of bordetellosis in naturally occurring disease, it is more frequently accompanied by Newcastle disease virus, *Mycoplasma* spp., and opportunistic bacteria such as *E. coli*.

Intervention Strategies

Management Procedures

Bordetella avium is transmitted by direct contact and through contamination of water, feed, and litter. It is highly contagious, making strict biosecurity measures necessary to prevent infection of clean flocks. Rigorous clean-up procedures are required to eliminate the organism from contaminated premises, including complete removal of litter, thorough washing of all surfaces, disinfection of watering systems and feeders, and the application of a disinfectant to thoroughly washed surfaces. Although restrictions apply to its use, either formaldehyde fumigation or application of a dilute formaldehyde solution to all surfaces may be needed to completely eliminate the organism. *B. avium* is easily spread from one facility to another, so the use of disinfectant foot baths, clean outer clothing, and a shower between visits to different houses and locations is essential. Because the severity of bordetellosis is exacerbated by adverse environmental and infectious factors, optimal temperature, humidity, and air quality should be maintained while avoiding or delaying the use of live attenuated vaccines in infected flocks.

Vaccination

Vaccines available commercially for bordetellosis in turkeys include a live, temperature-sensitive (ts) mutant of *B. avium* derived from a virulent isolate following nitrosoguanidine treatment (Art Vax, Merck Animal Health, Madison, NJ) (18) and a live strain of *B. avium* attenuated by undisclosed means (Snick Guard, ARKO Laboratories, Ltd., Jewell, IA). Art Vax is administered by spray at 1 day of age and via drinking water 2 weeks later. Snick Guard is given in drinking water at 10 and 24 days of age. A whole-cell bacterin (ADJUVAC-ART) previously available from Sanofi Animal Health, Inc. (Overland Park, KS) is no longer manufactured. Art Vax was shown to colonize the nasal mucosa and induce moderate levels of serum antibodies when given intranasally or in drinking water (18). Subsequent studies reported inconsistent efficacy and variation in antibody response, depending on dosage, turkey age, and environmental factors (7, 44, 49, 53, 61). In some instances, substantial protection was achieved but in others only a moderate reduction in lesion severity and delayed onset of clinical signs was observed. Turkeys less than 3 weeks of age may respond poorly to the vaccine.

Several studies indicate that breeder hen vaccination may be useful for prevention of bordetellosis in progeny poults (9, 48, 89). Vaccination with either heat-killed (48) or formalin-killed (9) adjuvanted bacterins delayed the onset and severity of clinical disease in challenge-exposed poults. Passive immunization of 3-week-old poults with convalescent serum reduces adherence of *B. avium* to the tracheal mucosa in a dose- and time-dependent manner (5). Taken in total, these studies suggest that maternal antibody of the IgG class may confer temporary immunity to newly hatched poults.

Because *B. avium* and *B. hinzii* are antigenically related, Jackwood and Saif (62) designed experiments to determine whether poults infected with nonpathogenic isolates of *B. hinzii* would develop immunity to *B. avium*. *B. hinzii* failed to persist for a significant period in the respiratory tract and failed to induce either a serologic response or protection to *B. avium* challenge.

Treatment

Treatment of bordetellosis with antibiotics administered in the water, by injection, or by aerosol generally produces minimal clinical improvement. In experimentally infected poults, parenteral administration of long-acting oxytetracycline had no apparent effect on *B. avium* infection (115). Treatment of experimental bordetellosis with oxytetracycline-HCl administered by aerosol caused a transient reduction of bacteria in the trachea and a delay in clinical signs and lesion development (134). However, by 4 days after treatment, bacterial numbers and disease severity were similar in treated and nontreated groups.

Administration of tetracycline-HCl and penicillin-G in drinking water for 3 days produced clinical improvement within 24 hours in an infected breeder flock (66). Treatment of bordetellosis in young turkeys with an aerosol of oxytetracycline-HCl reduced mortality associated with subsequent Newcastle disease vaccination compared with untreated flocks (30). Although these observations suggest a favorable response to treatment, it remains unclear whether clinical improvement results from antibacterial effects against *B. avium* or effects on secondary pathogens such as *E. coli*.

Other treatments, including the addition to drinking water of niacin (140) or a 0.016% oxy-halogen formulation (92) were reported to reduce clinical signs of bordetellosis.

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20

Infectious Coryza and Related Bacterial Infections

Pat J. Blackall and Edgardo Soriano-Vargas

Summary

Agent, Infection, and Disease. Infectious coryza (IC), a respiratory disease that occurs wherever chickens are raised, is caused by *Avibacterium paragallinarum*, an organism that typically requires nicotinamide dinucleotide (NAD) for *in vitro* growth. The organism can be classified into 3 Page serovars (A, B, and C) or 9 Kume serovars (A-1 to A-4, B, and C-1 to C-4). The disease is characterized by poor growth performance and a marked reduction (10%–40%) in egg production in layers. NAD-independent forms of *Av. paragallinarum* occur in a number of regions and pose a challenge for diagnostic laboratories. A range of other *Avibacterium* species (with and without a requirement for NAD) also occur in chickens, although there is little to no evidence for any role in disease.

Diagnosis. The preferred diagnostic tests for IC are traditional culture, with media that meet the need for NAD by use of a nurse colony or which are supplemented with NAD and serum. Culture allows the performance of antimicrobial sensitivity assays as well as serotyping. For laboratories with limited culture experience, the use of a polymerase chain reaction (available in both conventional and real-time formats) is recommended. Serology has been used but no existing assay has been confidently shown to be universally relevant for application to disease detection or monitoring vaccination programs.

Intervention. Both commercial and autogenous bacterins are widely used to reduce the economic losses associated with IC. Because these bacterins are serovar specific, a knowledge of serovars in a region is a critical component of an effective vaccination program.

Introduction

Definition and Synonyms

Infectious coryza (IC) is an acute respiratory disease of chickens caused by the bacterium known as *Avibacterium paragallinarum* (15). The genus *Avibacterium* also contains the species *Av. gallinarum*, once known as *Pasteurella gallinarum* (15), as well as 3 other species – *Av. avium, Av. endocarditidis* and *Av. volantium* (8, 15). Hence, this chapter has integrated our current knowledge on all bacterial species within the genus *Avibacterium*. It is generally accepted that *Av. paragallinarum* is a primary pathogen whereas all other members of the genus are either opportunistic pathogens (*Av. endocarditidis, Av. gallinarum*) or not recognized as pathogens (*Av. avium, Av. volantium*).

The clinical manifestation of IC has been described in the early literature as roup, contagious or infectious catarrh, cold, and uncomplicated coryza (142). The disease was named infectious coryza because it was infectious and primarily affected the nasal passages (5). No specific syndrome name has been allocated to the disease conditions associated with *Av. gallinarum* and *Av. endocarditidis*.

Economic Significance

The greatest economic losses associated with IC result not only from poor growth performance in growing birds and marked reduction (10%–40%) in egg production in layers, but also because control requires intense vaccination. The disease can have a much greater impact than the relatively simple scenario described above. As an example, an outbreak of the disease in older layer birds in California,

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which was not associated with any other pathogen, caused a total mortality of 48% and a drop in egg production from 75% to 15.7% over a 3-week period (28).

Infectious coryza can have significant impact in meat chickens. In California, 2 cases of IC, 1 complicated by the presence of *Mycoplasma synoviae*, caused increased condemnations, mainly caused by airsacculitis, that varied from 8.0% to 15% (50). In Alabama, an IC outbreak in broilers that was not complicated by any other disease agent caused a condemnation rate of 69.8%, virtually all caused by airsacculitis (66).

When IC occurs in chicken flocks in developing countries, the added presence of other pathogens and stress factors can result in disease outbreaks that are associated with marked economic losses. In China, outbreaks of IC have been associated with morbidities of 20%–50% and mortalities of 5%–20% (41). IC has also been reported in kampung (village) chickens in Indonesia (101, 125).

Outbreaks of disease associated with *Av. gallinarum* have not been commonly reported. Mortalities in chickens have been reported at 5%–10% (29) and 10%–34% (52). In turkeys, mortalities of 18%–26% have been reported (7). In most cases of disease associated with *Av. gallinarum*, the possibility that other infectious agents such as viruses and mycoplasmas were involved in the overall disease complex remains a possibility.

Avibacterium endocarditidis has been associated with valvular endocarditis in broiler breeders (6, 8).

Public Health Significance

There is no public health significance to *Av. paragallinarum* or *Av. endocarditidis*. There are 3 reports of *Av. gallinarum* being a possible cause of disease in humans (1–3). However, these reports lack definitive molecular or phylogenetic data and some have been suggested to be misidentifications (58). On the balance of the available evidence, *Av. gallinarum* does not appear to have public health significance.

History

As early as 1920, Beach (4) believed that IC was a distinct clinical entity. The etiologic agent eluded identification for a number of years, because the disease was often masked in mixed infections and with fowl pox in particular. In 1932, De Blieck (48) isolated the causative agent and named it *Bacillus hemoglobinophilus coryzae gallinarum*.

The first report to draw attention to organisms that appear to resemble the organism *Av. gallinarum* was Schneider (118). The description of the species *P. gallinarum* occurred in 1955 (63). *Av. endocarditidis* has only recently been recognized (8).

Etiology

Classification

A full review of the various early names used for the causative agent of IC (*Haemophilus gallinarum*; *Haemophilus paragallinarum)* is provided by Blackall and Soriano (25). Within the genus *Avibacterium, Av. paragallinarum* is the only primary pathogen in the genus (15). An extensive phylogenetic analysis has shown that whereas 5 species are currently recognized with the genus *Avibacterium* (*avium, endocarditidis, gallinarum, paragallinarum*, and *volantium*), there may only be 2 or 3 species (9). The current recommendation is that organisms that do not match the described species by genotypic or phenotypic methods should be described as *Avibacterium* spp. (9). For the purpose of this review, where possible, the *Avibacterium* terminology will be used regardless of the names used in the original study.

There has been a long history of confusion over the requirement for X-(hemin) and V-(nicotinamide adenine dinucleotide - NAD) factors for *in vitro* growth by *Av. paragallinarum* (25). It is now accepted that both V-factor dependent and V-factor independent isolates of *Av. paragallinarum* exist, with the independent form present in South Africa, Mexico, and Peru (56, 61, 90).

Morphology and Staining

Avibacterium paragallinarum and *Av. gallinarum* are both Gram-negative nonmotile bacteria. In 24-hour cultures, both appear as short rods or coccobacilli 1–3 mm in length and 0.4–0.8 mm in width, with a tendency for filament formation. A capsule may be demonstrated in virulent strains of *Av. paragallinarum* (65, 114). *Av. paragallinarum* undergoes degeneration within 48–60 hours, showing fragments and ill-defined forms. Subcultures to fresh medium at this stage will again yield the typical rod-shaped morphology.

Growth Requirements

Either NAD (20–100 µg/mL) (108) or the reduced form (NADH) (1.56–25 µg/mL medium) (98, 105) is necessary for the *in vitro* growth of most isolates of *Av. paragallinarum*. The exceptions are the isolates described in South Africa, Mexico and Peru that are NAD independent (56, 61, 90). Sodium chloride (NaCl) (1.0–1.5%) (105) is essential for growth of *Av. paragallinarum*. Chicken serum (1%) is required by some strains (65), whereas others merely show improved growth with this supplement (23). A medium that was developed for the isolation of *Av. paragallinarum* in the face of contaminating Gram-positive organisms has been described

(127). Complex media are often used to obtain dense growth of organisms for characterization studies (13, 102). Some isolates of *Av. paragallinarum* still show satellitic growth even on a medium that is designed to provide all necessary and known growth requirements (16). The pH of various media varies from 6.9 to 7.6. A number of bacterial species excrete V-factor that will support growth of *Av. paragallinarum* on media that lack V-factor (98).

In contrast, *Av. gallinarum* has no need for NAD for *in vitro* growth and grows on a range of basic media such as blood agar (93). Similarly, *Av. endocarditidis* grows well on blood agar with no need for NAD (8).

The determination of the growth factor requirements of the avian haemophili is not an easy process. Commercial growth factor disks used for this purpose may yield a high percentage of cultures that falsely appear to be both X- and V-factor dependent (19). For classical X- and V-factor testing, the use of purified hemin and NAD as supplements to otherwise complete media also may be considered.

Avibacterium paragallinarum is commonly grown in an atmosphere of 5% carbon dioxide. *Av. gallinarum* also does not require carbon dioxide but a more uniform colony development does occur if isolates are incubated under an atmosphere of 5%–10% carbon dioxide (15). *Av. endocarditidis* does not need carbon dioxide (8).

For *Av. paragallinarum*, the minimal and maximal temperatures of growth are 25 °C and 45 °C, respectively; the optimal range being 34 °C–42 °C. *Av. endocarditidis, Av. gallinarum* and *Av. paragallinarum* are commonly grown at 37 °C–38 °C.

Colony Morphology

Avibacterium paragallinarum typically gives tiny dewdrop, nonhemolytic colonies up to 0.3 mm in diameter on suitable media. In obliquely transmitted light, mucoid (smooth) colonies are iridescent, whereas rough colonies are noniridescent (64, 102, 110).

Avibacterium gallinarum colonies on serum or dextrose-starch agar are iridescent, circular, smooth and entire and may reach up to 1.5 mm after 24 hours incubation (particularly if in a 5%–10% carbon dioxide) (37). A grayish-yellow pigment is typically produced (15).

Avibacterium endocarditidis colonies on blood agar are nonhemolytic, circular, smooth and entire with a greyish tinge and may reach 1.5 mm after 24 hours incubation (8).

Biochemical Properties

The ability to reduce nitrate to nitrite, and ferment glucose without the formation of gas, is common to all members of the genus *Avibacterium*. Oxidase activity and a failure to produce indole or hydrolyse urea or gelatin are also uniform characteristics (15). Both agar and liquid medium for carbohydrate fermentation tests have been used (13, 127). Table 20.1 presents those properties that allow a full identification of all members of the genus *Avibacterium*. The failure of *Av. paragallinarum* to ferment either galactose or trehalose and a lack of catalase clearly separate this organism from the other members of the genus. As noted earlier, there is now doubt that all 5 described species in this genus are true species and

Table 20.1 Differential tests for the genus *Avibacterium*.

Taxon	*Avibacterium gallinarum*	*Avibacterium paragallinarum*	*Avibacterium volantium*	*Avibacterium avium*	*Avibacterium sp. A.*
Catalase	+	–	+	+	+
Symbiotic growth	–	V	+	+	+
ONPG	V	–	+	–	V
Acid from					
L-arabinose	–	–	–	–	+
D-galactose	+	–	+	+	+
Maltose	+	+	+	–	V
D-mannitol	–	+	+	–	V
D-sorbitol	–	+	V	–	–
Trehalose	+	–	+	+	+
α-glucosidase	+	–	+	+	+

All species are Gram-negative and nonmotile. All species reduce nitrate, are oxidase positive, and ferment glucose. Most isolates of *Avibacterium paragallinarum* require enriched carbon dioxide (5%–10%) atmosphere, and most will show an improved growth in the presence of 5%––10% chicken serum. Most isolates of *Avibacterium gallinarum* show an improved growth in an enriched carbon dioxide (5%–10%) atmosphere. OPNG, ortho-nitrophenyl-β-galactoside; +, positive; -, negative; V, variable.

isolates with intermediate properties have been reported (11). Hence, if using phenotypic identification, isolates with properties that do not match those shown in Table 20.1 are best reported as *Avibacterium* spp.

Susceptibility to Chemical and Physical Agents

Avibacterium paragallinarum is a delicate organism that is inactivated rather rapidly outside the host. Infectious exudate suspended in tap water is inactivated in 4 hours at ambient temperature; when suspended in saline, the exudate is infectious for at least 24 hours at 22°C. Exudate or tissue remains infectious when held at 37°C for 24 hours and, on occasion, up to 48 hours; at 4°C, exudate remains infectious for several days. At temperatures of 45°C –55°C, hemophili are killed within 2–10 minutes. Infectious embryonic fluids treated with 0.25% formalin are inactivated within 24 hours at 6°C, but the organism survives for several days under similar conditions when treated with thimerosal, 1:10,000 (144).

Six to 7-day-old chicken embryos may be inoculated with single colonies or broth cultures via the yolk sac; yolk from embryos dead in 12–48 hours will contain a large number of organisms which may be frozen and stored at -20°C to -70°C or lyophilized (142). After any storage, whether frozen or lyophilized, revival should include inoculation of a suitable liquid growth medium (egg inoculation is ideal) as well as an agar medium.

There is little specific knowledge on the susceptibility of *Av. gallinarum* or *Av. endocarditidis* to either chemical or physical agents. Cultures of *Av. gallinarum* have been shown to survive well when held at room temperature on Dorset egg medium (93).

Strain Classification

Antigenicity

Page (98, 99) classified *Av. paragallinarum* with the plate agglutination test using whole cells and chicken antisera into serovars A, B, and C. It is now recommended to use a hemagglutination inhibition (HI) test to serotype isolates by the Page scheme (17). This HI test uses fixed chicken erythrocytes and results in fewer nontypable isolates than the original agglutination technology (17).

The distribution of Page serovars differs from country to country. A full review of serovar distribution was provided in the previous editions of *Diseases of Poultry* (25, 26). Although a multiplex polymerase chain reaction (PCR) assay and a PCR-restriction fragment length polymorphism assay to recognize the Page serovars was proposed (106), further studies have indicated significant problems in this approach (89, 133) and the assay cannot be recommended as a replacement for Page serotyping.

An alternative serologic classification of *Av. Paragallinarum*, the Kume scheme, based on an HI test using potassium thiocyanate-treated and -sonicated cells, rabbit hyperimmune serums, and glutaraldehyde-fixed chicken erythrocytes has been developed (77). The modified Kume scheme consists of serogroups A, B, and C which match the Page serovars of A, B, and C (18). The 9 currently recognized Kume serovars are A-1, A-2, A-3, A-4, B-1, C-1, C-2, C-3, and C-4 (18). Many isolates that were nontypable in the Page scheme by agglutination tests were easily typed using the Kume scheme (55).

The Kume scheme has not been widely applied, because it is technically demanding to perform. Previous editions of *Diseases of Poultry* (25, 26) have provided a detailed listing of the known Kume serovar distribution.

Two serotyping schemes for *Av. gallinarum* have been reported (91, 93) but there appears to have been no adoption of either of these serotyping schemes.

Immunogenicity or Protective Characteristics

Infectious coryza is relatively unique among common bacterial infections in that a bacterin (inactivated whole cell vaccine) is protective against the disease when the bacterin is adequately prepared. There is a correlation between Page serovars and immunovar specificity; chickens vaccinated with a bacterin prepared from 1 Page serovar are protected only against homologous challenge from that Page serovar (24, 79, 104). There is evidence that the cross-protection within Page serovar B is only partial (137).

A complete cross-protection study using the reference strains of the 9 serovars of *Av. paragallinarum* has been completed (121). Within Kume serogroup A, serovars A-1, A-2, and A-3 are strongly cross-protective whereas there is good cross-protection between serovars A-1 and A-4. Within Kume serogroup C, there was a good level of cross-protection for serovars C-1, C-2, and C-3, with some exceptions. Kume serovars C-1, C-2, and C-3 all provided protection against a C-1 challenge. In contrast, with the serovar C-2 and C-3 challenge, the respective homologous group was significantly better protected than the heterologous groups. The only instance of a vaccine being able to provide cross-protection across Kume serogroups that was at the same level as the homologous level was for the serovar C-4 vaccine and the serovar B-1 challenge. This study thus broadly confirmed the widely accepted dogma that serogroups A, B, and C represent 3 distinct immunovars.

Although there is only 1 serovar, B-1, within Kume serogroup B, there are reports of undefined heterogeneity within the Kume serogroup B/Page serovar B. Bivalent vaccines containing Page serovars A and C provide protection against Page serovar B strain Spross, but not against 2 South African isolates of Page serovar B (137). Furthermore, there is only partial cross-protection

within various strains of Page serovar B (137). Poor vaccine protection against IC caused by serovar B strains in Argentina has been suggested to be because of antigenic differences between field isolates and the "standard" serovar B strains in commercial vaccines from North America or Europe (128). These difficulties have resulted in at least 1 commercial vaccine that contains multiple Page serovar B strains to provide better protection (74). Vaccination/challenge exposure studies are needed to study the antigenicity and immunospecificity of recent serovar B isolates.

There have been suggestions that Kume serovar C-3 as well as other serovars of NAD-independent *Av. paragallinarum* are so antigenically different that they are causing vaccine failure (31, 54). However, it has been shown that a commercial vaccine, specified as containing serovars A, B and C without details of the actual strains, provided acceptable levels of protection against NAD-independent isolates of Page serovar A and Kume serovar C-3 (75). It is possible that differences in challenge models may explain these differing results. Clearly in those regions of the world with NAD-independent forms of *Av. paragallinarum*, there is a need for careful consideration when selecting a vaccine for a prevention and control program.

There is little knowledge about the immunogenicity of *Av. gallinarum*. Autogenous vaccines have been used (93) although there is no literature on the effectiveness of these products or the existence of different immunovars.

Molecular Techniques

DNA fingerprinting by restriction endonuclease analysis has been shown to be a suitable typing technique, providing useful insights in epidemiologic studies of both *Av. paragallinarum* (22) and *Av. gallinarum* (7). Ribotyping is another molecular technique that has proven useful, providing insight into the links between the NAD-independent *Av. paragallinarum* isolates from South Africa (86), the epidemiologic relationships among Chinese isolates of *Av. paragallinarum* (84), and the heterogeneity and epidemiological links amongst *Av. gallinarum* isolates (7, 42). ERIC-PCR, a DNA fingerprinting method that uses the PCR technique, has been shown to be capable of typing *Av. paragallinarum* isolates (122).

These nucleic acid techniques (including the species-specific PCR assays discussed later) are advancing to the stage where they offer a rapid and convenient method for identification and typing. These techniques are likely to replace time-consuming and cumbersome cultural, biochemical, and serological means of identification and typing in the near future.

Pathogenicity

As a general observation, the pathogenicity of *Av. paragallinarum* can vary according to factors such as the growth conditions, passage history of the isolate and the state of the host. There is now considerable specific evidence of variation in pathogenicity amongst *Av. paragallinarum* isolates. The reference strains of Kume serovars A-1, A-4, C-1, C-2, and C-3 showed higher virulence than the strains for serovars A-2, A-3, B-1, and C-4 (120). On the basis of field observations in South Africa, it has been suggested that the NAD-independent isolates may cause airsacculitis more commonly than the classic NAD-dependent *Av. paragallinarum* isolates (67). In contrast, experimental infections have shown that South African NAD-dependent isolates are more virulent than NAD-dependent isolates (33, 34, 54). The virulence of the NAD-dependent serovar C-3 strains has been suggested as explaining the large number of coryza outbreaks in vaccinated flocks in South Africa (30). Within a serovar, variation in virulence has also been reported. Yamaguchi et al. (138) found that 1 of 4 strains of *Av. paragallinarum* serovar B failed to produce clinical signs. This variation within a serovar may explain the report that a South African serovar B isolate failed to cause clinical signs in experimentally infected chickens (54).

The early experimental infections of chickens with *Av. gallinarum* resulted in little mortality (63). Field isolates from Israel have caused swollen wattles in 6-week-old chickens (93) whereas both an American field isolate and the type strain (ATCC 13361) have caused endocarditis in mature leghorn chickens given high intravenous doses (129). Intramuscular injection of chickens with an Argentinean isolate resulted in severe myositis at the inoculation site (126). An American field isolate, when given by the intramuscular route, caused severe myositis at the inoculation site as well as pericarditis, perihepatitis, airsacculitis, and synovitis (53, 119). Because the type strain failed to give similar results, there is evidence of strain variation in pathogenicity (53, 119).

Virulence Factors

A range of factors has been associated with the pathogenicity of *Av. paragallinarum*. Considerable attention has been paid to hemagglutinin HA antigens. In both Page serovar A and C, mutants lacking HA activity have been used to demonstrate that the HA antigen plays a key role in colonization (110, 141). The HA antigen is now known to be a 201 kDa protein (HMTp210) that is a trimeric autotransporter adhesin that confers hemagglutination, cell adherence and biofilm formation activities (134).

The capsule has also been associated with colonization and has been suggested to be the key factor in the lesions associated with IC (110, 116). The capsule of *Av. paragallinarum* has been shown to protect the organism against the bactericidal activity of normal chicken serum (112). The loss of capsule increases the hemagglutination and adhesive properties of *Av. paragallinarum* but reduces the virulence of the organism (131).

Avibacterium paragallinarum is capable of acquiring iron from chicken and turkey transferrin, suggesting that iron sequestration may not be an adequate host defense mechanism (97). In contrast, 2 strains of *Av. avium* were unable to acquire iron from these transferrins despite apparently having the same receptor proteins (97).

Crude polysaccharide extracted from *Av. paragallinarum* is toxic to chickens and may be responsible for the toxic signs that may follow administration of bacterin (70). The role, if any, of this component in the natural occurrence of the disease is unknown.

Avibacterium paragallinarum has been shown to produce an RTX-toxin that has strong cytotoxic activity for an avian macrophage-like cell line (81). This toxin, AvxA, is encoded on a classical RTX operon structure that consists of the activator gene *avxC*, the structural toxin gene *avxA*, and the genes for a type I secretion system, *avxBD* (81).

There is no specific knowledge on any virulence factors associated with *Av. gallinarum* or *Av. endocarditidis*.

Pathobiology and Epizootiology

Incidence and Distribution

Infectious coryza occurs wherever chickens are raised. The disease is a common problem in the intensive chicken industry. Although most of the US layer industry is coryza-free, IC is highly prevalent in layer flocks of almost all countries of Latin America, many countries in Asia and Africa and selected regions of the European Union. The disease has also been reported in other, less intensive situations such as kampong (village) chickens in Indonesia (101) and hobby flocks in Great Britain (135).

Although reports are scarce and scattered, *Av. gallinarum* is probably present wherever chickens are raised. A review of outbreaks of disease associated with *Av. gallinarum* is provided in the previous edition of *Diseases of Poultry* (26).

Natural and Experimental Hosts

The chicken is the natural host for *Av. paragallinarum*. There are reports that the village chickens of Africa (36) and Asia (101, 146) are as susceptible to IC as normal commercial breeds. Although there have been reports of IC caused by *Av. paragallinarum* in a number of bird species other than chickens, reviewed by Yamamoto (143), these reports need to be interpreted carefully. Because a range of hemophilic organisms, none of which are *Av. paragallinarum*, have been described in birds other than chickens (49, 62, 100), only studies with detailed bacteriology and/or molecular characterization can be regarded as definitive proof of the presence of *Av. paragallinarum* in birds other than chickens. The following species are refractory to experimental infection: turkey, guinea fowl, pigeon, sparrow, duck, crow, rabbit, guinea pig, and mouse (36, 142, 144). The observation that *Av. paragallinarum* isolates are resistant to 6 hours treatment with chicken serum but not turkey or guinea fowl serum may help explain the host specificity of the organism (36).

Avibacterium gallinarum has been consistently associated with chickens (42). Outbreaks of disease associated with this species have also been reported in guinea fowl in Africa (87) and turkeys in Europe (7). A single isolate has been reported from a healthy duck (92) and a goose of unspecified health status (93).

Age of Host Most Commonly Affected

All ages of chickens are susceptible to *Av. paragallinarum* (143), but the disease is usually less severe in juvenile birds. The incubation period is shortened, and the course of the disease tends to be longer in mature birds, especially hens with active egg production.

Transmission, Carriers, and Vectors

Chronic or healthy carrier birds have long been recognized as the main reservoir of IC infection. The application of molecular fingerprinting techniques has confirmed the role of carrier birds in the spread of IC (22). IC seems to occur most frequently in fall and winter, although such seasonal patterns may be coincidental to management practices (e.g., introduction of susceptible replacement pullets onto farms where IC is present). On farms where multiple-age groups are brooded and raised, spread of the disease to successive age groups usually occurs within 1–6 weeks after such birds are moved from the brooder house to growing cages near older groups of infected birds (44). IC is not an egg-transmitted disease. Epidemiologic studies have suggested that *Av. paragallinarum* may be introduced onto isolated ranches by the airborne route (145).

There is no knowledge on the routes of transmission, carrier status or vectors for *Av. gallinarum* or *Av. endocarditidis*.

Incubation Period

The characteristic feature of IC is a coryza of short incubation that develops within 24–48 hours postinoculation of chickens with either culture or exudate. The latter will more consistently induce disease (102). Susceptible birds exposed by contact to infected cases may show signs of the disease within 24–72 hours. In the absence of a concurrent infection, IC usually runs its course within 2–3 weeks.

Clinical Signs

The most prominent features of IC are an acute inflammation of the upper respiratory tract including involvement of the nasal passage and sinuses with a serous to mucoid nasal discharge, facial edema, and conjunctivitis. Figure 20.1 illustrates the typical facial edema. Swollen wattles may be evident, particularly in males. Rales may be heard in birds with infection of the lower respiratory tract.

A swollen head-like syndrome associated with *Av. paragallinarum* has been reported in broilers in the absence of avian pneumovirus, but in the presence or absence of other bacterial pathogens such as *M. synoviae* and *M. gallisepticum* (50, 107). Arthritis and septicemia have been reported in broiler and layer flocks respectively. The presence of other pathogens contributed to the disease complex (107).





Figure 20.1 Chickens artificially infected with *Avibacterium paragallinarum*. (A) Mature male with coryza and facial edema. (B) Mature female showing conjunctivitis, nasal discharge and open-mouth breathing.

Birds may have diarrhea, and feed and water consumption usually is decreased. There will be an increased number of culls in growing birds, and a reduced egg production (10%–40%) in laying flocks. A foul odor may be detected in flocks in which the disease has become chronic and complicated with other bacteria.

The most common sign seen in outbreaks of disease where *Av. gallinarum* has played a potential role have been those of an acute respiratory disease, coughing and sneezing, with some outbreaks involving periorbital swelling and keratoconjunctivitis. Swollen wattles have been reported in cases in Israel (93) and Africa (87).

Morbidity and Mortality

Infectious coryza is usually characterized by low mortality and high morbidity. Variations in age and breed may influence the clinical picture (12). Complicating factors such as poor housing, parasitism, and inadequate nutrition may add to severity and duration of the disease. When complicated with other diseases such as fowl pox, infectious bronchitis, laryngotracheitis, *Mycoplasma gallisepticum* infection, and pasteurellosis, IC is usually more severe and prolonged, with resulting increased mortality (107, 142). Even in the absence of any other pathogen, older birds can suffer a high mortality as shown by an outbreak in California where the total mortality reached 48% (28).

Although not common, high mortalities have been seen associated with *Av. gallinarum* in broilers (up to 34%) (52) and in turkeys (up to 26%) (7).

Pathology

Gross

Avibacterium paragallinarum produces an acute catarrhal inflammation of mucous membranes of nasal passages and sinuses. There is frequently a catarrhal conjunctivitis and subcutaneous edema of face and wattles. Typically, pneumonia and airsacculitis are rarely present; however, reports of outbreaks in broilers have indicated significant levels of condemnations (up to 69.8%) caused by airsacculitis, even in the absence of any other recognized viral or bacterial pathogens (50, 66).

The lesions associated with *Av. gallinarum* infections have been diverse and include airsacculitis, conjunctivitis, pericarditis, perihepatitis and sinusitis (42) (Figure 20.2). The only pathological condition that has been associated with *Av. endocarditidis* has been valvular endocarditis (6).

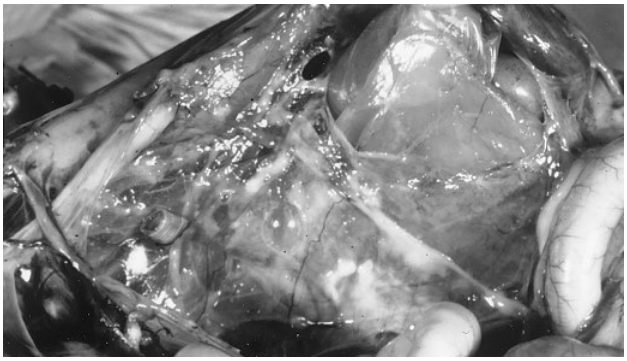


Figure 20.2 Field infection with infectious coryza showing caseopurulent air sac lesions.

Microscopic

Fujiwara and Konno (59) studied the histopathologic response of chickens from 12 hours to 3 months after intranasal inoculation with *Av. paragallinarum*. Essential changes in the nasal cavity, infraorbital sinuses, and trachea consisted of sloughing, disintegration, and hyperplasia of mucosal and glandular epithelia, and edema and hyperemia with heterophil infiltration in the tunica propria of the mucous membranes. Pathologic changes first observed at 20 hours reached maximum severity by 7–10 days, with subsequent repair occurring within 14–21 days. In birds with involvement of the lower respiratory tract, acute catarrhal bronchopneumonia was observed, with heterophils and cell debris filling the lumen of secondary and tertiary bronchi; epithelial cells of air capillaries were swollen and showed hyperplasia. Catarrhal inflammation of air sacs was characterized by swelling and hyperplasia of the cells, with abundant heterophil infiltration. In addition, a pronounced mast cells infiltration was observed in the lamina propria of the mucous membrane of the nasal cavity (117). The products of mast cells, heterophils, and macrophages may be responsible for the severe vascular changes and cell damage observed with IC. A dissecting fibrinopurulent cellulitis similar to that seen in chronic fowl cholera has been reported in broiler and layer chickens (50). Extensive hemorrhages, varying from slight to severe, have been observed in the lumen, mucous membranes, and lamina propria of the nasal cavity of chickens experimentally infected with *Av. paragallinarum* serovar C-1 (130).

Shivaprasad and Droual (119) examined the microscopic lesions following an experimental infection with the *Av. gallinarum* strain associated with severe mortality in broilers in California. The lesions caused by this field isolate were more severe than those associated with the type strain and included severe subacute to chronic pyogranulomatous pneumonia, airsacculitis, pericarditis, perihepatitis, synovitis and myositis. There was severe lymphoid depletion of the bursa of Fabricius (119).

Immunity

Chickens that have recovered from active infection with *Av. paragallinarum* possess varying degrees of immunity to re-exposure. Pullets that have experienced IC during their growing period are generally protected against a later drop in egg production. Resistance to re-exposure among individual birds may develop as early as 2 weeks after initial exposure by the intrasinusal route (109).

It has been shown that experimentally infected chickens develop a cross-serovar (Page scheme) immunity (103). In contrast, as discussed earlier, bacterins provide only serovar-specific immunity (104). This suggests that cross-protective antigens are expressed *in vivo* that are either not expressed or expressed at very low levels *in vitro*.

The protective antigens of *Av. paragallinarum* have not been definitively identified. It has been suggested that the capsule of *Av. paragallinarum* contains protective antigens (111). Using both Page serovar A and C strains, a crude polysaccharide extract was shown to provide serovar-specific protection (70).

Considerable attention has been paid to the role of HA antigens as protective antigens. It has been long noted that for Page serovar A organisms, there is a close correlation between HI titer and both protection (80) and nasal clearance of the challenge organism (76) in vaccinated chickens. Purified HA antigen from a Page serovar A organism has been shown to be protective (71). Takagi et al. have shown that a monoclonal antibody specific for the HA of Page serovar A provides passive protection, and that the HA antigen purified by use of this antibody is also protective (123, 124). Two hemagglutinin epitopes, HPA5.1 and HPC5.5 (serovar A and C derived respectively), have been shown to provide serovar specific protection, suggesting the possibility of novel IC vaccines (95, 96, 136). It is worth noting that vaccines that do not stimulate HI titers can still be protective (43, 60, 121) suggesting that other antibodies do have a role in protection.

Although autogenous bacterins of *Av. gallinarum* have been used and have been shown to stimulate antibody production (93), there is no detailed understanding of the efficacy of these products or the key antigens for protection.

Diagnosis

Isolation and Identification of Causative Agent

Although *Av. paragallinarum* is considered to be a fastidious organism, it is not difficult to isolate, requiring simple media and procedures. Specimens should be taken from 2 or 3 chickens in the acute stage of the disease (1–7 days' incubation). The skin under the eyes is seared with

a hot iron spatula and an incision made into the sinus cavity with sterile scissors. A sterile cotton swab is inserted deep into the sinus cavity where the organism is most often found in pure form. Tracheal and air sac exudates also may be taken on sterile swabs. Where transport delays are expected, swabs should be placed in a commercial transport medium containing supplements to improve viability of the organism (35, 132). The swab is streaked on a blood agar plate, which is then cross-streaked with a *Staphylococcus* culture and incubated at 37 °C in a large screw-cap jar in which a candle is allowed to burn out. *Staphylococcus epidermidis* (98) or *S. hyicus* (23), which are commonly used as "feeders," should be pretested because not all strains actively produce the V-factor (Figure 20.3). The characteristic feature of satellitism allows the recognition of the possible presence of *Av. paragallinarum*. Terzolo et al. (127) have reported the successful use of an isolation medium that contains selective agents that inhibit the growth of Gram-positive bacteria. This medium has the advantage of not using either a "feeder" organism or additives such as NADH. The drawback with the use of a complete medium for isolation is that there is no characteristic marker for the presence of *Av. Paragallinarum*, forcing the diagnostic bacteriologist to sift among numerous colonies for possible *Av. paragallinarum*.

At the simplest level, IC may be diagnosed on the basis of a history of a rapidly spreading disease in which coryza is the main manifestation, combined with the isolation of a catalase-negative bacterium showing satellitic growth.

Better equipped laboratories should attempt a more complete biochemical identification as described earlier. Additional studies of this nature are essential when isolates of NAD-independent *Av. paragallinarum* are suspected. To perform this biochemical testing, the suspect isolates are best grown in pure culture on medium that does not require the addition of a nurse colony. Although many different media have been developed to support the growth of *Av. paragallinarum*, the medium called TM/SN (23) has proven very effective. The medium described by Terzolo et al. (127) is particularly suited for those laboratories where the cost of such ingredients as NADH and albumin is too expensive. The carbohydrate fermentation tests shown in Table 20.1 can be performed in either a phenol red broth base (102), in an agar plate format (13) or an agar tube method (127).

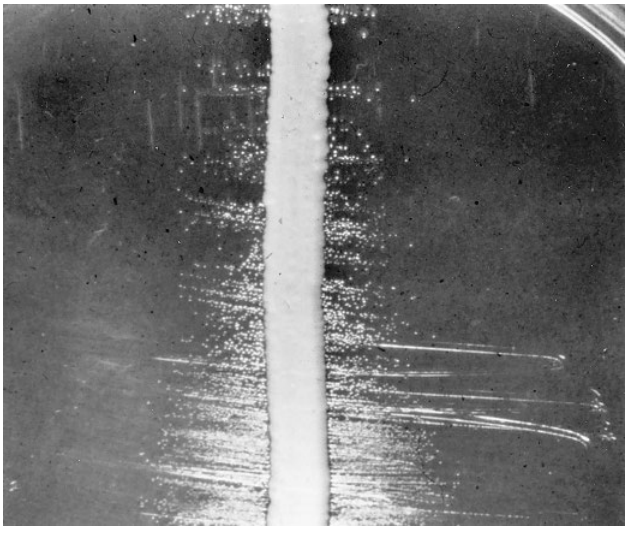


Figure 20.3 Satellite phenomenon. Tiny dewdrop colonies of *Avibacterium paragallinarum* growing adjacent to *Staphylococcus* culture (broad streak) on a blood agar plate.

Two PCR tests, one a conventional PCR (39) and the other a real-time PCR (46), both based on the same target, have been developed for *Av. paragallinarum*. Both PCRs are specific for *Av. paragallinarum*, are rapid and able to detect all known variants. The conventional PCR, termed the HP-2 PCR, has been validated for use on colonies on agar or on mucus obtained from squeezing the sinus of live birds (39). When used directly on sinus swabs obtained from artificially infected chickens in pen trials performed in Australia, the HP-2 PCR has been shown to be the equivalent of culture, but much more rapid (39). When used in China, direct PCR examination of sinus swabs outperformed traditional culture when used on routine diagnostic submissions (38). The problems of poor samples, delayed transport and poor quality (but expensive) media mean that culture will have a higher failure rate in developing countries than in developed countries, making the PCR an attractive diagnostic option. The real-time PCR has been shown to have an increased analytical sensitivity (10- to 100-fold) over the conventional PCR (46).

The HP-2 PCR is a robust test: sinus swabs stored for up to 180 days at 4 °C or -20°°C were 80% positive in the PCR, whereas culture failed after 3 days of storage at 4 °C (40). The HP-2 PCR has proven very useful in South Africa where the diagnosis of IC is complicated by the presence of NAD-independent *Av. paragallinarum* and *Ornithobacterium rhinotracheale* as well as the traditional form of NAD-dependent *Av. paragallinarum* (85).

Isolation of *Av. gallinarum* and *Av. endocarditidis* is best performed using sheep blood agar plates incubated at 37 °C with the plates for *Av. gallinarum* being under a 5%–10% carbon dioxide atmosphere. Phenotypic tests (see Table 20.1) should be performed using conventional methodologies. No molecular diagnostic test has been developed for *Av. gallinarum* or *Av. endocarditidis*.

Serology

There is no totally suitable serological test for the diagnosis of IC. However, despite this absence of a "perfect" test, serological results are often useful for retrospective/epidemiological studies in the local area. A review of the

techniques that have been used in the past is presented by Blackall et al. (21).

At this time, the best available test methodology is the HI test. Although a range of HI tests have been described, 3 main forms of HI tests have been recognized: simple, extracted, and treated HI tests (27). Full details of how to perform these tests are available elsewhere (27). In the following text, the advantages and disadvantages of the 3 HI tests are briefly and critically discussed.

The simple HI is based on whole bacterial cells of Page serovar A *Av. paragallinarum* and fresh chicken erythrocytes (73). Although simple to perform, this HI test can only detect antibodies to serovar A. The test has been widely used to both detect infected as well as vaccinated chickens (21). A variation of this test (whole bacterial cells and glutaraldehyde-fixed chicken erythrocytes) has been shown to detect antibodies caused by all 9 Kume serovars in vaccinated chickens (121).

The extracted HI test is based on KSCN-extracted and sonicated cells of *Av. paragallinarum* and glutaraldehyde-fixed chicken erythrocytes (115). This extracted HI test has mainly been validated for the detection of antibodies to Page serovar C organisms. The test has been shown to be capable of detecting a serovar-specific antibody response in Page serovar C vaccinated chickens (115). A major weakness with this assay is that, in chickens infected with serovar C, the majority of the birds remain seronegative (140).

The treated HI test is based on hyaluronidase-treated whole bacterial cells of *Av. paragallinarum* and formaldehyde-fixed chicken erythrocytes (139). The extracted HI has not been widely used or evaluated. It has been used to detect antibodies to Page serovars A, B, and C in vaccinated chickens with only serovar A and C vaccinated chickens yielding high titres (137). The test has been used to screen chicken sera in Indonesia for antibodies arising from infection with serovars A and C (125).

Vaccinated chickens with titers of 1:5 or greater in the simple or extracted HI tests have been found to be protected against subsequent challenge (115). There is not enough knowledge or experience yet to draw any sound conclusions on whether there is a correlation between titer and protection for the treated HI test.

Overall, the serological test of choice remains either the simple HI test (73) for either infections or vaccinations associated with serovar A, the extracted or treated HI tests (115, 139) for vaccinations associated with serovar C and the treated HI test (139) for infections associated with serovar C. There has been so little work performed on serological assays for infections or vaccinations associated with serovar B that it is not possible to recommend any test.

Both plate agglutination and gel precipitin tests have been described for the detection of antibodies to *Av. gallinarum* (93) but there has been no apparent use of these assays.

Differential Diagnosis

Infectious coryza must be differentiated from other diseases such as chronic respiratory disease, chronic fowl cholera, fowl pox, ornithobacteriosis, swollen head syndrome (caused by turkey rhinotracheitis), and A-avitaminosis, which can produce similar clinical signs. Because *Av. paragallinarum* infections often occur in mixed infections, one should consider the possibility of other bacteria or viruses as complicating IC, particularly if mortality is high and the disease takes a prolonged course (see Pathogenicity; Morbidity and Mortality).

Because the conditions linked with *Av. gallinarum* are generally linked with upper respiratory disease, a similar range of disease conditions as those listed previously need to be considered. For *Av. endocarditidis*, a range of other bacteria have been associated with endocarditis: *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus zooepidemicus* and *Streptococcus pluranimalium* (6).

Intervention Strategies

Management Procedures

Recovered carrier birds are the main source of IC; therefore, practices such as buying breeding males or started chicks from unknown sources should be discouraged. Only day-old chicks should be secured for replacement purposes unless the source is known to be free of IC. Isolation rearing and housing away from old stock are desirable practices. To eliminate the agent from a farm, it is necessary to depopulate the infected or recovered flock(s) because birds in such flocks remain reservoirs of infection. After cleaning and disinfection of the equipment and houses, the premises should be allowed to remain vacant for 2–3 weeks before restocking with clean birds.

Suitable disinfectants, given via drinking water and spraying, have been shown to support the efficacy of vaccines but are not a replacement for vaccines (32, 69).

Vaccination

Types of Vaccines

Commercial IC bacterins are widely available. Because the literature of the various factors influencing the efficacy of bacterins has been reviewed (14), only key points are considered here. Most commercial products are currently based on broth-grown cultures. They must contain at least 10^8 colony-forming units/mL to be effective (82).

There is disagreement in the literature as to the effect of different inactivating agents on the efficacy of bacterins with a full review provided by Blackall and Soriano (25).

Overall, the published literature suggests that although vaccines containing formalin as the inactivating agent can be protective, it is possible that a similar vaccine containing thimerosal would be even more efficient.

A number of adjuvants have been shown to be effective for IC bacterins, in particular aluminium hydroxide gel, mineral oil, and saponin (20). As with any bacterin that contains adjuvants, particularly mineral oil, the potential adverse reaction at the site of injection (51) should be considered when using such products. Research studies have shown that modern generation adjuvants are effective in IC bacterins (54).

Inactivated IC bacterins provide, at best, protection only against the Page serovars included in the vaccine; it is vital that bacterins contain the Page serovars present in the target population. The confirmed existence of Page serovar B as a true serovar with full pathogenicity, as well as its widespread occurrence, means that this serovar must be included in inactivated bacterins in areas where serovar B is present (57). However, because different strains of serovar B provide only partial cross-protection among themselves (137), it may be necessary to prepare an autogenous bacterin for use in areas where the B serovar is endemic or consider commercial bacterins that contain multiple serovar B strains (74). The finding that some Kume serovars within serogroup C are not fully cross-protective (88, 121) needs to be considered in areas where multiple Kume C serovars are known to exist.

Because dissociation of *Av. paragallinarum* has been reported (113), care should be taken in selecting the proper seed culture, media, and incubation period to obtain the most immunogenic product.

A range of mixed bacterins containing inactivated viruses and *Av. paragallinarum* have been described, as previously reviewed (25). There is one report that a combined *Av. paragallinarum–M. gallisepticum* bacterin suppressed the immune response to the *Av. paragallinarum* component (83).

There appears to have been no widespread use of *Av. gallinarum* vaccines.

Field Vaccination Protocol and Regimes

Infectious coryza bacterins are generally injected in birds between 10 and 20 weeks of age and yield optimal results when given 3–4 weeks prior to an expected natural outbreak. Two injections given approximately 4 weeks apart before 20 weeks of age seem to result in better performance of layers than a single injection. When administered to growing birds, the bacterin reduces losses from complicated respiratory disease. Both subcutaneous and intramuscular routes have been effective (24, 47, 82). Injection of the bacterin into the leg muscle gave better protection than when injected into the breast muscle (72). The intranasal route was not effective (24). Oral delivery of an IC bacterin was effective, but this route required 100 times as many cells as with the parenteral route (94). Significant immunity has been demonstrated for about 9 months following vaccination (24, 78, 82).

Because IC has been a problem in broilers in some parts of the world (50, 107), there is an interest in the possibility of day-old vaccination in broilers. A limited level of protection has been achieved in broilers vaccinated at 1 day of age and challenged at day 31 (45).

Treatment

Various sulfonamides and antibiotics are useful in alleviating the severity and course of IC and have been reviewed (21). It should be noted that drug resistance in *Av. paragallinarum* does occur (10). Indeed, a multidrug-resistant plasmid has been reported to be common in isolates of *Av. paragallinarum* from Taiwan, with the plasmid conferring resistance to streptomycin, sulfonamides, kanamycin and neomycin (68). Relapse often occurs after treatment is discontinued and the carrier state is not eliminated (144). Erythromycin and oxytetracycline are 2 commonly used antibiotics.

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21

Mycoplasmosis

Introduction

Naola Ferguson-Noel

Summary

Agent, Infection, and Disease. Avian mycoplasmosis is a collection of diseases of worldwide distribution caused by bacteria in the genus *Mycoplasma* affecting several bird species. They are vertically and horizontally transmitted and the clinical signs vary greatly with the *Mycoplasma* species, the strain, and the species of bird infected. Respiratory disease, synovitis, poor performance, skeletal deformities, or embryo mortality have all been associated with *Mycoplasma* infection.

Diagnosis. Diagnostic tests include serological screening by serum plate agglutination tests or enzyme-linked immunosorbent assays for preliminary diagnosis. Infection may be confirmed by polymerase chain reaction or isolation of the organisms.

Intervention. Elimination of specific avian *Mycoplasma* species from breeding stock avoids vertical transmission to progeny. Vaccination and antimicrobials have been used to control infection and prevent disease.

Introduction

Mycoplasmas (class Mollicutes) are found in humans, many animal species, plants, and insects. These prokaryotes are characterized by their very small size, small genome and complete absence of cell walls; mycoplasmas are bound by a plasma membrane only (54). This accounts for the "fried egg" type of colony morphology, resistance to antibiotics that affect cell wall synthesis, and complex nutritional requirements. Mycoplasmas tend to be quite host-specific; some infect only a single species of animal, whereas others may have the ability to infect several different host species. In general, mycoplasmas colonize mucosal surfaces and most species are noninvasive. However, some species, including *Mycoplasma gallisepticum* (69) and *Mycoplasma synoviae* (21), are known to have the ability to penetrate cells. The primary avian Mycoplasma pathogens of concern to poultry include *M. gallisepticum, M. synoviae, Mycoplasma iowae* and *Mycoplasma meleagridis*.

Classification

Mycoplasmas are members of the class Mollicutes, Order Mycoplasmatales. The genus *Mycoplasma* has more than 120 species, a DNA G + C content of 23%–40%, a genome size of 580–1350 kb, requires cholesterol for growth, occurs in humans and animals, and has a usual optimum growth temperature of 37 °C. The genus *Ureaplasma* is differentiated on the basis of hydrolysis of urea. Acholeplasmas are classified in Order Acholeplasmatales, family Acholeplasmataceae, genus *Acholeplasma*. They are characterized by lack of a growth requirement for cholesterol (54). Phylogenetic analysis of the *16S ribosomal RNA (16S rRNA)* gene and 16S-23S rDNA spacer sequences have proven to be useful to analyze genetic relationships and to identify and classify mycoplasmas (13, 53, 65, 68). The complete genome sequences of several *Mycoplasma* species have been reported and as more *Mycoplasma* genomes become available, the analysis and comparison of these genomes allows further characterization and investigation of the genetic basis of *Mycoplasma* biology and evolutionary relationships (5).

Earlier serotype designations for avian mycoplasmas (20) have been replaced by species names. A current listing

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Table 21.1 Characteristics of avian mycoplasmas.

Species	Usual Host	Glucose Fermentation	Arginine Hydrolysis	Reference
A. laidlawii[1]	Various	+	–	(58)
M. anatis	Duck	+	–	(55)
M. anseris	Goose	–	+	(11)
M. buteonis	Buteo hawk	+	–	(52)
M. cloacale	Turkey, goose	–	+	(9)
M. columbinasale	Pigeon	–	+	(35)
M. columbinum	Pigeon	–	+	(57)
M. columborale	Pigeon	+	–	(57)
M. corogypsi	Black vulture	+	–	(50)
M. falconis	Saker falcon	–	+	(52)
M. gallinarum	Chicken	–	+	(28)
M. gallinaceum	Chicken	+	–	(35)
M. gallisepticum	Chicken, turkey, house finch, other	+	–	(22)
M. gallopavonis	Turkey	+	–	(35)
M. glycophilum	Chicken	+	–	(26)
M. gypis	Griffon vulture	–	+	(52)
M. imitans	Duck, goose, partridge	+	–	(8)
M. iners	Chicken	–	+	(22)
M. iowae	Turkey	+	+	(35)
M. lipofaciens	Chicken	+	+	(10)
M. meleagridis	Turkey	–	+	(70)
M. pullorum	Chicken	+	–	(35)
M. sturni	European starling	+	–	(27)
M. synoviae	Chicken, turkey	+	–	(35, 49)
M. tullyi	Penguin	+	–	(71)
U. gallorale[2]	Chicken	–	–	(39)

[1] *Acholeplasma* species do not require sterols for growth.
[2] *Ureaplasma* species are characterized by splitting of urea.

of avian *Mycoplasma* species is found in Table 21.1. In addition, there are numerous *Mycoplasma* isolates from various species of birds, including strain 1220, a pathogen of domestic geese (59), isolates from various avian species including ratites and penguins, as well as unidentified isolates from domestic poultry.

Direct staining of *Mycoplasma* colonies on agar surfaces or colony imprints with specific fluorescent antibody (19, 61) has been commonly used to determine the species of avian *Mycoplasma* isolates. Additional methods include growth inhibition (18), immunodiffusion (48), and others. More recently, molecular methods such as sequencing of the *16S rRNA* gene (31) and polymerase chain reaction (PCR) (41, 47, 74) have been used with increasing frequency.

The most up-to-date listing of *Mycoplasma* species can be found on the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The minimum requirements for the description of new species of *Mycoplasma* are determined by the International Committee on Systematics of Prokaryotes Subcommittee on the Taxonomy of Mollicutes (13).

Morphology and Staining

In Giemsa-stained preparations or dark-field examinations, *M. gallisepticum, M. synoviae, M. meleagridis* and *M. iowae* cells generally appear as pleomorphic coccoid or coccobacillary bodies approximately 0.2–0.5 µm in diameter (66, 70, 72) but slender rods, filaments, and ring forms have been described. Based on electron microscopy studies, the organisms appear round, pear-shaped or may show a filamentous or flask-shaped polarity of the cell body. In *M. gallisepticum*, polarity is

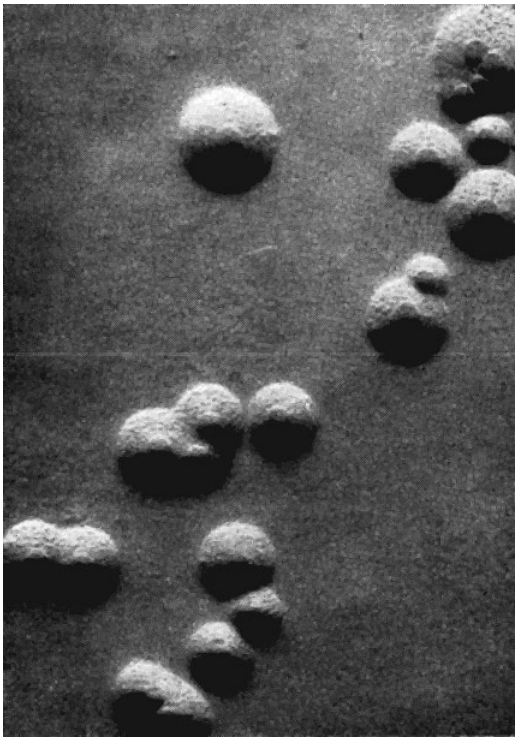


Figure 21.1 Electron micrograph of *Mycoplasma gallisepticum* (MG) cells attaching to a tracheal epithelial cell. The MG cells have flask-shaped morphology and lack of cell wall. The dark elongated tips of the flask-shaped organisms are the MG attachment organelles. (Image courtesy of Dr. Stanley H. Kleven)

caused by the presence of well-organized attachment organelles (blebs or tip structures) (14) (Figure 21.1) and this polarity appears prior to division (4, 46). Such structures are involved in motility, chemotaxis (37, 40, 45), host–pathogen interactions (e.g., cytadherence – hence also termed attachment organelles), and pathogenicity. Some studies suggest the presence of an attachment organelle in *M. iowae* (2) which would be consistent with its phylogenetic placement and with the demonstration of attachment organelle ortholog genes in *M. iowae* strain 695 (67). Ultrastructural studies showed that *M. meleagridis* did not possess bleb structures typical of *M. gallisepticum*, but had thicker fibrils in the central nuclear area (64). Some species of *Mycoplasma* (including *M. gallisepticum* (60), *M. synoviae* (1), *M. iowae* (2), and *M. meleagridis* (32)) are capable of expressing an extracellular surface layer (capsule).

Growth Requirements

Mycoplasma species from avian sources generally require a protein-rich medium containing 10%–15% added animal serum. In general, swine serum should be used in media for *M. synoviae*, and either horse or swine serum for *M. gallisepticum* or *M. meleagridis*. Further supplementation with some yeast-derived component is often beneficial. Growth of *M. synoviae* requires the addition of nicotinamide adenine dinucleotide (NAD) (15, 16). A medium described by Frey (30) or a medium described by Bradbury (7) is commonly used for the cultivation of avian mycoplasmas, although no single medium formulation has been universally accepted as optimum for the growth of all avian *Mycoplasma* species. A broth medium designated SP-4, containing cell culture medium components, also supports excellent growth of several mycoplasmas (63). A satisfactory broth for growth of *M. meleagridis* consists of *Mycoplasma* broth powder (2.1%), yeast autolysate (1%), and heat-inactivated (56 °C for 30 minutes) horse serum (15%). For solid medium, Bacto agar (1.2%) is added to the formulation; the pH of the final medium is 7.5–7.8. A modification of Frey's medium is detailed in Table 21.2. All components except cysteine, NAD, serum, and penicillin may be sterilized by autoclaving at 121 °C for 15 minutes. This should be cooled to 50 °C and aseptically added to the above components, which have been sterilized by filtration and warmed to 50 °C. Pour plates to a depth of approximately 5 mm. Phenol red may be eliminated from agar plates.

Table 21.2 Modified Frey's medium.

Mycoplasma broth base (BBL)	22.5 g
Glucose	3 g
Swine serum	120 mL
Nicotinamide adenine dinucleotide (NAD)	0.1 g
Cysteine hydrochloride	0.1 g
Phenol red (1%)	2.5 mL
Thallium acetate (10%)[1]	5 mL
Potassium penicillin G[1]	1,000,000 units
Distilled H_2O	1000 mL
Adjust pH to 7.8 with 20% NaOH and filter sterilize.	

[1] For potentially contaminated specimens, an extra 20 mL of 1% thallium acetate and 2,000,000 units of penicillin per liter may be added. Ampicillin (200 mg/L to 1 g/L) may be substituted for penicillin.

Mycoplasma organisms tend to grow rather slowly, usually prefer 37 °C –38 °C (some strains of *M. iowae* grow best at 41 °C– 43 °C) (31) and are rather resistant to thallium acetate and penicillin, which are frequently employed in media to retard growth of contaminant bacteria and fungi. On primary isolation, tissue antigens, toxins, and antibodies may be present; therefore, a small inoculum, transferred within 24 hours, or making dilutions of the inoculum in broth may improve results. Transfers are made with a pipette using a 10% inoculum. Colonies form on agar media after 3–10 days at 37 °C;

however, nonpathogenic species such as *M. gallinarum* and *M. gallinaceum* may develop colonies within 1 day (*M. gallinarum* and *M. gallinaceum* are frequently isolated as contaminants during attempts to isolate pathogenic avian mycoplasmas). Most mycoplasmas are facultative anaerobes and although aerobic incubation is sufficient, CO_2 may be added (3, 35).

For *Mycoplasma* species that ferment glucose the resulting lowering of the pH causes the incorporated phenol red indicator to change from red to orange/yellow, making it possible to visually detect growth in broth. *M. synoviae* is sensitive to low pH; therefore, cultures incubated for more than a few hours after the phenol red indicator has changed to yellow (pH less than 6.8) may no longer be viable. Broth cultures should be incubated until a color change is noted; the culture should then be transferred to an agar plate and subcultured into another broth culture.

Inoculated plates should be covered and incubated at 37 °C in a closed container in a moist atmosphere and may require at least 3–7 days of incubation before typical mycoplasmal colonies are sufficiently large to be observed at low magnification (24). For agar plates, use of 1% of a purified agar such as Noble agar, Difco purified agar, or Bacto agar is recommended. Direct plating onto agar plates may result in colonies at 3–5 days of incubation, but isolation in broth is more sensitive. For *M. iowae*, recovery from tissues may be more successful by direct plating on agar than via broth (3); and isolates *M. meleagridis* do not always adapt readily to broth media (23).

The presence and quality of yeast extract are important, and some field isolates may be intolerant of certain media components. Fresh yeast extract (29) may be substituted for the dehydrated product.

Mycoplasmas may also be isolated or propagated in embryonating chicken eggs.

Colony Morphology

Typical colonies are small (0.1–1.0 mm), smooth, circular, and somewhat flat with a denser central elevation ("fried egg" appearance) (see Figure 21.2). Variations in colony morphology have been described, but cannot be relied upon to differentiate the various species. Evidence of colony growth is best studied at low magnification with the aid of a dissecting microscope and indirect light.

Biochemical Properties

Biochemical characteristics of several avian *Mycoplasma* species have been described (2, 16, 20, 34, 35, 54, 62, 70, 72). In general, fermentation of carbohydrates is variable, but all species may be divided into those that ferment glucose with acid production and those that do not.

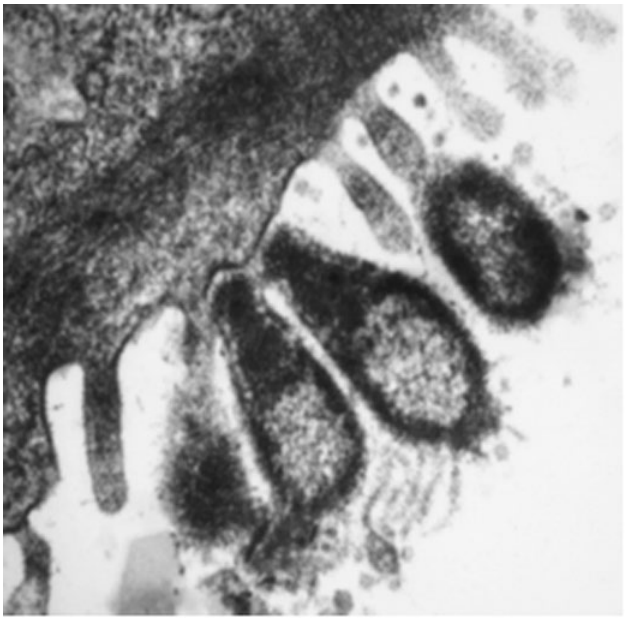


Figure 21.2 Colonies of *Mycoplasma gallisepticum* on agar. ×40. (Hofstad)

Glucose is frequently added to broth media to enhance growth of the carbohydrate-fermenting species and to provide an indication of growth when glucose fermentation produces acid in media containing added phenol red. Phosphatase activity is often present, as is arginine decarboxylase. Most species that do not ferment glucose use the amino acid arginine as their major source of energy. *M. iowae* and some other species, however, ferment glucose and hydrolyze arginine (35). Unusually for a *Mycoplasma*, *M. iowae* also grows in the presence of 0.5%–1% bile salts (56). *M. gallisepticum* was identified as the first bacterium in which messenger RNA is not polyadenylated (51).

A useful characteristic of *M. gallisepticum*, *M. meleagridis*, and *M. synoviae* is hemagglutination of erythrocytes from chickens or turkeys. Hemagglutinating antigens are used for hemagglutination-inhibition serologic tests for these 3 pathogenic species. Some strains of *M. iowae* also hemagglutinate avian erythrocytes, but the property is unstable.

Susceptibility to Chemical and Physical Agents

It is assumed that most of the commonly employed chemical disinfectants are effective against avian *Mycoplasma* species. Inactivation has been produced by phenol, formalin, β-propiolactone, and thimerosal. Resistance to penicillin and a low concentration (1:4,000) of thallous acetate make these valuable additives to *Mycoplasma* culture media as inhibitors of bacterial and fungal contamination, respectively.

Broth cultures maintained frozen at –70 °C or lyophilized cultures maintained at 4 °C are viable for several years (72). Freshly seeded cultures on agar will survive for days at room temperature (36). Some decrease in titer may be anticipated during storage and loss of viability in liquids may depend on the strain, medium, or diluent, and temperature. Live *M. gallisepticum* vaccine stability in various diluents and temperatures has been studied (12, 38). Investigation into the survival of *M. meleagridis* in turkey semen showed that the organism does not decline in substantial numbers in turkey semen during cryopreservation and subsequent thawing (25).

Mycoplasma gallisepticum and *M. synoviae* were inactivated in infected chicken hatching eggs that reached 45.6 °C –45.8 °C during a 12- to 14-hour heating procedure (73). *M. meleagridis* infection in turkey embryos was reduced by preincubation heat treatment of turkey eggs at 46.2 °C–47.6 °C for 11.5–12.5 hours, although there was a reduction in hatchability (33).

Mycoplasma gallisepticum and *M. synoviae* have been detected in environmental samples, including feathers, dust, feed, drinking water, and droppings by culture and by PCR (42, 43). Positive reverse transcription-PCR and culturable *M. synoviae* organisms were found in the environment of a depopulated isolator unit for 3–5 days after depopulation of *M. synoviae*-infected chickens (44). *M. iowae* may survive better than *M. gallisepticum* or *M. synoviae* in the environment (17). Under experimental conditions all 3 *Mycoplasma* species survived on feathers for at least 2 days. Another study showed that *M. gallisepticum* and *M. meleagridis* survived for at least 6 hours in the air (6).

Mycoplasma gallisepticum Infection

Natalie K. Armour

Summary

Agent, Infection, and Disease. *Mycoplasma gallisepticum* (MG) causes chronic respiratory disease in chickens and infectious sinusitis in turkeys. MG is egg transmitted, and economic losses result from processing condemnations, reduced egg production and feed efficiency, and costs of control. MG is worldwide in distribution.

Diagnosis. Serum plate agglutination or enzyme-linked immunosorbent assays are used for screening, and reactors are generally confirmed by hemagglutination inhibition. Diagnosis is confirmed by isolation and identification and/or by polymerase chain reaction, sometimes followed by molecular techniques for strain differentiation.

Intervention. Rearing MG-clean stock with good biosecurity and monitoring programs is necessary to prevent infection. Medication and vaccination with live attenuated or inactivated vaccines are interventions to mitigate clinical disease and production losses when maintaining MG-clean flocks is not considered feasible.

Introduction

Definition and Synonyms

Mycoplasma gallisepticum (MG) infections are commonly known as chronic respiratory disease (CRD) of chickens and infectious sinusitis of turkeys. MG disease is characterized by respiratory rales, coughing, nasal discharge, and conjunctivitis, and frequently infraorbital sinusitis in turkeys. Clinical manifestations are usually slow to develop and the infection or disease may have a long course. Complicated CRD or "air sac disease" describes a severe airsacculitis that is the result of MG or *Mycoplasma synoviae* (MS) infection complicated by a respiratory virus infection (e.g., infectious bronchitis or Newcastle disease) and usually *Escherichia coli*.

Economic Significance

Mycoplasma gallisepticum is the most pathogenic and economically significant mycoplasmal pathogen of poultry. Airsacculitis in chickens or turkeys resulting from MG infections, with or without complicating pathogens, causes increased condemnations at processing. Economic losses from condemnations or downgrading of carcasses, reduced egg production and feed efficiency, and drops in hatchability make MG one of the costliest infectious diseases confronting commercial poultry production worldwide. Prevention and control programs, which may include surveillance, medication, vaccination and elimination of infected breeding stock, account for additional costs.

Public Health Significance

Mycoplasma gallisepticum infects exclusively avian host species and has no public health significance.

History

In 1935, Nelson (202) described coccobacilliform bodies associated with an infectious coryza of slow onset in chickens. Markham (180) isolated and identified pleuropneumonia-like organisms (PPLO) as the etiologic agents of CRD in chickens and infectious sinusitis in turkeys in 1952. The species designation *M. gallisepticum* was made in 1960 by Edward and Kanarek (57). See Yoder and Hofstad (274) and prior editions of *Diseases of Poultry* for reviews of the historical MG literature.

Etiology

Classification

Mycoplasma gallisepticum is an avian pathogen within the genus *Mycoplasma* (class Mollicutes) (226), which was previously designated as serotype A avian *Mycoplasma* (138). *M. gallisepticum* is phylogenetically classified in the *Pneumoniae* subgroup of mycoplasmas, which is named for the human pathogen *M. pneumoniae* (258). Sequence analysis of the *16S rRNA* gene has been used for taxonomic classification, phylogenetic studies, and species identification of mycoplasmas (258). The complete genome sequence has been determined for MG strains R_{low} (217), R_{high}, F (245), S6 (78), and 8 house finch MG strains (254). Strain R_{low} has a genome size of 1,012,800 bp and a G + C content of 31 mol% (217, 245).

Antigenic Structure and Toxins

The plasma membrane of MG, which plays a strategic role in host–pathogen interactions, contains approximately 200 polypeptides (118). A number of MG surface lipoproteins have known and putative functions in motility, adhesion to host cells (cytadhesion), surface antigenic variation and nutrient acquisition, all of which are considered important virulence factors of MG (25, 194, 284).

Variable lipoprotein hemagglutinin (VlhA) (previously pMGA) proteins are immunodominant surface lipoproteins and major hemagglutinins of MG (12, 181). VlhA lipoproteins are abundantly expressed in variant forms by alternating transcription of over 40 *vlhA* genes, which constitute 10.4% of the genome of strain R_{low}, and represent the largest paralogous gene family in the genome (204, 217). Rapid and reversible phase variation in VlhA expression and antigenic switching plays an important role in the generation of MG phenotypic diversity, which is thought to facilitate immune evasion and chronic infection (96, 175, 182, 204).

Other surface-exposed proteins with known or putative functions in cytadherance also contribute to the phenotypic diversity of MG. The primary MG cytadhesin GapA (or Mgc1) and the accessory cytadhesin CrmA (or Mgc3) are coexpressed and are required for MG cytadhesion and motility (102, 117, 216). The putative cytadhesin PvpA is surface-exposed and localized on the attachment (terminal) organelle (18). GapA, CrmA (concurrently with GapA), and PvpA undergo high-frequency phase variation in expression (18, 265, 278). PvpA also exhibits size variation among strains (18). Mgc2, another cytadhesin protein which localizes on the attachment organelle (110) is required for motility (117).

Mycoplasma gallisepticum surface proteins that bind the extracellular matrix (ECM) molecules of the host may facilitate adherence and colonization (113). Several MG proteins with ECM binding potential have been identified, including the fibronectin binding proteins PlpA and Hlp3 (185), the heparin binding OsmC-like protein MG1142 (121), and the plasminogen binding protein alpha-enolase (34)

Potent toxins have not been identified for MG. See Virulence Factors.

Strain Classification

Certain isolates of MG are known by their isolate or other designations and are sometimes called strains. MG strains may differ markedly in their antigen profiles and their virulence-related properties (233). Some MG isolates were described as "variant" or "atypical" because they were difficult to isolate and were less pathogenic, transmissible, and immunogenic than typical field isolates (52, 131, 272).

Antigenicity

Antigenic variation of MG strains and isolates has been demonstrated using serologic assays (46, 149, 169) and monoclonal antibodies in colony immunostaining, immunoblotting, and flow cytometry assays (81, 159, 184, 233). Significant antigenic variability between MG strains can affect the sensitivity and specificity of serologic tests, and presents a challenge to the development and optimization of these assays. Higher antibody titers in sera from chickens infected with MG strains homologous with the test antigen strain has been reported for the hemagglutination inhibition (HI) test (149) and for a ts-11 pMGA enzyme-linked immunosorbent assay (ELISA) (205).

In addition to antigenic differences between MG strains, phenotypic diversity within strains occurs, and is due, at least in part, to high frequency phase variation and antigenic switching (81, 96, 159, 175, 265, 278). This increasingly recognized intrastrain antigenic variability may affect the performance of various serodiagnostic assays (204, 233). The use of recombinant MG antigens

in serologic assays ensures the consistency of prepared antigens and circumvents the problem of variable antigen expression in MG cultures (11, 204). See Antigenic Structure and Toxins, Virulence Factors, and Pathogenesis of the Infectious Process.

Immunogenicity or Protective Characteristics

The attenuated virulence, immunogenicity, and protective characteristics of the MG strains F (231), ts-11 (261, 262), and 6/85 (62) have been applied to their commercial development as live vaccines. See Live Attenuated Vaccines. Immunogenic and protective characteristics also have been described for some other MG strains that may be vaccine candidates, including the naturally attenuated strains K5054 (72) and K-strain (75), and the genetically modified strains GT5 (218) and Mg 7 (83).

Antigenic variability between MG strains does not seem to be sufficient to necessitate the use of multivalent or strain-specific vaccines (259). In vaccination-challenge studies in chickens with heterologous and homologous MG strains, the virulence of the vaccine strain, rather than its homology to the challenge strain, appeared to be the most important determinant of its protective efficacy (169). See Immunity and Vaccination.

Genetic or Molecular

Highly discriminatory and reproducible molecular techniques have largely replaced traditional serologic and protein analysis techniques for MG strain differentiation, facilitating the differentiation of field and vaccine strains, epidemiological studies, and outbreak investigations.

Mycoplasma gallisepticum strains have been differentiated from one another by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (136), by restriction fragment length polymorphism (RFLP) (144), and by Southern blot hybridization using ribosomal RNA gene probes (277).

Random amplified polymorphic DNA (RAPD) or arbitrary primed polymerase chain reaction (AP-PCR) is a DNA fingerprinting method which has proven very useful for strain differentiation (32, 64, 87). RAPD banding patterns are, however, prone to variability and are difficult to reproduce and interpret. Amplified fragment length polymorphism (AFLP) has provided accurate and reproducible strain differentiation (112), but is somewhat complex. The isolation of MG in pure culture is a preliminary requirement for these DNA fingerprinting techniques.

PCR-based techniques that do not necessitate MG isolation have been increasingly used for strain differentiation. PCRs targeting various cytadherance-related genes followed by RFLP (PCR-RFLP) (176, 179) and PCR with high-resolution melting curve analysis (PCR-HRM) (93) have been used for MG strain identification. Targeted sequencing of single or multiple genomic loci (*mgc2*, *pvpA*, *gapA*, *MGA_0319* and 16S-23S rRNA IGSR) has become the preferred approach for MG strain differentiation (genotyping) in epidemiologic studies and outbreak investigations, facilitating the development of sequence databases and interlaboratory comparison (5, 70, 92, 147, 224, 239).

Pathogenicity

Isolates and strains of MG vary widely in their relative virulence, depending on the genotypic and phenotypic characteristics of the isolates, method of propagation, number of passages through which they have been maintained, and challenge route and dosage.

The pathogenic reference MG strains most commonly used for *in vitro* and *in vivo* studies are strains S6, A5969 and R. The neurotropic S6 strain was isolated from the brain of a turkey with nervous signs (42, 280), whereas the A5969 and R strains were isolated from chickens with CRD (250). The R strain has been widely used for bacterin production (276) and as a virulent strain for MG challenge studies (98, 100, 142, 145, 232, 247). The genotypic and phenotypic properties of low- (R_{low}) and high- (R_{high}) passage R strain have been intensively studied. R_{low} is capable of cytadherence and cell invasion, and is pathogenic, whereas R_{high} shows diminished capacities in comparison (218, 219, 266).

The commercially available live attenuated MG vaccine strains differ in relative virulence. F strain vaccine has proven relatively more virulent for turkeys than chickens (172, 232). The 6/85 and ts-ll vaccine strains are less virulent for chickens and turkeys than the F strain (1, 62, 145, 261, 262).

House finch and house finch-like strains of MG have shown relatively low virulence for chickens and turkeys (71, 72, 210, 221). See Virulence Factors and Vaccination.

Virulence Factors

Virulence factors associated with MG include motility, cytadhesion, phenotypic variation, nutrient acquisition, the ability to invade host cells, and the ability to modulate the host's immune response to infection (immunomodulation) (25, 194, 243, 284). The pathogenic effects of MG are thought to be primarily related to the host's immune response to infection (immunopathology) rather than to the production of toxins (226). Sequencing of the complete genomes of several MG strains, and comparative genomic hybridization analyses of attenuated vaccine strains and the virulent strain R_{low}, has facilitated the identification of a number of virulence-associated genes (217, 245, 254).

Mycoplasma gallisepticum organisms move by gliding motility, which allows the organism to access target tissues and to breach host physical barriers, such as respiratory mucus and ciliary activity (20). Attachment of MG

to host cells (cytadhesion) is a prerequisite for successful colonization and subsequent pathogenesis (226). MG cytadhesion is mediated by the attachment or terminal organelle, which is the leading end in gliding motility (117, 194). GapA (or *M. gallisepticum* cytadhesin 1, Mgc1) is the primary MG cytadhesin, and is co-expressed with the accessory cytadhesin CrmA (or Mgc3) (102, 129, 216). Loss of either GapA or CrmA expression results in impaired MG cytadhesion and motility and drastic changes in cellular morphology, with significantly reduced virulence in inoculated chickens (102, 116, 117, 216). GapA, CrmA, and HatA (a component of a high-affinity transporter system) are expressed by the virulent strain R_{low}, but not by its avirulent derivative R_{high} (216, 219). The cytadhesin Mgc2 and the putative cytadhesin PvpA are also located in the terminal organelle; Mgc2 was also reported to be required for motility (18, 110, 117).

Several MG cytadhesins and putative cytadhesins have recognized functions in the generation of phenotypic variation, which is considered to be an important virulence factor, because it is thought to facilitate evasion of the host's immune response and chronic infection. GapA, CrmA (concurrently with GapA) and PvpA undergo high-frequency phase variation in expression (18, 265, 278). Immunodominant variable lipoprotein hemagglutinin (VlhA, previously pMGA) proteins are encoded by a large multigene family comprising 30–70 genes, only 1 of which is expressed at any given time (12, 97, 175, 181). Phase variation in expression of *vlhA* genes and antigenic switching generates VlhA phenotypic variation (96). VlhA phase variation occurred *in vitro* in response to VlhA-specific antibodies, but preceded antibody detection *in vivo* (95, 96). Gene expression studies revealed a pattern of dominant *vlhA* gene expression over time in independent infected chickens (222).

The ability of various surface-exposed MG proteins to bind components of the ECM is thought to facilitate adherence and colonization of MG. *Pneumoniae*-like protein A (PlpA) and HMW3-like protein (Hlp3) bind the ECM protein fibronectin, and are present in the virulent MG strain R_{low}, but absent or aberrant in its attenuated derivative R_{high} (185). MG1142, an OsmC-like protein of MG, binds heparin (121) and MG alpha-enolase binds plasminogen (34), indicating the potential role of these proteins in MG cytadherence. MG1142 was also reported to be an organic hydroperoxide resistance protein, which may play a role in the detoxification of endogenous and exogenous peroxides (122).

The virulence potential of several MG genes encoding proteins with known and putative functions in nutrient transport and metabolism has been demonstrated. Inactivation of the genes *lpd* (encoding dihydrolipoamide dehydrogenase, an essential subunit of the pyruvate dehydrogenase complex) (115), *mslA* (encoding *Mycoplasma*-specific lipoprotein A, a polynucleotide binding protein) (183, 244), *malF* (encoding MalF, a predicted ABC sugar transport permease) (253), and *oppD* (encoding a predicted ATP-binding protein), and 2 putative peptidase genes (252) in pathogenic MG strains by transposon mutagenesis generated mutants with attenuated virulence for chickens, suggesting a role of these genes in the virulence of MG.

Several other putative virulence factors have been identified for MG. Sialidase knockout mutants of MG were significantly attenuated in virulence *in vivo* compared with the virulent MG strain R_{low}, although complementation of the genetic lesion did not restore wild-type virulence (186). The putative MG lipoprotein MGA_0676 was found to be a membrane-associated cytotoxic nuclease capable of inducing apoptosis in cultured chicken embryo fibroblasts (270).

The ability of some strains of MG to invade host cells has been demonstrated *in vitro* and *in vivo*, and has been hypothesized to facilitate evasion of host defenses and antibiotic therapy, chronic infection, and systemic invasion (198, 257, 266). MG invaded cultured cell lines and survived within the intracellular space for at least 48 hours (266). *In vitro* and *in vivo*, the virulent MG strain R_{low} demonstrated significantly greater cell invasion potential than the avirulent strain R_{high} (198, 257, 266).

Immunopathology is recognized as playing an important role in the virulence of MG infection, and there is evidence that MG is capable of modulating the host response during infection through both immunostimulation and immunosuppression, thereby facilitating chronic infection (25, 243). Immunomodulation is achieved at least in part through the stimulation or suppression of chemokines and cytokines (197, 243). MG infection resulted in upregulated expression of several cytokine and chemokine genes, including CXCL13, lymphotactin, RANTES (CCL5), and MIP-1β, but in downregulated expression of others, including CCL20, IL-8, and IL-12 (150, 152, 197). MG possesses a gene encoding a putative cysteine protease and was found to be capable of digesting chicken IgG, potentially indicating a novel mechanism for prolonged MG survival in the face of an active host antibody response (39). See Antigenic Structure and Toxins, Pathogenesis of the Infectious Process, and Immunity.

Pathobiology and Epizootiology

Incidence and Distribution

Mycoplasma gallisepticum infections have resulted in important flock health challenges in chickens and turkeys in all areas of commercial production, and are worldwide in distribution (158).

The application of national monitoring and control programs (e.g., the National Poultry Improvement Plan (NPIP) in the United States (3) and *M. gallisepticum* control and eradication programs in the Netherlands (154)) has significantly reduced the incidence of MG infection in some countries, particularly in primary and multiplier breeding stock (3, 154).

Mycoplasma gallisepticum infection in breeding stock is uncommon in countries with well-developed poultry industries, and infected flocks are typically eliminated because of the risk of vertical and horizontal transmission (140, 154). In these countries, the prevalence of MG infection in commercial meat chickens and turkeys is generally low. However, occasional outbreaks in commercial flocks are considered highly significant because of potentially severe economic consequences. In other countries, MG infection is prevalent in breeding and meat chickens and turkeys (92, 174, 237).

Mycoplasma gallisepticum infection is relatively common in commercial multi-age egg layer farms in many countries (140), and endemically infected flocks represent potential reservoirs of infection. Decreased MG prevalence has, however, been reported in commercial egg laying chickens subsequent to their inclusion in national control programs (154).

Surveillance of backyard, hobby and free-range/village poultry flocks in a number of countries has provided evidence of prevalent MG infection in these birds (30, 63, 104, 187, 192), indicating that they may serve as sources of infection for commercial flocks.

Beginning in 1994, MG was identified as the cause of conjunctivitis in free-ranging house finches and some other songbird species in the eastern United States and Canada (163). By 2002–2005, MG infection and disease in house finches had expanded to the bird's western range (56, 166).

Natural and Experimental Hosts

Mycoplasma gallisepticum infections naturally occur primarily in gallinaceous birds, particularly chickens and turkeys. However, MG has also been isolated from naturally occurring infections in pheasants, chukar partridge, grey partridge, peafowl, bobwhite quail, and Japanese quail (15, 41, 53, 256). MG has also been isolated from naturally infected ducks and geese (16, 26), greater flamingos (59), peregrine falcons (223), and from an Amazon parrot (19), and was detected by PCR in rooks (220). MG has been isolated from sparrows and pigeons living in close proximity to chickens (14, 236), and experimental infections of house sparrows and pigeons indicated that they may be transiently infected, but develop no or only mild clinical disease (91, 146). Red-legged partridges (80), budgerigars (19), and domestic canaries (107) have been experimentally infected with MG. Infrequent MG isolations from wild turkeys (*Meleagris gallopavo*) have been reported (48, 123).

Mycoplasma gallisepticum was isolated as the etiologic agent of an epidemic of conjunctivitis in free-ranging house finches (*Haemorhous* [formerly *Carpodacus*] *mexicanus*), which began in 1994 in the eastern United States (163, 177), and quickly became widespread, affecting house finches throughout their entire eastern range and negatively impacting their population (51, 203). By 2002–2005, the disease in house finches had extended to their western range (56, 166). Subsequent to the house finch MG epidemic, studies have reported MG detections by culture and/or PCR in 21 songbird and other passerine species in North America (49, 106, 164, 178, 193), as well as in downy woodpecker and mourning doves (49, 66). Although conjunctivitis associated with MG infection has primarily been observed in house finches, similar disease has been reported in naturally infected American goldfinch, purple finch, evening grosbeak, pine grosbeak, and blue jay (106, 162, 193) and in experimentally infected pine siskin and tufted titmouse (66). RAPD analyses of MG isolates revealed similar banding patterns for isolates from house finches and those from other wild songbirds, suggesting that these may have originated from house finches (162).

Studies have reported the detection of MG isolates from commercial turkeys that were genetically very similar to house finch isolates (71, 111) and of an MG isolate in house finches genetically related to poultry isolates (111). Phylogenetic analysis of isolates from domestic poultry, house finches and other songbirds indicated that MG has undergone multiple transfers between poultry and house finches, with only a single successful lineage persisting in house finches (111). House finch MG isolates were reported to be attenuated in virulence for chickens and turkeys (71, 210, 221).

Age of Host Commonly Affected

Mycoplasma gallisepticum can probably infect susceptible birds at any age, although very young birds are seldom submitted with naturally occurring disease. In broiler flocks, most outbreaks occur after 4 weeks of age, and signs are frequently more marked than those observed in mature flocks. Younger birds are generally considered to be more susceptible to experimental infections; in one study, chickens younger than 4 weeks of age developed significantly more severe clinical disease than 4- or 6-week-old chickens following virulent MG challenge (84).

Transmission, Carriers, and Vectors

Horizontal transmission of MG occurs readily by direct or indirect contact of susceptible birds with clinically or subclinically infected birds, resulting in high

infection/disease prevalence within flocks. The upper respiratory tract and/or conjunctiva are portals of entry for the organism in aerosols or droplets. There are strain differences in the rates of MG horizontal transmission (52, 238), and transmission rates increase with increasing population density (190). Feberwee *et al*. (68) described an experimental model of horizontal transmission in chickens to study the transmission dynamics of MG and the efficacy of intervention strategies (67, 69).

Clinically or subclinically infected carrier birds are essential to the epizootiology of MG disease because *M. gallisepticum* seldom survives for more than a few days outside of a host. Backyard flocks (63, 187), multiple-age commercial layer flocks (196), and some wild bird species (164) are potential reservoirs of MG infection. Good management and biosecurity practices are necessary to ensure that MG infections are not introduced to MG-clean flocks from these and other sources.

The ability of MG to survive for up to several days on contaminated fomite materials (31, 38), including airborne dust, droplets, or feathers, provides an important mechanism for indirect horizontal transmission and more widespread disease outbreaks. *M. gallisepticum* remained viable in chicken feces for 1–3 days and in egg yolk for 6–7 weeks at 20°C (31) and survived in the human nasal passage for 24 hours; on straw, cotton, and rubber for 2 days; on human hair for 3 days; and on feathers for 4 days (38). The ability of some strains of *M. gallisepticum* to produce biofilms may facilitate their survival in the environment (33). In experimental studies, indirect MG transmission was demonstrated from infected fomites to naïve house finches (50), and low-level MG transmission occurred between groups of chickens separated by short distances in the same room (40, 68).

Mycoplasma gallisepticum can be transmitted vertically from naturally infected hens to their progeny, and vertical (transovarian or egg) transmission has been induced following experimental infections of susceptible chickens (4, 100, 101, 170, 213, 234). The highest rates of transmission occur during the acute phase of the disease when MG levels in the respiratory tract peak; thereafter, egg transmission rates decline as the postinfection interval lengthens (100, 101, 170). In six separate studies, peak egg transmission of the virulent R strain of MG occurred between 3 and 8 weeks after challenge and ranged from 14% to 53% (4, 100, 101, 170, 213, 234). Egg transmission rates during chronic infections under field conditions are likely to be lower than those reported for experimental infection. However, even low rates of vertical transmission may result in high flock infection levels as a result of horizontal transmission of MG from infected progeny that hatch (158). MG control programs must focus on primary and multiplier breeder flocks because of the severe epidemiological consequences of egg transmission.

Incubation Period

In experimental infections of chickens or turkeys with uniform and high dosages, the MG incubation period varies from 6 to 21 days. Sinusitis often develops in experimentally inoculated turkeys within 6–10 days. However, the onset and extent of clinical signs following a known exposure can be very variable depending on MG strain virulence, complicating infections, bird age, and environmental and other stressors (52, 84, 141). Chickens and turkeys often develop clinical infections near the onset of egg production, suggesting a subclinical infection that becomes clinical in response to stressors. Seropositivity may be the first indicator of MG infection with less virulent strains in older birds (272).

Clinical Signs

Chickens

The most characteristic signs of naturally occurring MG disease in adult flocks are tracheal rales, nasal discharge, and coughing. Feed consumption is reduced, and birds lose weight. In laying flocks, egg production declines but is usually maintained at a lowered level (195). However, flocks may have serologic evidence of infection with no obvious clinical signs, especially if they are recovered carriers. Male birds may have the most pronounced signs, and the disease is often more severe during winter (141). Severe outbreaks with high morbidity and mortality observed in broilers are frequently caused by concurrent infections and environmental factors (141). Cases of keratoconjunctivitis caused by MG infection in commercial layer pullets were characterized by facial and eyelid swelling, increased lacrimation, and conjunctival congestion (209). See Morbidity and Mortality.

Turkeys

Turkeys are more susceptible to MG than chickens, commonly developing more severe clinical signs, including sinusitis (Figure 21.3), tracheal rales, coughing, dyspnea, listlessness, decreased feed intake, and weight loss. As in chickens, more severe outbreaks with high morbidity and mortality frequently follow the involvement of complicating factors such as colibacillosis or environmental stressors (141). Nasal discharge and foamy eye secretions often precede swelling of the infraorbital sinuses, which may result in partial to complete eye closure. Feed consumption may remain normal if sight is not affected, but progressive disease ultimately results in poor weight gain and weight loss. Encephalitic forms of MG have been reported in 8- to 16-week-old commercial meat turkeys displaying torticollis and/or opisthotonos (37, 268, 280). In breeding flocks, there may be a drop in egg production. See Morbidity and Mortality.

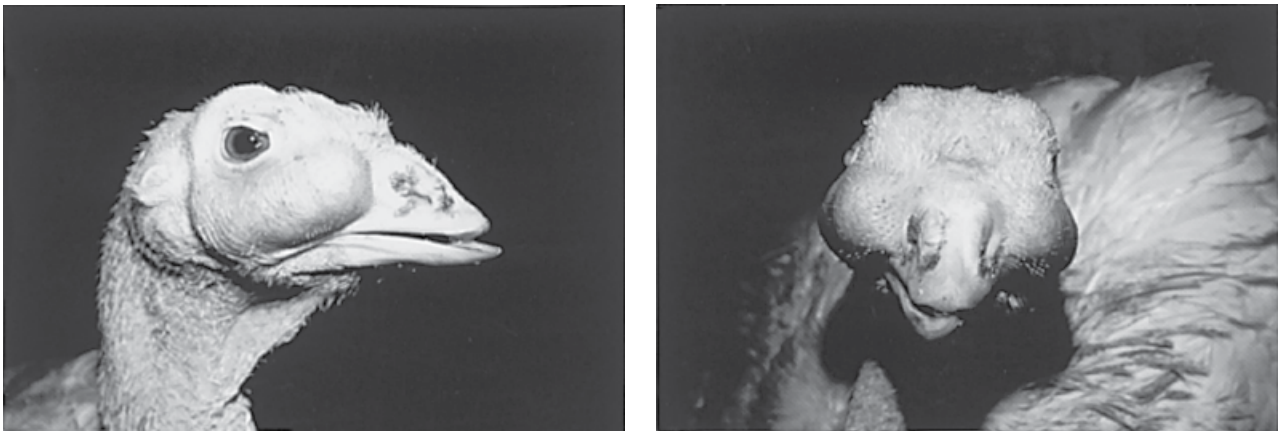


Figure 21.3 Turkey with advanced case of infectious sinusitis showing marked swelling of infraorbital sinuses and nasal exudate.

Morbidity and Mortality

Embryos. Embryo mortality resulting from egg transmission of MG (4, 100, 213) results in the reduced hatchability observed following MG infection of breeders (20, 174). Inoculation of broth cultures or exudates containing *M. gallisepticum* into 7-day-old embryonating chicken eggs via the yolk sac route usually results in embryo deaths within 5–7 days, with dwarfing, generalized edema, liver necrosis, and splenic enlargement. MG strains varied in their *in ovo* virulence, and no correlation was found between *in ovo* virulence and other *in vivo* or *in vitro* methods for virulence evaluation (157). Inoculation of embryonating eggs is rarely employed for the primary isolation of avian mycoplasmas now that adequate culture media are available.

Chickens. *Mycoplasma gallisepticum* typically infects most chickens in a flock, but clinical disease is variable in severity and duration. It tends to be more severe during the cold months (141) and in younger birds (84), although there may be significant egg production losses in laying flocks (195).

Although MG is considered the primary cause of CRD, other organisms frequently cause complications, precipitating severe air sac infection, often designated complicated CRD or "air sac disease." Field or live vaccine strains of Newcastle disease or infectious bronchitis viruses may exacerbate MG infection, which is frequently complicated by *E. coli* (103, 141, 232). Concurrent infections with turkey rhinotracheitis virus in turkeys (201) and low pathogenic avian influenza virus in chickens (241) also resulted in more severe MG disease.

Mortality may be negligible in adult laying flocks, but there can be a reduction in egg production (29, 195). In broilers the mortality may range from low in uncomplicated disease to as much as 30% in complicated outbreaks, especially during the colder months. Retarded growth and carcass condemnations, and downgrading at processing constitute additional losses.

Turkeys. *Mycoplasma gallisepticum* infection of turkeys causes disease in most birds in a flock, which may last for months in untreated flocks. Turkeys do not consistently exhibit sinusitis, and the lower respiratory form of infection may be most prominent (52). Clinical signs, morbidity, and mortality associated with MG infection in turkeys may be highly variable. Typically, meat turkeys experience outbreaks between 8 and 15 weeks of age. Mild respiratory signs may progress in 2–7 days to a severe cough in 80%–90% of the flock, followed by the development of sinus swelling with nasal discharge in 1%–70% of birds in affected flocks (52). Condemnations at processing result from airsacculitis and related systemic effects.

Pathology

Gross

Gross lesions consist primarily of mucosal congestion and catarrhal exudate in nasal and paranasal passages, trachea, bronchi, and air sacs. Sinusitis with mucoid to caseous exudate accumulation is usually most prominent in turkeys, but may also be observed in chickens and other affected avian hosts. Air sacs frequently contain caseous exudate that may be focal, multifocal, or diffuse. Some degree of pneumonia may be observed. In severe and chronic respiratory infections in chickens or turkeys, caseous airsacculitis and fibrinous pericarditis and perihepatitis result in high mortality and extensive condemnations at processing. These lesions are not, however, pathognomonic for MG. Commercial layer chickens with MG keratoconjunctivitis had marked facial and eyelid edema with occasional corneal opacity (209). Conjunctivitis with periocular swelling and inflammation are characteristics of MG in house finches (Figure 21.4) and other songbirds (106, 163, 193) and have been seen in chukar partridges (189). Salpingitis has been associated with decreased egg production in MG-infected flocks (54, 207).

Microscopic

Microscopic pathology caused by MG infection in chickens and turkeys is characterized by marked thickening of the mucous membranes of affected respiratory tract tissues as a result of infiltration with mononuclear cells (primarily lymphocytes) and lymphoid follicle hyperplasia (238, 259, 263) (Figures 21.5, 21.6, 21.7). Metaplasia of the respiratory epithelium from pseudo-stratified ciliated columnar to nonciliated low cuboidal or squamous has been described (238) (Figure 21.6). Increased tracheal mucosal thickness is commonly used as a measure of MG disease severity (208, 260). Lungs may have pneumonic areas, lymphofollicular changes, and granulomatous lesions. Detailed examinations of MG-infected chicken air sacs via light microscopy, scanning electron microscopy, and histomorphometric evaluation have been published (251) (Figure 21.7).

Keratoconjunctivitis in layer chickens associated with MG infection is characterized by epithelial hyperplasia, marked lymphocytic infiltration with the formation of germinal centers, and subepithelial edema, resulting in marked thickening of the eyelids (209).



Figure 21.4 House finch (*Haemorhous mexicanus*) with conjunctivitis caused by MG infection. Reproduced with permission of the American Association of Avian Pathologists.

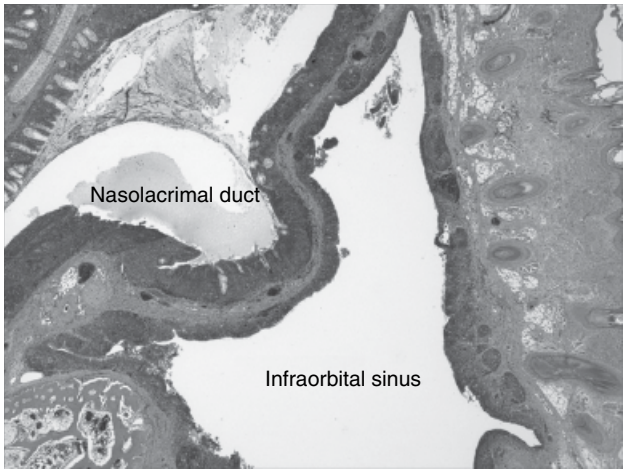


Figure 21.5 Cross-section of the nasal cavity showing the infraorbital sinus. The epithelium of the infraorbital sinus is increased in thickness, and there is a nodular proliferation of lymphoid cells in the connective tissue beneath the lining epithelium. Exudate is present in the sinus.

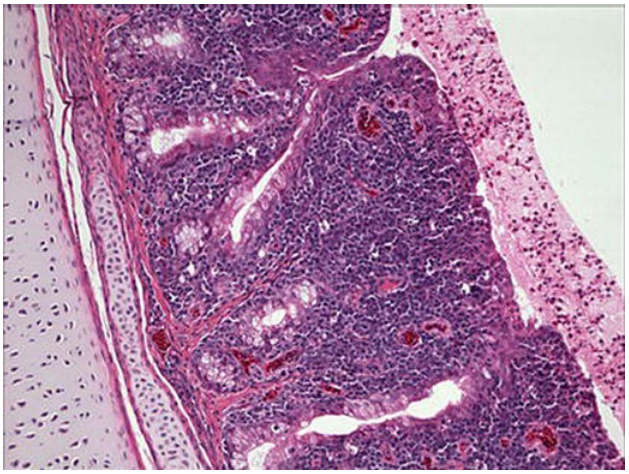


Figure 21.6 Lymphocytic tracheitis in a chicken induced by experimental MG infection. The tracheal mucosa is increased in thickness, primarily caused by lymphocytic infiltration in the lamina propria, and the epithelium is deciliated and cuboidal. A layer of mucus containing numerous heterophils is observed in the tracheal lumen. (O. Fletcher)

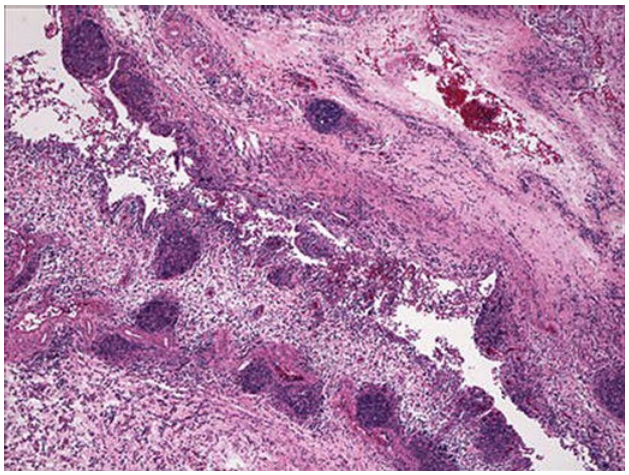


Figure 21.7 Severe, chronic, lymphoplasmacytic airsacculitis in an MG-infected chicken. The air sac is markedly increased in thickness by fibrinoheterophilic, lymphoplasmacytic infiltration, germinal centers, and fibroplasia. The air sac epithelium is hyperplastic, and the lumen contains fibrinoheterophilic exudate and necrotic cell debris. (O. Fletcher)

Histologic examination of turkey brains in cases of encephalitic MG revealed acute to subacute encephalitis with lymphocytic cuffing of vessels, vasculitis, focal to multifocal parenchymal necrosis, and leptomeningitis (37, 42, 268).

Salpingitis associated with reduced egg production in layer chickens was characterized by marked thickening of the oviductal mucosa caused by epithelial hyperplasia and marked lymphoplasmacytic infiltration (207).

Ultrastructural

Ultrastructural details of MG interaction with the tracheal epithelium of chickens have been elucidated by electron microscopic examination (151, 160, 246). Tracheal mucosal lesions were closely associated with the presence of mycoplasmas, and were characterized by deciliation, surface erosion, inflammatory cellular infiltration, edema, and catarrhal changes (151, 160, 246). Mycoplasmas were predominantly found extracellularly, and were only rarely detected in phagocytic vacuoles of epithelial cells (246). Mycoplasmas were attached to epithelial cells by their attachment organelles close to the host cell membrane (246). See Pathogenesis of the Infectious Process.

Pathogenesis of the Infectious Process

Except for infections acquired by egg transmission, the upper respiratory tract and/or conjunctiva are generally accepted to be the portals of entry for naturally acquired MG infections. *M. gallisepticum* is considered to be primarily a surface pathogen of the respiratory tract and conjunctiva, although detection in the bloodstream (257), and spread to other organs, e.g., brain (37, 268) and oviduct (207), indicates that systemic infections can occur.

Gliding motility of MG facilitates access to target tissues and breach of host physical defenses (20). Attachment of MG to host cells (cytadhesion), a prerequisite for successful colonization and subsequent pathogenesis, is mediated by the attachment or terminal organelle and its associated cytadhesive surface lipoproteins (25). The MG surface lipoprotein pMGA1.2 (VlhA1.2) was recently reported to interact with chicken alipoprotein A-1 (ApoA-1) during *in vitro* infection, suggesting a possible role of ApoA-1 as a host receptor for VlhA1.2 (114).

Edema, ciliostasis, deciliation, surface erosion, and catarrhal changes occur subsequent to MG attachment, and are important in the pathogenesis of infection (35, 151, 160, 246). MG-induced ciliostasis was demonstrated in tracheal organ cultures (35). In *in vivo* studies, tracheal edema, deciliation, and catarrhal changes were observed as early as 3 days after virulent MG R strain infection (160).

The robust lymphoproliferative host immune response and ensuing tissue damage (immunopathology) elicited by MG attachment and colonization is considered key in the pathogenesis of MG disease (25, 243). The ability of MG to modulate the host's immune response through immunostimulation or immunosuppression has been demonstrated, and may be achieved through the stimulation or suppression of chemokines and cytokines, and possibly by IgG digestion (39, 150, 152, 197).

The establishment of chronic infection despite the presence of an active immune response is a feature of MG disease, which may be achieved through several recognized mechanisms, including the aforementioned ability of MG to modulate the host's immune response to infection. Phenotypic variation generated by phase variable expression of the MG lipoproteins VlhA, GapA, and PvpA (204), and the ability of some MG strains to invade host cells (198, 257, 266) may also facilitate immune evasion and chronic infection. The cell invasion potential of some MG strains may be a mechanism for their systemic spread (257).

The pathogeneses of egg production drops and egg transmission induced by MG infection have not been fully elucidated. Salpingitis with ovarian regression and oviductal atrophy was associated with MG colonization of the oviduct and egg production drops (74, 207). The detection of MG in eggs has been associated with the presence of air sac lesions and the isolation of MG from the air sacs and/or oviducts (230, 234).

Complicating bacterial and viral infections (especially with *E. coli* and respiratory viruses), immune suppression, poor environmental conditions and other stressors result in more severe MG disease (141). See Antigenic Structure and Toxins, Virulence Factors, Immunity, and Morbidity and Mortality.

Immunity

Active

Mycoplasma gallisepticum infection is characterized by a robust lymphoproliferative response, involving infiltrations of heterophils and macrophages, followed later by large numbers of lymphocytes (243). The tissue damage resulting from this exuberant immune response (immunopathology) is considered to be a primary cause for the pathogenic effects of MG infection, and the ability of MG to modulate the host's immune response to infection (immunomodulation) is an increasingly recognized virulence mechanism.

Cell-mediated immunity is thought to facilitate MG clearance and host resistance to some extent, although the mechanisms remain to be fully elucidated (243). Significant lymphoproliferation with the production of interferon and nitric oxide, occurred as early as 1 week postinfection (227). An influx of $CD8^+$ and $CD4^+$ T lymphocytes

was detected in the trachea 1 week after infection in unvaccinated chickens, but recovery from infection corresponded with the detection of B lymphocytes 3 weeks after infection (85).

The importance of bursal-dependent lymphoid cells in protection against MG infection is well established. Bursectomized chickens had lower antibody titers, worse air sac lesions, and high mortality after inoculation with a virulent MG strain compared with intact chickens (2). The protective effect of immunization against MG challenge was abolished in bursectomized, but not in thymectomized chickens (153).

Mucosal antibodies are particularly important for protection against infection and subsequent disease (6, 120, 218, 271). Recovery of birds from MG infection was associated with the presence of antibodies in tracheal washings (36, 271). Mucosal antibodies appear to confer protection primarily by blocking MG attachment to host epithelial cells (6). Protection against virulent MG challenge correlated with lower numbers of infiltrating B cells and $CD4^+$ and $CD8^+$ cells and higher numbers of MG-specific IgG- and IgA-secreting plasma/B cells, and was associated with discrete lymphofollicular aggregates in the tracheas of vaccinated compared with nonvaccinated chickens (120, 264). In contrast to the importance of mucosal antibodies, circulating antibody levels do not correlate well with protection against MG infection (206, 247, 259).

Although chickens or turkeys that have recovered from clinical MG disease have some degree of immunity, recovered birds may still carry the organism (13, 85), and can transmit infections to susceptible birds by horizontal or vertical (egg) transmission. See Antigenic Structure and Toxins, Virulence Factors, and Vaccination.

Passive

Maternal antibody was demonstrated to provide very little protection against MG challenge, and did not interfere with day-of-age vaccination with the F strain of MG (173). Embryo mortality caused by virulent MG was completely blocked in the presence of maternal antibodies, but MG could still be isolated from the yolk sac, leading investigators to postulate that the presence of maternal antibody could promote the hatching of infected eggs (157).

Diagnosis

Isolation and Identification of Causative Agent

Isolation and identification of the organism is the gold standard for MG diagnosis and is essential for experimental infections. For MG culture, swabs taken from the trachea or choanal cleft (palatine fissure) should be inoculated directly to *Mycoplasma* broth and/or agar media (73). When present, airsacculitis lesions may be sampled; however, MG organisms tend to disappear from lesions after a few weeks but persist in the upper respiratory tract (73). For details on sampling, culture media and isolation methods see the Introduction to Mycoplasmosis and Ferguson-Noel (73).

Detection of Causative Agent Genetic Material

PCR allows rapid, sensitive, and specific detection of MG DNA. Both conventional and real-time PCR techniques are widely used for MG detection, and can be performed directly on clinical samples, without the requirement for culture. Several conventional PCR techniques have been developed for species identification of MG (82, 155, 224). PCR with restriction fragment length polymorphism (PCR-RFLP) has also been used for MG detection (65).

Real-time PCR facilitates rapid, sensitive and quantitative detection of MG DNA. Several techniques have been described (27, 28, 191, 225), and kits are commercially available. Multiplex PCR protocols have been developed, allowing for the simultaneous detection of MG, *M. synoviae* and other organisms (119, 240).

Some MG DNA detection methods do not involve PCR. DNA and ribosomal RNA gene probes have been used to detect MG (77, 135), but have largely been superseded by PCR-based procedures. MG detection by isothermal amplification of target DNA using loop-mediated isothermal amplification (LAMP) has been reported (282).

See Strain Classification for MG strain differentiation techniques.

Serology

Serologic procedures are useful for flock monitoring in MG control programs (3) and to aid in diagnosis when infection is suspected. The serum plate agglutination (SPA) or ELISA tests are used for serologic screening, whereas the HI test is generally used to confirm SPA or ELISA reactors (3, 73). Serologic diagnosis should be confirmed by MG isolation and identification and/or by PCR.

The SPA test is highly efficient in detecting IgM antibody, and infected birds test positive as soon as 7–10 days after infection (73, 139). Because the SPA test is rapid, inexpensive and highly sensitive, and the antigen is commercially available, it has been widely used as an initial screening test for flock monitoring and serodiagnosis (3, 73). However, nonspecific SPA reactors are not uncommon, and have been attributed to MS infection (caused by cross-reactive antigens) (10) and recent vaccination with oil-emulsion vaccines and/or vaccines of tissue-culture origin against various agents (99, 273).

ELISAs are commonly used serologic screening tests for MG, and kits are commercially available. A number of techniques have been described and have been used for the detection of MG in serum, egg yolk, and respiratory secretions (61, 108, 130, 212). Multiplex ELISAs facilitate the simultaneous detection of MG, MS, and other organisms (11, 212). MG antibodies are detected earlier in infection by ELISA than by HI (73). False positive ELISA reactions do occur, and ELISA reactors should be confirmed by HI or using antigen detection methods (3).

Mycoplasma gallisepticum SPA and ELISA reactors are commonly confirmed by the HI test (3). The HI test is highly specific but is less sensitive and more time consuming than the SPA and ELISA tests. Infected birds test positive 2–3 weeks or longer after infection by the HI test (73), which detects IgG (or IgY) antibody. Antibody titers were higher when antigen used in the HI test was homologous to the challenge strain compared with heterologous HI antigen, indicating that antigenic variation between different MG strains may affect HI results (149).

The use of certain antimicrobials, especially early in the course of infection, may affect the development of a detectable antibody response (158).

Differential Diagnosis

Mycoplasma gallisepticum infections of poultry must be differentiated from other respiratory diseases, taking into consideration that clinical MG disease often occurs in conjunction with complicating respiratory infections (73, 141). Specific agent identification and/or serologic procedures are needed to differentiate MG from other microbial causes of disease in chickens and turkeys.

In chickens, MG should be differentiated from Newcastle disease, infectious bronchitis, and colibacillosis, which may be present as separate entities or as part of the complicated CRD syndrome. Other differentials for MG in chickens include infectious coryza (*Avibacterium paragallinarum*), fowl cholera (*Pasteurella multocida*), *Ornithobacterium rhinotracheale* infection, avian metapneumovirus, and respiratory disease caused by mildly virulent strains of infectious laryngotracheitis and avian influenza (73). *M. synoviae* may cause similar respiratory disease to MG, and may be present alone or in coinfections with MG.

In turkeys, the respiratory disease and sinusitis induced by MG infection must be differentiated from low pathogenic avian influenza, Newcastle disease, avian metapneumovirus (turkey rhinotracheitis), fowl cholera, *Bordetella avium* (turkey coryza), *O. rhinotracheale* infection, chlamydiosis, respiratory cryptosporidiosis, aspergillosis, and MS infection (73, 124).

Intervention Strategies

Management Procedures

Because MG can be egg transmitted, and because there is no effective way to reliably eliminate MG from infected flocks, maintaining flocks free of MG infection is only possible by obtaining replacement stock from mycoplasma-free sources, and then rearing them with adequate biosecurity to prevent introduction of the organism (140). Frequent testing according to a monitoring program is important to facilitate early detection of MG infection, and to prevent horizontal and vertical transmission (140). Serologic monitoring of breeder flocks at short intervals (e.g., every 3–4 weeks in turkeys and every 2–3 weeks in chickens) will optimize the ability to detect and prevent the consequences of egg transmission.

Because of the risks of vertical and horizontal transmission from infected flocks, MG infection is usually not tolerated in commercial breeding stock in countries with well-developed poultry industries. In these countries, infected breeding flocks are typically isolated and eliminated (generally by early marketing/slaughter), their hatching eggs destroyed, and biosecurity and surveillance increased complex-wide (140, 154). Depopulated farms are restocked with MG-clean replacement stock following complete house cleaning and disinfection and extended premises downtime.

However, multiple biosecurity challenges facing poultry companies worldwide, including trends towards multi-age production complexes and increased poultry population densities involving various types of poultry, may make maintaining MG-free poultry flocks very difficult (158). In situations where preventing MG infection is not considered feasible or economically viable, appropriate antimicrobial therapy may be used as a short-term intervention to reduce morbidity, mortality, production losses, and MG transmission. Vaccination may be considered as a longer-term intervention in some situations.

Vaccination

The primary objectives of MG vaccination are to provide protection against respiratory disease, drops in egg production and egg transmission, and, in some cases, to displace virulent wild-type strains on a premises with milder vaccine strains (259). Vaccination prior to wild-type exposure is essential (158). Inactivated, live attenuated, and recombinant MG vaccines are commercially available.

In countries with MG-clean breeding stock, vaccination with inactivated or live attenuated vaccines is typically only used in commercial egg-type pullets destined for placement on multi-age, MG-infected farms (140, 154). In countries with enzootic MG infection in breeding

stock, inactivated and/or live MG vaccines are often routinely used for the immunization of breeder replacement pullets.

Types of Vaccines

Inactivated Vaccines. *Mycoplasma gallisepticum* bacterin vaccines typically comprise inactivated MG organisms suspended in aqueous oil emulsion or in aluminum hydroxide adjuvants, and are administered by the intramuscular or subcutaneous route (259).

Mycoplasma gallisepticum bacterin vaccines have demonstrated efficacy at significantly reducing ovarian regression, egg production losses and egg transmission of MG (8, 74, 100, 101, 109, 234, 276), although these protective effects were not apparent in all studies (137, 259).

Reports indicating the ability of bacterin vaccines to provide protection against respiratory disease induced by virulent MG have been varied. Although some authors reported significant protection from respiratory disease in bacterin-vaccinated MG-challenged chickens (74, 109, 128), others have reported that bacterin-vaccinated chickens had no detectable protection against airsacculitis (1).

Chickens vaccinated with bacterins were marginally more resistant to challenge and had somewhat lower MG tracheal loads than unvaccinated chickens after MG challenge. However, these effects were considered to be of limited practical significance in reducing horizontal transmission and in controlling MG infection in the field (69, 140, 142, 247).

Because bacterin vaccines do not contain live MG organisms, there is no risk of vaccinal transmission or reversion to virulence; however, drawbacks include cost, the requirement for individual bird administration, and the occurrence of local vaccine reactions (43, 55, 140). To enhance the performance of inactivated MG vaccines, various inactivating agents and adjuvants have been investigated (8, 60, 168).

Live Attenuated Vaccines. The 3 commercially licensed live MG vaccines currently in common use worldwide are F strain, ts-11, and 6/85.

Although F strain vaccine is a relatively mild MG strain, the original F strain was reported to be a strain of moderate virulence (140, 232). F strain vaccines have been used extensively worldwide for the immunization of long-lived chickens, particularly commercial egg-type pullets prior to placement in multi-age production complexes. F strain vaccines are lyophilized and are labeled for spray or drinking water application, although eyedrop application is commonly practiced in the field (140, 259).

F strain vaccines have demonstrated efficacy at protecting chickens against respiratory disease caused by virulent MG challenge (1, 74, 169, 171, 231). F strain-vaccinated chickens had increased resistance to infection and reduced tracheal colonization of the challenge strain (1, 44). Protection against vertical transmission of MG (100) and MG-induced ovarian regression (74), egg production losses (29, 100, 174, 195), and egg quality and hatchability losses (174) were reported in chickens vaccinated with F strain vaccines.

F strain persists in the tracheas of vaccinated chickens for the life of the flock, inducing a consistent serologic response (1, 143). F strain was able to displace the virulent MG strain R from the tracheas of experimentally infected chickens (145). Displacement of a field strain of MG by F strain in a multi-age commercial layer flock was reported (148). However, F strain continued to cycle among flocks on the farm after vaccination was discontinued (255).

F strain is mildly virulent to chickens and is more reactive than the ts-11 and 6/85 vaccines (1, 23, 231, 232). F strain vaccines are too pathogenic for use in turkeys (172). Vertical and horizontal transmission of F strain has been demonstrated experimentally (143, 170) and epidemiological studies have provided evidence for F strain transmission both within and between farms (92, 94, 134, 161).

The ts-11 vaccine originated from an Australian MG field isolate (strain 80083) of moderate virulence that was exposed to chemical mutagenesis and selected for temperature-sensitivity (normal growth at 33 °C and reduced growth at 39.5 °C) (238, 262). The ts-11 MG vaccine has minimal or no virulence for chickens and turkeys (1, 17, 24, 84, 262). The attenuation of ts-11 is not dependent on the temperature sensitive (ts^+) phenotype (259). The ts-11 vaccine strain lacks expression of the GapA cytadhesin (199). However, GapA expression has been observed in reisolates from infected chickens, and a $GapA^+$ ts-11 vaccine was apathogenic (235). The ts-11 vaccine is distributed as a frozen product for eye-drop application in chickens (259, 261).

The ability of ts-11 vaccine to induce protection against respiratory disease resulting from virulent MG challenge in chickens has been demonstrated (1, 17, 84, 206, 261). Protection was also provided against MG-induced ovarian regression and egg production drops, and against vertical transmission of MG (7, 261). The ts-11 vaccine does not effectively colonize turkeys (158, 262, 264); however, immunogenicity and protection were recently reported for a $GapA^+$ ts-11 vaccine in turkeys (264).

The ts-11 MG strain persists in the upper respiratory tract of vaccinated chickens for the life of the flock and induces a long-lived protective immunity to MG despite a weak systemic antibody response (1, 206, 259, 261). The ts-11 vaccine was not able to displace the virulent MG strain R from the tracheas of experimentally infected chickens (145). However, displacement, followed by eradication of circulating MG F strain on a commercial

layer farm was achieved by ts-11 vaccination of replacement pullets (255).

Horizontal transmission of ts-11 vaccine to commingled birds has been demonstrated in pen studies (40, 165). Field cases of apparent reversion to virulence and vertical transmission of ts-11 vaccine have been reported (58). The virulence and egg transmission potential of an isolate genotyped as ts-11 from the broiler progeny of a ts-11 vaccinated breeder flock was subsequently demonstrated (4, 58).

The 6/85 strain of MG originated in the United States and is regarded as a strain of minimal or no virulence to chickens and turkeys (1, 22, 62, 165, 279). The 6/85 vaccine is lyophilized and is recommended for application by fine spray (62, 259).

The ability of 6/85 vaccine to induce protection against respiratory disease caused by virulent MG challenge has been demonstrated (1, 62). In a comparative *in vivo* protection study, protection induced by 6/85 vaccine was similar to that afforded by ts-11 vaccine, but less than with F-strain vaccination (1). In experimental studies, 6/85 vaccine elicited little or no detectable serologic response, and was detected in the upper respiratory tract of 20% of vaccinated chickens for up to 60–105 days after vaccination (1, 62, 165).

The 6/85 vaccine was not able to displace the virulent R strain of MG in the tracheas of challenged birds (145). In pen trials, 6/85 vaccine did not transmit to comingled pullets or turkeys, or to sentinel birds (165, 279). The isolation of 6/85-like MG from unvaccinated, clinically ill commercial layers and turkeys has been reported (147, 249).

Recombinant Vaccines. A recombinant fowlpox-MG vaccine is available; its safety has been established (156, 283), and its efficacy evaluated (74).

Other Vaccines. A naturally low virulent MG isolate (K5054) from turkeys, genotypically similar to the house finch strain, has shown potential for use as a vaccine in chickens and turkeys (71, 72). K-strain, a naturally attenuated MG isolate from layer chickens, was recently shown to be a safe and efficacious vaccine in chickens, inducing significant protection from respiratory disease, MG colonization and ovarian regression in R strain challenged chickens (75, 76).

A modified live MG vaccine designated GT5 was constructed by reconstitution of the avirulent high passage R strain (R_{high}) with the gene encoding the major cytadhesin GapA (120, 218, 219). The experimental vaccine Mg 7 was developed by transposon disruption of the dihydrolipoamide dehydrogenase gene of the virulent MG strain R_{low} (83, 115).

The development of subunit vaccines using MG surface proteins has been investigated (47, 188, 242).

Treatment

Mycoplasma gallisepticum is inherently resistant to beta-lactam antibiotics such as penicillin and cephalosporins, which act by inhibiting cell wall synthesis (140). MG has shown sensitivity *in vitro* and *in vivo* to several antibiotics including macrolides, pleuromutilins, tetracyclines, and fluoroquinolones (21, 45, 98, 125, 126, 133, 248).

Mycoplasma gallisepticum may develop resistance to commonly used antibiotics, and cross-resistance between antibiotics has been demonstrated (21, 86, 88–90, 215, 281). The molecular mechanisms of MG resistance to fluoroquinolones, macrolides, pleuromutilins, and other antibiotics were studied (88, 167, 229, 267, 269). Guidelines for minimum inhibitory concentration (MIC) testing to evaluate antimicrobial sensitivity have been published (105).

Various antibiotics, including tylosin, tilmicosin, tylvalosin, tiamulin, valnemulin, oxytetracycline, chlortetracycline, enrofloxacin, danofloxacin, and lincomycin-spectinomycin have demonstrated efficacy for the treatment of MG respiratory diseases, reducing the severity of clinical signs and gross lesions, and lowering mortality and performance losses (9, 79, 98, 125–127, 132, 248). Antibiotic treatment may reduce MG populations in the respiratory tract, potentially reducing MG shedding and lowering the risk of horizontal transmission to neighboring flocks (45).

Reductions in egg production losses were reported following in-feed tylosin medication of commercial layers (214). Medication of hens with tylosin, enrofloxacin, or lincomycin-spectinomycin reduced egg transmission of MG (213, 275). Injection or dipping of hatching eggs with antibiotics (e.g., tylosin and erythromycin) under a temperature or pressure differential have been used to reduce or eliminate MG egg transmission (140, 200, 211, 275).

Tylosin and tetracycline antibiotics are commonly used worldwide for the treatment of MG disease. Other macrolide antibiotics (e.g., tilmicosin) and pleuromutilins (e.g., tiamulin) and have also proven useful for MG control in countries where they are approved for use in poultry. Fluoroquinolone antibiotics are highly effective (140), but their use is prohibited in food-producing animals in many countries. Regulations controlling the use of antimicrobials in poultry vary considerably among jurisdictions and should be consulted just prior to treatment for verification and currency.

Although antimicrobial therapy has been successfully used to ameliorate MG disease and production losses, it cannot be relied upon to completely eliminate MG from infected flocks (140, 158, 228). In addition, continuous antibiotic usage may result in the development of antibiotic resistance. Antimicrobial treatment should therefore be regarded as a short-term intervention and not as a long-term solution for MG control (140, 158).

Mycoplasma synoviae Infection

Naola Ferguson-Noel and Amir H. Noormohammadi

Summary

Agent, Infection, and Disease. *Mycoplasma synoviae* infects several bird species including chickens and turkeys, and causes subclinical respiratory disease, lameness, and reduced egg production and eggshell quality. The agent is transmitted both horizontally and vertically and infection has been reported in commercial poultry in most countries around the globe.

Diagnosis. Infection is often detected by serological monitoring of the poultry flocks and confirmed by polymerase chain reaction. Isolation of the organism is primarily attempted for further identification of the isolate by research work.

Intervention. Antibiotic treatment is used for alleviation of clinical signs, but it does not eliminate infection. Vaccination with a live attenuated vaccine appears to be the method of choice in several countries for prevention and long-term control.

Introduction

Mycoplasma synoviae (MS) infection most frequently occurs as a subclinical upper respiratory infection. It may cause air sac lesions when combined with Newcastle disease, infectious bronchitis, or both. MS may also become systemic and results in infectious synovitis, an acute to chronic infectious disease of chickens and turkeys, involving primarily the synovial membranes of joints and tendon sheaths producing an exudative synovitis, tenovaginitis, or bursitis.

Mycoplasma synoviae colonies were first observed as satellites adjacent to *Micrococcus* colonies by Chalquest and Fabricant (16), who identified the requirement for nicotinamide adenine dinucleotide (NAD). It was designated as serotype S by Dierks et al. (20). Olson et al. (97) proposed the name *M. synoviae*, which was subsequently confirmed as a separate species (52). See Ferguson-Noel *et al.* (31) for historical MS literature references. The complete genome sequence of at least 2 strains of MS has been published (79, 121), whereas incomplete genome sequences of multiple other strains are also available (21).

Antigenic Structure

Mycoplasma synoviae has 2 major phase- and size variable antigens, MSPA and MSPB (94). MSPA has been shown to be a hemagglutinin, but MSPB has not been linked with any function so far. Cultures of the hemagglutinin negative phenotype expressed truncated versions of MSPB and were less pathogenic than hemagglutination positive cultures (85). Sequence variation of a 12 amino acid sialoreceptor binding motif within MSPA is believed to be responsible for differences observed between MS strains in their capacity to adhere to the host cells (77). MSPA and MSPB are expressed as a prolipoprotein, variable lipoprotein hemagglutinin (VlhA), from a single gene named *vlhA* (91), but the product is then cleaved to form MSPB and MSPA (5, 91). MS vlhA has a high degree of identity with the vlhA 4.10 (*pMGA1.7* gene) of *M. gallisepticum* (91) which provides a possible explanation for occasional cross-reactivity between these organisms in serological testing. There is only a single complete copy of the *vlhA* gene associated with a promoter region in the MS genome, but there are a large number of incomplete copies (pseudogenes) present in a cluster adjacent to the complete *vlhA* copy (92, 121). Variability in expression of vlhA is thought to be controlled by homologous recombination events between the complete copy of *vlhA* and pseudogenes (92).

Strain Classification

Currently, analysis of the conserved domain (approximately 400 bp of the 5′ end) of the *vlhA* gene by direct nucleotide sequencing or other techniques is frequently used for identification of MS strains (3, 25, 40, 43, 46, 95, 123). Three separate studies (19, 21, 24) have found that multilocus sequence typing analysis was superior to *vlhA*-based genotyping techniques although the set of genes found suitable for strain identification were totally different in these studies. Also, comparison of individual genes in all these studies found that the single copy conserved 5′ end of the *vlhA* gene provided the highest discrimination power amongst all genes examined. Analysis of the whole or partial genomic sequences of MS strains has allowed for the development of rapid strain identification techniques that can differentiate between live vaccines and field strains (22, 61, 113).

Virulence Factors

There is considerable variation among isolates in their ability to produce disease; some isolates cause little or no clinical disease although virulent strains and field cases have been reported in several countries (53, 111). The pathogenicity and virulence of field isolates have been

compared experimentally (68, 83) but differences in virulence could not be explained by potential virulence factors such as hemagglutination and hemadsorption, attachment to cells, or ciliostasis (69). However, the hemagglutination positive phenotype of MS induces infectious synovitis lesions more frequently than does the hemagglutinin negative phenotype (85). Sialidase activity (8, 78), nitric oxide production (66) and cell invasion (12, 23) have been linked to MS virulence. It is also believed that antigenic variation of MSPA and MSPB contributes to the virulence of MS by providing a means for the organism to evade the host immune system (87). The development of new molecular tools (including an origin of replication plasmid) for targeted gene disruption and gene complementation is expected to facilitate future investigations into virulence factors of MS (112).

Pathobiology and Epidemiology

Incidence and Distribution

Infectious synovitis was observed primarily in growing birds of 4–12 weeks of age in broiler-growing regions of the United States during the 1950s and 1960s. Since the 1970s the synovitis form has been less frequently observed in chickens in the United States, but the respiratory form has been seen more frequently. Infection without apparent clinical signs is not unusual. MS infection occurs frequently in multi-age commercial layers (82, 99). Infectious synovitis usually appears in turkeys when they are 10–20 weeks old. MS is worldwide in distribution.

Natural and Experimental Hosts

Chickens and turkeys are the common natural hosts of MS. Ducks (6), geese (7), guinea fowl (102), pigeons (2, 106), Japanese quail (2), pheasants (10), red-legged partridge (103), ostriches (117), a lesser flamingo (14), pigeons, sparrows, and other wild birds (80, 103) have been found to be naturally infected. Pheasants and geese (10), ducks (124), and budgerigars (9) are susceptible by artificial inoculation. Kleven and Fletcher (58) found that although sparrows could be artificially infected, they were quite resistant and are likely to be transient carriers when naturally infected.

Natural infection in chickens has been observed as early as 1 week, but acute infection is generally seen when chickens are 4–16 weeks old and turkeys are 10–24 weeks old. Acute infection occasionally occurs in adult chickens. Chronic infection follows the acute phase and may persist for the life of the flock. The chronic stage may be seen at any age and in some flocks may not be preceded by an acute infection.

Transmission

Lateral transmission occurs readily by direct contact. Birds are infected for life and remain carriers. In many respects, the spread appears to be similar to that of *M. gallisepticum* except that it is more rapid. However, slow-spreading infections have been reported (122). Transmission occurs via the respiratory tract, and usually 100% of the birds become infected, although it is possible for only a few to develop clinical signs. Infection may also occur as a result of environmental contamination or fomites (18, 75, 76).

Vertical transmission plays a major role in spread of MS in chickens and turkeys; however, several flocks hatched from infected breeders may remain free of infection. Experimental infection of broiler breeders resulted in MS infection in the trachea of day-old progeny, infertile eggs, and dead-in-shell embryos 6–31 days postinoculation (118). When commercial breeder flocks become infected during egg production, the egg-transmission rate appears to be highest during the first 4–6 weeks after infection; transmission thereafter may cease, but infected flocks may shed at any time.

Incubation Period

Infectious synovitis has been seen in 6-day-old chicks, suggesting that the incubation period can be relatively short in birds infected by egg transmission. The incubation period following contact exposure is generally 1–21 days. Antibodies may be detected before clinical disease becomes evident. In birds experimentally infected at 3–6 week of age, the incubation period varies from 2 to 20 days, depending on the route of administration. Intratracheal inoculation results in infection of the trachea and sinus as early as 4 days and readily spreads to contact birds. Air sac lesions are at a maximum 17–21 days after aerosol challenge (59). The incubation period also varies with titer and pathogenicity of the inoculum.

Clinical Signs

Chickens

The first observable signs in a flock affected with infectious synovitis are pale comb, lameness, and retarded growth. As the disease progresses, feathers become ruffled and the comb shrinks. In some cases, the comb is bluish red. Swellings usually occur around joints and breast blisters may occur. Hock joints and foot pads are principally involved, but in some birds most joints are affected. Birds are occasionally found with a generalized infection but without apparent swelling of the joints. Birds become listless, dehydrated, and emaciated. Although birds are severely affected, many continue to eat and drink if placed near feed and water. Acute signs

described above are followed by slow recovery; however, synovitis may persist for the life of the flock. In other instances, the acute phase is absent or not noticed, and only a few chronically infected birds are seen in a flock. Chondrodystrophy was noted in the opposite leg of chickens inoculated via the foot pad. This may have been caused by increased weight-bearing stress on the leg opposite the affected leg (83).

Outbreaks of MS in brown egg layers in the Netherlands were associated with amyloid arthropathy, which was reproduced experimentally (63).

Chickens affected by the respiratory form of MS may show slight rales in 4–6 days or may be asymptomatic. Progeny of MS-infected breeders may have increased air sac condemnations, reduced weight gains, and reduced feed efficiency.

Experimental inoculation of hens with MS resulted in a detectable drop in egg production in 1 week postchallenge; by 2 weeks production dropped 18%, and by 4 weeks production returned to normal (70). With naturally occurring infection of adults, however, there may be little or no effect on egg production or egg quality (81), although instances of egg production losses in commercial layers have been observed. MS has been implicated as a contributing factor in the development of *Escherichia coli* peritonitis syndrome and associated mortality in commercial layers (104). MS has also been linked with abnormalities of the apical eggshell in a number of countries (13, 27, 47). Broiler breeder hens appear to be less susceptible to producing eggs with abnormalities after MS infection (28).

Turkeys

Mycoplasma synoviae generally causes the same type of signs in turkeys as in chickens. Lameness may be the most prominent sign. Warm flocculent swellings of 1 or more joints of lame birds are usually found. Occasionally, there is enlargement of the sternal bursa. Severely affected birds lose weight.

Respiratory signs are not usually observed in turkeys, but MS has been isolated from sinus exudates obtained from turkey flocks exhibiting a very low incidence of sinusitis, and from tracheas and choanal clefts of turkeys exhibiting increased mortality and pneumonia. Airsacculitis may at times occur in day-old and older turkeys in MS-infected flocks. Rhoades (108) described a synergistic effect of MS and *Mycoplasma meleagridis* in producing sinusitis in turkeys.

Morbidity and Mortality

Chickens. Morbidity in flocks with clinical synovitis varies from 2% to 75%, with 5%–15% being most usual. Respiratory involvement may be asymptomatic, but 90%–100% of the birds may be infected. Mortality is usually less than 1%, ranging up to 10%.

Turkeys. Morbidity in infected flocks is usually low (1%–20%), but mortality from trampling and cannibalism may be significant.

Pathology

Gross

Chickens. In the early stages of the infectious synovitis form of the disease, chickens frequently have a viscous creamy to gray exudate involving synovial membranes of the tendon sheaths, joints, and keel bursa, and hepatosplenomegaly (Figure 21.8). Kidneys are usually swollen, mottled, and pale. As the disease progresses caseous exudate may be found involving tendon sheaths, joints, and extending into muscle and air sacs. Articular surfaces, particularly of the hock and shoulder joints, become variably thinned to pitted over time (Figure 21.9).

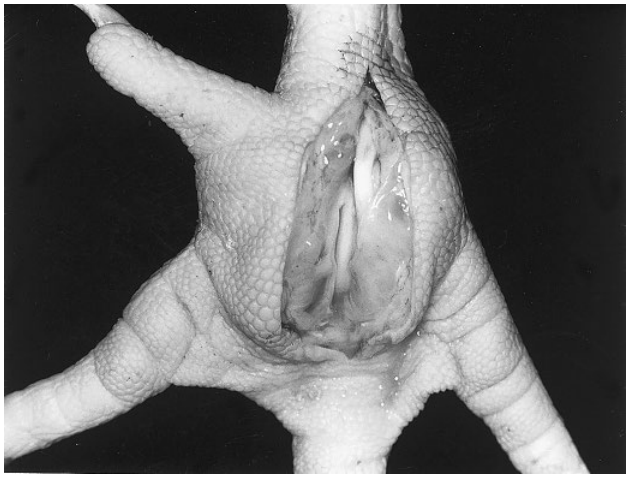


Figure 21.8 Incised swollen foot pad of an 8-week-old turkey with granulation tissue and purulent exudate surrounding digital flexors. Similar lesions can be seen in chickens.

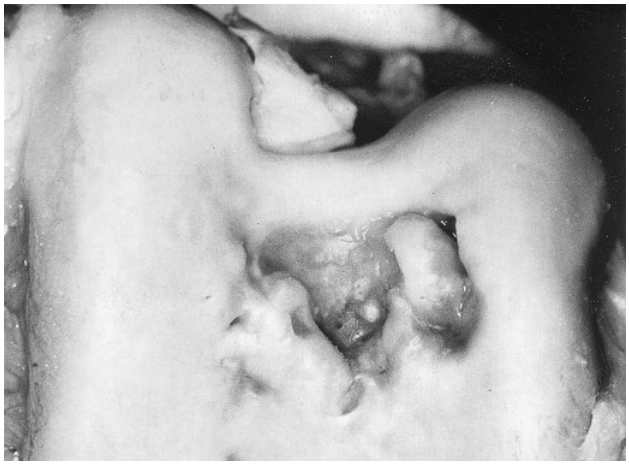


Figure 21.9 Ulceration of articular surface of distal tibiotarsus from a chicken with infectious synovitis.

Generally, no gross lesions are seen in the upper respiratory tract. In the respiratory form of the disease, airsacculitis may be present.

Turkeys. Swellings of the joints may not be as prominent as in chickens, but fibrinopurulent exudate is frequently present when the joints are opened. Lesions in the respiratory tract are variable.

Microscopic

The histopathology of infectious synovitis (54) in chickens and respiratory disease caused by MS in chickens (33) and turkeys (107) has been described.

The joints, particularly of the foot and hock, have an infiltrate of heterophils and fibrin into joint spaces and along tendon sheaths. The synovial membranes are hyperplastic with villous formation and a diffuse to nodular subsynovial infiltrate of lymphocytes and macrophages (Figure 21.10). Cartilage surfaces, over time, become discolored, thinned, or pitted. Air sacs may have a mild lesion consisting of edema, capillary proliferation, and the accumulation of heterophils and necrotic debris on the surface, to more severe lesions with hyperplasia of epithelial cells, a diffuse infiltrate of mononuclear cells and caseous necrosis. Other lesions reported to be associated with infectious synovitis are: hyperplasia of the macrophage–monocyte system associated with the sheathed arteries of the spleen; lymphoid infiltrates in the heart, liver, and gizzard; and thymic and bursal atrophy.

Pathogenesis of the Infectious Process

The route of MS infection may play a significant part in the resulting disease. Natural infection may occur vertically *in ovo* or horizontally by direct contact or airborne spread. Pathogenicity of MS strains generally involves attachment and colonization of the upper respiratory tract plus additional unidentified factors associated with systemic invasion and lesion production.

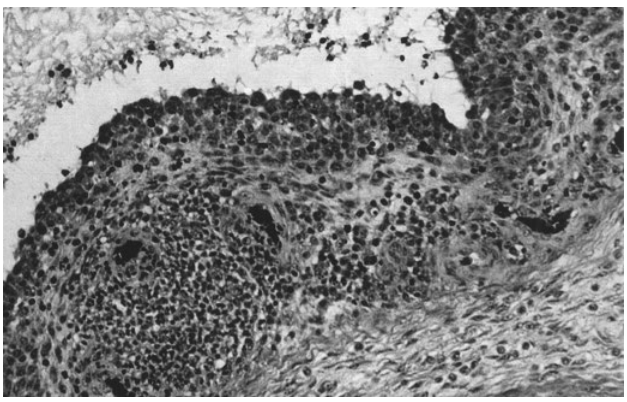


Figure 21.10 Hyperplastic synovial membrane with multiple subsynovial lymphoid aggregates from a 7-week-old turkey with infectious synovitis.

Mycoplasma synoviae isolated from air sac lesions are more apt to cause airsacculitis, whereas those isolated from synovia are more apt to produce synovitis (57). Airsacculitis is exacerbated by Newcastle disease-infectious bronchitis vaccination (59) or any respiratory infection. The severity of the airsacculitis depends on the virulence of the infectious bronchitis virus used in conjunction with MS (44). Air sac lesions are greatly enhanced by cold environmental temperatures (127). Infectious bursal disease causes immunosuppression in chickens and dual infection with MS results in more severe air sac lesions (36). Nervous signs with lesions of meningeal vasculitis have been seen in MS-infected turkeys displaying severe synovitis (17).

Immunity

Chickens inoculated intranasally with a temperature-sensitive mutant of MS were protected against airsacculitis for at least 21 weeks (86). Resistance to lesions induced by MS is bursa dependent (62, 119), whereas thymus-dependent lymphocytes may be needed for the development of macroscopic synovial lesions (62). Specific antibodies of the IgG, IgM, and IgA classes have been detected in the oviduct and albumen of chickens naturally infected by MS, as well is in their developing embryos (4).

Diagnosis

Isolation and Identification

Positive diagnosis may be made by isolation and identification of MS. MS may be isolated from lesions during the acute phase of the disease, but infection of the upper respiratory tract is usually permanent (59). In the chronic stages of infection viable organisms may be no longer present in lesions; isolation from the upper respiratory tract is more reliable in chronically infected birds. (For medium and isolation methods, see Introduction.) Historical methods of identification of MS colonies including growth inhibition and DNA probes have been described in Ferguson-Noel et al. (31) and previous editions of this chapter. Polymerase chain reaction (PCR)-based techniques are now available in many laboratories for simple, rapid, and sensitive detection of MS DNA in tissues or culture medium (45, 65, 105, 115). The PCR procedures are comparable (or in some instances superior) in sensitivity to isolation and identification (110).

Serology

Serum plate agglutination (SPA) test antigens are commercially available. Antigen should be tested with known positive and negative serums in each test. Approximately

2–4 weeks are required for antibodies to develop in infected birds (96). The SPA test may be insensitive in some instances (26). Nonspecific reactors occur in some flocks when using the SPA test (37, 125), especially in flocks that have been vaccinated with oil emulsion vaccines against various agents. *M. gallisepticum* antigen may be agglutinated on occasion, but reaction is somewhat delayed and usually lower in titer (98). To confirm specificity of the reaction, the hemagglutination inhibition (HI) test is used (120).

Enzyme-linked immunosorbent assay (ELISA) (42, 100) is commonly used for routine testing of flocks, and may replace SPA as the primary serologic test. ELISA kits are available commercially. It should be noted that performance (specificity and sensitivity) of ELISA systems is highly dependent on the reagents used, especially the nature and quality of the antigen or antibody used in their preparations. A recombinant antigen containing a highly antigenic domain of MSPB (94) has been used as a serodiagnostic reagent (93). The recombinant MSPB antigen from vaccine strain MS-H was more efficient in detecting antibodies in vaccinated chickens than was a similar antigen prepared from strain WVU 1853, suggesting some antigenic variability in that region (88). Antigenic variability among MS strains results in homologous reactions (using antigen prepared from the same strain used to infect the chickens) that tend to be stronger than heterologous reaction (antigen prepared from a different strain) (56). ELISA has also been used to detect antibodies in the yolk of eggs from commercial layers (38).

In a comparison of diagnostic tests for MS, all serological procedures studied exhibited some false-positive activity (29). Further confirmation of serologic results may be made by isolation and identification of MS from the upper respiratory tract (109) or by PCR.

Turkeys produce a low level of antibody following respiratory infection; therefore, SPA may not be effective in determining the MS status of a flock (60). Individual infected turkeys may not develop detectable antibodies (101). Culture, PCR, ELISA, and HI testing may be required in some cases to detect infection.

Differential Diagnosis

A presumptive diagnosis may be made on the basis of lameness, breast blisters, and enlarged but compressible foot pads or hock joints containing exudates. Other bacteria that may cause synovitis or arthritis must be eliminated by bacteriologic procedures. These include (but are not limited to) *Staphylococcus aureus*, *Escherichia coli*, pasteurellae, and salmonellae may also be present as primary causes of synovitis.

Fibrosis of metatarsal extensor or digital flexor tendons and lymphocytic infiltration of the myocardium associated with the viral arthritis agent help to differentiate it from MS (71). Serum from viral tenosynovitis-infected chickens does not agglutinate MS antigen, but one must bear in mind that MS agglutinins may be present without obvious joint involvement.

In cases with respiratory involvement, *M. gallisepticum, Avibacterium paragallinarum*, *Pasteurella multocida*, and other causes of respiratory disease should be eliminated.

Intervention Strategies

Management Procedures

Mycoplasma synoviae is egg transmitted and the most effective method of control is to select chickens or turkeys from MS-free flocks. In countries where primary breeding stocks are free of infection, MS-free sources of replacement breeding stocks should be available. Effective biosecurity measures should be used to prevent introduction of the infection. See Kleven (55) for a review of avian *Mycoplasma* control strategies in commercial poultry.

Outbreaks of MS infection in broilers can often be traced to a specific breeder flock. By the time the infected breeder flock is found, egg transmission may be low or no longer of clinical significance. The decision to slaughter infected parent breeder flocks is often made on an economic basis. If such flocks are kept for egg production, progeny should be hatched separately and isolated from MS-free flocks. Antibiotic treatment of breeders is not effective in eliminating MS infection, although the level of egg transmission may be reduced.

Vaccination

An inactivated, oil emulsion bacterin has been commercially available, but its role in the control of MS has not been adequately studied. It is commonly believed that these vaccines induce a strong humoral antibody response, but this may not be necessarily correlated with protection against infection. A live temperature-sensitive MS vaccine strain, MS-H, was selected by mutagenesis of a field isolate from Australia (84). Its safety and efficacy have been established under laboratory (72, 73) and field (74) conditions. Vaccine doses of 4.8×10^5 cfu/mL were protective (48); protective immunity was detected after 3–4 weeks postvaccination (50) and persisted for at least 40 weeks (49). The vaccine has been shown to be effective in reducing apical egg shell abnormalities caused by MS infection (30). The temperature-sensitivity phenotype of the MS-H vaccine is believed to be mediated by a point mutation in its GTP-binding protein Obg (114), although factors other than the temperature-sensitive phenotype appear to be

involved in the attenuation of the MS-H vaccine strain (90). This vaccine has received wide use in Australia and many major poultry-producing countries but registration in the United States is pending. The safety and efficacy of MS-H vaccine have also been assessed in turkeys (89).

Treatment

Mycoplasma synoviae is susceptible *in vitro* to several antibiotics, including chlortetracycline, danofloxacin, enrofloxacin, lincomycin, oxytetracycline, spectinomycin, spiromycin, tetracycline, tiamulin, tilmicosin, aivlosin, and tylosin (11, 15, 35, 41, 51, 64). Soluble lincomycin-spectinomycin (2 g/gallon of drinking water) (39) and tiamulin in the drinking water (0.006%–0.025%) have been shown to be effective in preventing clinical signs (1). Generally, suitable medication is of value in preventing airsacculitis or synovitis, but treatment of existing lesions is less effective. Antibiotic medication is not thought to eliminate MS infection from the flock, but reports have been somewhat variable (32, 67, 116), maybe because of differences in treatment and MS strains.

In contrast to *M. gallisepticum*, MS isolates appear to be resistant to erythromycin (11). High-level resistance to erythromycin and tylosin developed rapidly after low-level exposure *in vitro*, but enrofloxacin resistance developed more gradually. No resistance to tiamulin or oxytetracycline was shown (34).

Treatment of eggs with antibiotics such as tylosin by egg dipping, or egg inoculation with tylosin and gentamycin, or heat treatment (126) of hatching eggs has been used in breeding flocks to prevent egg transmission of MS. Exposure of breeders before the onset of egg production with virulent MS will reduce egg transmission. This should only be used in flocks in which infection will almost certainly occur.

Mycoplasma iowae Infection

Mohamed El-Gazzar and Janet M. Bradbury

Summary

Agent, Infection, and Disease. Late embryo mortality and leg problems in young turkeys are associated with *Mycoplasma iowae*. It can be vertically transmitted and not all breeders are free, which has economic and commercial implications. However, there is a gap in knowledge about its pathogenesis and virulence factors.

Diagnosis. New molecular assays have made diagnosis and epidemiological investigations easier.

Interventions. Elimination of *M. iowae* from breeding stock avoids vertical transmission to progeny. Vaccines are not available. Antimicrobials have been used to control infection and prevent disease.

Introduction

Mycoplasma iowae has been associated with reduced hatchability and embryo mortality as well as leg problems in young turkeys.

Economic Significance

Poult hatchability may reduce by 2%–5%. Vertical transmission can cause mortality and reduced performance during the first few weeks of a commercial turkey's life (13, 34, 50).

History

The type strain, Iowa 695, was characterized and designated avian serotype I (52, 53). Serotypes I, J, K, N, Q, and R were later assigned to 1 related group and named *Mycoplasma iowae* (26). Turkey type 8 strains were also *M. iowae* (1).

Etiology

Classification

Mycoplasma iowae belongs to the class Mollicutes, family Mycoplasmataceae, with characteristic colonial morphology, no cell wall, and a growth requirement for sterol. Phylogenetic analysis of the 16S rRNA places it in the pneumoniae group with *Mycoplasma gallisepticum*, *Mycoplasma imitans* and *Mycoplasma pneumoniae* (11).

Morphology and Staining

Giemsa staining or dark-field examination reveals coccobacillary organisms with pleomorphism. Ultrathin sections reveal a cell membrane but no wall (26). A putative attachment organelle is consistent with its phylogenetic placement in the *Mycoplasma muris* cluster (1, 29, 37) and recent study on these organelles in *M. iowae* and its close relative *Mycoplasma penetrans* proposed a role in gliding motility (29).

Growth Requirements

Incubation is usually at 37 °C, although some strains grow best at 41 °C–43 °C and growth occurs aerobically or with added CO_2 (26). Isolation may be more successful on agar than in broth. Several media can be used and the presence and quality of yeast extract are important (4).

Colony Morphology

Colonies on agar have the typical fried egg appearance with diameters of 0.1–0.3 mm (53).

Biochemical Properties

Mycoplasma iowae ferments glucose and hydrolyses arginine (26). Unusually for Mycoplasma, it grows in the presence of 0.5%–1% bile salts (45). Some strains hemagglutinate avian erythrocytes (52, 53), but the property is unstable.

Susceptibility to Chemical and Physical Agents

Mycoplasma iowae survival may exceed that of *M. gallisepticum* or *Mycoplasma synoviae* (15). It survived 5 days on feathers and 6 days on human hair and other materials. However, *M. iowae* appears to be inactivated by proper cleaning and disinfection.

Antigenic Structure

There is significant antigenic variation among *M. iowae* strains (38, 54). Immunoblotting using monoclonal antibodies demonstrated considerable diversity (38). Colony immunoblots probed with a monoclonal antibody revealed phenotypic variation of surface antigens (19, 44).

Strain Classification

Antigenicity

Mycoplasma iowae strains give poor antibody response in chickens and turkeys. Little is known about its cellular immunity. Raising chicken antibodies to 12 different avian mycoplasmas proved most difficult with *M. iowae* (1). Furthermore, no single antiserum from hyperimmunized rabbits detected all strains by immunofluorescence (1).

Immunogenicity or Protective Characteristics

Little is known on these topics.

Genetic or Molecular Characteristics

The *M. iowae* genome consists of 1,195,147 bp, one of the largest within the genus, with an average G + C content of 20%. It includes 151 tandem repeats, 11 pseudogenes, 2 rRNA loci, and 29 tRNA genes (51). Heterogeneity in the DNA is evident using restriction enzyme analysis and by restriction fragment length polymorphism using southern hybridization with 16S rRNA probes (32). The 16-23S rRNA intergenic spacer region sequences of 16 *M. iowae* field strains and the 6 serotypes showed 100% similarity (42). Arbitrarily primed polymerase chain reaction (PCR) is the most used method for genotyping *M. iowae* field strains (17). Amplified fragment length polymorphism (AFLP) (24) and whole genome sequencing were proposed for intraspecific differentiation of *M. iowae*. Recently, multilocus sequence typing (MLST) has been developed for *M. iowae* (21) and provides reliable strain differentiation with universal, expandable nomenclature and a publicly accessible database.

Pathogenicity

Variability exists in pathogenicity and virulence of *M. iowae* strains (30, 53). Experimental infection causes dose-related mortality in chicken and turkey embryos (10, 36, 53). Under field conditions turkey embryo mortality and reduced hatchability may occur with widely variable losses.

Artificial challenge can induce mild to moderate airsacculitis in turkeys and leg lesions in chickens and turkeys (5–8, 53). Such problems are rare in the field, but some outbreaks in young turkeys with leg weakness and deformities have been reported (16, 34, 50) and an association with vertebral lesions noted (34). Unlike other avian mycoplasmas, *M. iowae* exhibits a predilection for the digestive tract (37).

Virulence Factors

Virulence factors of *M. iowae* are unknown, but availability of the genome sequence (40, 51) provided some insights. *M. iowae* genes encoding proteins similar to toxins identified in *M. pneumoniae* have been identified (39). Oxygen tension is suspected to play a role in gene regulation, which could explain increased virulence of *M. iowae* in tissues with higher O_2 concentration (39).

Pathobiology and Epizootiology

Incidence and Distribution

Mycoplasma iowae has been reported in North America, Western and Eastern Europe (2, 13, 14), Asia (35), but not in Australia.

Natural and Experimental Hosts

Turkeys of any age are the natural host but embryos in late incubation are most affected. Isolation of *M. iowae*

from chickens and geese is possible (2, 35, 53) with reports in parrots (3), grey partridge (14), and wild birds.

Transmission, Carriers, and Vectors

Avian species are the only known host for *M. iowae*, despite isolation from an apple seed in France. Egg transmission occurs in turkeys (36, 53) with lateral transmission in the hatchery via the meconium.

Horizontal transmission occurs although the organism does not spread rapidly in young flocks. Few birds in a flock may be culture-positive before maturity.

Infection can spread venereally through artificial insemination (31, 46). After onset-of-lay, following artificial insemination, a high percentage of females may become culture-positive. Although infected semen may play a role in lateral spread, vaginal contamination of hands at artificial insemination seems to be more important.

Clinical Signs

Clinical signs are rare in live turkeys, but some reports (16, 34, 50) have associated *M. iowae* with leg weakness in young poults and incidence of stunting, lameness, and swollen hocks. Eggs from infected turkey breeders may have reduced hatchability (generally 2%–5%). Affected embryos typically die during the last 10 days of incubation, often from days 18–24. Experimentally-infected embryos showed a significantly reduced embryo-to-egg-weight ratio (37).

Pathology

Gross

Embryos show stunting and congestion, with various degrees of hepatitis, edema, and splenomegaly (36, 37, 53), and sometimes swollen plumules. Chorioallantoic membranes of inoculated turkey embryos are edematous and sometimes hemorrhagic (37). Airsacculitis in inoculated chickens and turkeys is usually mild to moderate (53). Inoculation of day-old poults and chicks resulted in stunting, poor feathering, tenosynovitis, and a variety of leg abnormalities (Figure 21.11A–C) (6, 7, 9, 53). Inoculated poults may show cloacal bursal atrophy. Some similar lesions have been seen in naturally-infected turkeys (34, 50). A low incidence of wry neck (Figure 21.11D and E) and other vertebral lesions (Figure 21.11F–H) have been reported, as well as splenomegaly (34).

Microscopic

Chorioallantoic membranes of inoculated turkey embryos developed edema and cellular infiltration, whereas parenchymatous organs showed a heterophilic response (37).

Inoculated day-old poults developed changes in spleen, cloacal bursa, duodenum, ileum, and cecal tonsils (6). Microscopically, vertebral chondrodystrophy in turkeys has also been detailed (34).

Air sac inoculation of poults revealed membranes thickened with inflammatory cells, and fibrinous and mucosal exudate. Experimentally infected broiler-breeders developed tenosynovitis, hemorrhage, and tendon fiber degeneration, followed by chronic lymphocyte/plasma cell reaction and tendinous and peritendinous fibrosis (7).

Ultrastructural

Adherence of *M. iowae* to embryo intestinal mucosa occurred in artificial infection (37). Most organisms adhered to the microvilli, which were often swollen. The organisms were found in cloacal crypts and in secondary mucosal folds of turkey hen vagina by electron microscopy (48).

Pathogenesis

Little is known but the first step may be attachment to the embryo intestinal epithelial surface (37, 48), and may involve a 65-kDa polypeptide (18).

In strains that proliferate in the embryo, death probably results from acute inflammation of the chorioallantoic membrane and heterophilic reaction in the parenchymatous organs (37). Western blots of *M. iowae* reacted positively with antibodies to a 48-kDa *Mycoplasma* protein with immunomodulatory and hematopoietic activities (22).

Phenotypic variation plays a role by allowing the mycoplasmas to persist despite an immune response, and *M. iowae* may be mildly immunosuppressive (1).

Immunity

Active Immunity

Antibody responses are reported (53). Individuals in an infected breeder flock may resolve the infection within weeks or a few months with embryo mortality usually subsiding immediately before this. Growth-inhibiting and metabolism-inhibiting antibodies in serum of these turkey hens suggest immune response involvement.

Diagnosis

Isolation and Identification

Dead embryos contain higher numbers of *M. iowae* (10, 36), where esophageal or yolk sack swabs are preferred sites for sampling. After poult inoculation, isolation was

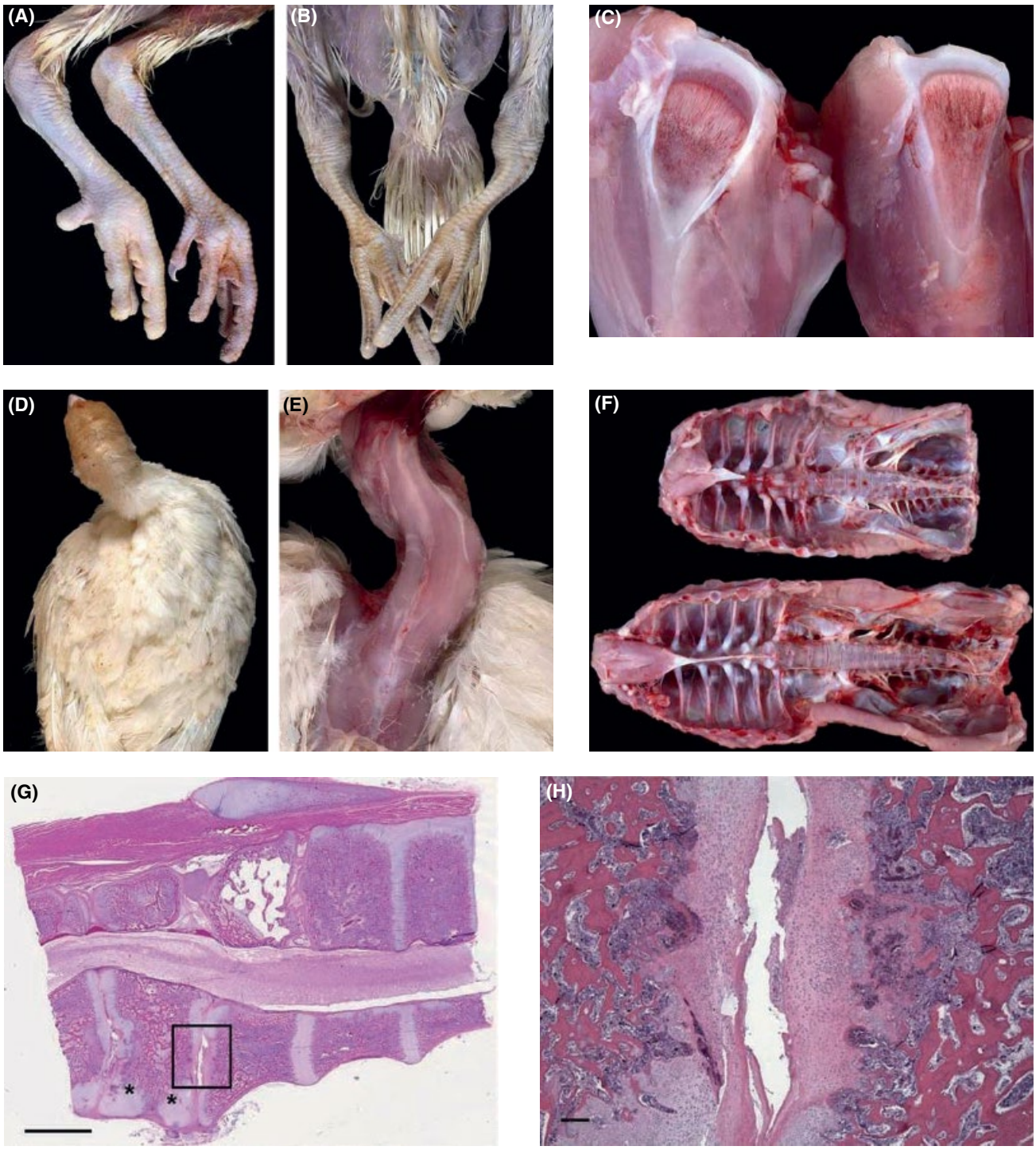


Figure 21.11 (A) Turkeys, 28 days, normal (left) vs. chondrodystrophic legs (right). Although weights were comparable, the chondrodystrophic turkey has short thick shanks and toes compared with the unaffected turkey. (B) Turkey, 42 days, bilateral chondrodystrophy. Legs are short, thick, with enlarged hocks, and there is marked medial bowing of both legs, with bilateral varus deformity. (C) Proximal tibiotarsi of the 28-day turkeys in (A). The affected bird (left) shows thickening and curvature of the bone and physis. (D) Turkey, 30 days, before and (E) after removal of skin. The neck is permanently deviated to the right and rotated. (F) Normal and chondrodystrophic spines from 2 similarly sized birds. The upper chondrodystrophic spine is shorter; the vertebral column is distorted and thickened, and the ribs are unevenly spaced. (G) Lesions in vertebral articular cartilage. Articular cartilage of the free thoracic vertebra is necrotic (box). Cartilage persists (*) in the ventral portions of the vertebra adjacent to the articular surface. Note the compression of the spinal cord. Bar = 20 mm. H & E. (H) A higher magnification of the region within the box in (G) shows degeneration and necrosis of cartilage with an irregular surface. Osteomyelitis is in the bone beneath the cartilage on the right. Bar = 20 μm. H & E. All pictures and comments are adapted from D.H. Ley et al. 2010. *Avian Pathol.* 39:87–93.

possible from gastrointestinal tract and cloaca, but became less frequent with age with no isolation after 12 weeks (6). Isolation from oviduct, semen, and phallus of adult chickens and turkeys has been reported (46, 53) and a combined oviduct/cloacal swab is useful towards the end of an eradication program. Samples on agar are incubated at 37 °C for 4–5 days or longer. Colonies can be identified by immunofluorescence, although use of polyclonal antisera to different serovars is needed to cover antigenic variation. Monoclonal antibodies are generally too specific to detect all isolates, but antibodies to a 45-kDa antigen reacted with all 22 field isolates tested (49).

PCR has been developed for detecting *M. iowae* DNA (12, 30, 41, 43). Others are referenced in previous editions. Using real-time PCR for clinical samples facilitated diagnosis (43). Additionally, MLST allowed *M. iowae* cases to be differentiated directly from clinical samples (21) without the need for isolation. Molecular diagnostics reduces the time of diagnosis and increases the ability for epidemiological investigations.

Serology

Although agglutination, metabolism inhibition, indirect hemagglutination, and ELISA have been used in experimental infections (30, 47, 53) the serologic response is weak (6, 9) and nonspecific reactions occurred with ELISA. Thus, there is no reliable serologic test for field use.

Differential Diagnosis

Mycoplasma iowae and *M. meleagridis* infection should be considered in cases of low hatchability in turkeys, especially if there is late embryo mortality. Embryo lesions may resemble certain nutritional deficiencies, whereas down abnormalities may mimic those produced by overheating of embryos during incubation. Although not recognized as a significant cause of clinical tenosynovitis, *M. iowae* should be considered in undiagnosed cases, especially in young turkeys.

Intervention Strategies

Management Procedures

Mycoplasma iowae was eradicated by certain primary turkey breeders by preincubation antibiotic treatment of hatching eggs, reinforced by monitoring (1), but this *Mycoplasma* is not currently included in the US National Poultry Improvement Plan or the European Union Council Directive 2009/158/EC.

There is no reliable serologic screening procedure for *M. iowae* and culture can be impractical and difficult before birds begin production. However, the use of PCR procedures enhances and accelerates diagnosis.

Mycoplasma iowae -free flocks can be maintained by preventing fomite transmission with special attention during artificial insemination. Residual site infection is not a known problem after proper terminal cleaning and disinfection.

Vaccination

There is no demand for vaccines against *M. iowae*.

Treatment

Antibiotics effectiveness in reducing infection was investigated (27, 28) and attempts made to reduce vertical transmission in commercial flocks to alleviate hatchability losses.

Mycoplasma iowae seems more resistant than other avian mycoplasmas to antimicrobials (23, 25, 33). It rapidly developed resistance during culture in subinhibitory amounts of erythromycin and tylosin (20). Resistance was found after culture with enrofloxacin, tiamulin, or oxytetracycline, and resistant mutants occurred more readily with *M. iowae* than with *M. gallisepticum* or *M. synoviae*.

The quinolone antibiotics, particularly enrofloxacin, have been effective when administered to laying hens in drinking water early in production. Eggs from quinolone-medicated turkeys were resistant to *in ovo* challenge with *M. iowae* (27). However, this product is not available for food animal use in some countries. Treatment consisting of eggs vacuum-dipped in antibiotic solution has been commonly employed.

Other Mycoplasmal Infections

Naola Ferguson-Noel

Summary

Agent, Infection, and Disease. Several bacteria in the class Mollicutes ("mycoplasmas") have been isolated from different bird species and although some of these infections have been associated with disease and economic losses, the association is often not well defined. *Mycoplasma meleagridis* (MM) has been identified as a pathogen of turkeys; other mycoplasmas of interest include *Mycoplasma imitans, Mycoplasma pullorum, Mycoplasma gallinarum*, ureaplasmas and mycoplasmas isolated from geese, ducks, and pigeons.

Diagnosis. *Mycoplasma meleagridis* has been extensively investigated and diagnostic tests have been developed including serology and polymerase chain reaction testing. Species-specific tests have not been developed for many of the other *Mycoplasma* species and diagnosis is dependent on isolation of the organisms.

Intervention. Antimicrobial treatments are used and may reduce clinical signs.

Mycoplasma meleagridis

Mycoplasma meleagridis (MM) (140) (N strain pleuropneumonia-like organism [4], H serotype [64]) is a specific pathogen of turkeys. The clinical syndrome of airsacculitis and/or associated skeletal abnormalities has been called day-old type airsacculitis (73), airsacculitis deficiency syndrome (97), and turkey syndrome-65 (TS-65) (129). During the early 1980s when the prevalence of MM was very high, the monetary cost to the US turkey industry resulting from MM-related hatchability losses and the cost of egg treatment to control egg-borne infections was estimated at $9.4 million/year (26). Currently, the economic losses caused by MM infection in turkeys have been reduced significantly with the availability of MM-free eggs and poults supplied by major turkey breeders. Genome sequences of the MM type strain and field strains have been published (108, 133, 134).

Antigenic Structure

Mycoplasma meleagridis is antigenically unrelated to other avian mycoplasmas. Use of hyperimmune polyclonal rabbit antiserum as well as monoclonal antibodies produced against MM and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed antigen heterogeneity among strains, and some epitopes were not expressed in all strains (34, 148). Arbitrarily primed PCR (AP-PCR) has also revealed intraspecies genotypic heterogeneity among isolates and strains (42). Hemagglutination activity (41, 102, 124, 140) and neuraminidase activity (11) are both variable. Hemagglutinating activity is not an essential component for virulence because strains lacking this activity may be pathogenic (140). Although the genes encoding major immunodominant antigens have not yet been isolated and characterized, an antigenic domain unique to MM has been detected (82) and a major surface nuclease (Mm19) has also been identified (132).

Strain Classification

Pathogenic and nonpathogenic strains of MM were described by Ghazikhanian and Yamamoto (52, 53). Strain variations may account for the variability in clinical manifestations attributed to this organism.

Incidence and Distribution

Early studies showed that MM was a common pathogen of turkeys with a worldwide distribution (27). These prevalence studies, together with the knowledge that MM was transmitted through the egg, led major primary breeders in the mid-1970s to initiate programs to eradicate the agent from their stocks (54). With the success of these programs, the prevalence of MM has been reduced significantly within the past 30 years in the major turkey-producing areas of the world.

Natural and Experimental Hosts

Mycoplasma meleagridis is a specific pathogen of turkeys. When injected into turkey embryos by the yolk sac route, the organism produces a high incidence of airsacculitis but causes minimal mortality (138). Turkeys of all ages are susceptible to air sac infection with MM when inoculated via the air sac or trachea (72, 91, 137). Chickens are generally refractory to infection or morbidity associated with MM (3, 74, 139, 144, 145). Recently, however, MM was isolated from chicken breeders located near a turkey breeding ranch (63). MM was reported to have been isolated from free-ranging birds of prey in Germany (77); antibodies were also detected in peafowl and lesser prairie chickens (56, 58).

Transmission, Carriers, and Vectors

Vertical Transmission

Mycoplasma meleagridis is perpetuated primarily through egg transmission. Infection of the female reproductive tract occurs as an endogenous infection during embryonic development (84), as an ascending infection from foci in the cloaca or cloacal bursa after the occluding plate is perforated at sexual maturity (85), or by insemination of hens with MM-containing semen (72, 91, 92, 141). The egg transmission rate among individual hens may vary from 10% to 60% (141). There is no regular pattern regarding the sequence of infected eggs laid (91) although transmission starts at a low rate during the first 2–3 weeks of lay, reaches a maximum at midseason, and gradually declines toward the end of the laying season (15, 72). A detailed review of early studies on the egg transmission of MM can be found in Chin (27).

Horizontal Transmission

Direct and indirect transmission of MM may occur at any stage of the bird's life. Direct transmission by the airborne route may occur within a hatchery (72) or flock (143), or on occasion between flocks separated by one-quarter mile (54).

Indirect transmission results from management practices including sexing, vaginal palpation, artificial insemination, and vaccination whereby mycoplasmas are carried manually from infected to noninfected turkeys via contaminated hands, clothing, and equipment (54, 91).

Airborne transmission apparently is of little significance because it pertains to vertical transmission after a bird has reached sexual maturity. Thus, egg transmission does not occur in noninfected females that have been placed in cages adjacent to infected females. Similarly, clean males held in the same room with phallus-infected males produce MM-free semen throughout the production period (135, 136).

Clinical Signs

Mycoplasma meleagridis causes late incubation (25–28 days) mortality in artificially (26) and naturally (37) infected turkey embryos. Despite a high rate of airsacculitis in poults originating from infected dams, respiratory signs are rarely observed. Lateral transmission that may occur by direct or indirect means in adult birds may lead to a high infection rate, but rarely to clinical disease. Thus, MM commonly occurs as a silent infection in adult birds. MM acts synergistically in producing severe airsacculitis with *M. iowae* (106) and sinusitis with *Mycoplasma synoviae* (107). Environmental conditions and co-infections may affect severity of airsacculitis (5, 109).

In affected flocks, MM-associated skeletal abnormalities (i.e., TS-65 syndrome) may be observed in poults between 1 and 6 weeks of age (129). Although not a consistent feature of the disease, the syndrome called TS-65 (also called airsacculitis deficiency syndrome) may be associated with MM egg-borne infection (27). The syndrome, which includes signs of bowing, twisting, and shortening of the tarsometatarsal bone and hock joint swelling, has been reproduced experimentally in MM-free poults (16, 130, 131, 142). Deformation of cervical vertebrae (24, 93), stunting, and abnormal feathering (16) are additional features of the disease.

Reproductive Performance

It has been estimated that MM causes a loss in hatchability of 5%–6% of fertile eggs set under commercial conditions (27).

Air Sac Lesions and Condemnations

During the mid-1960s, MM-associated airsacculitis was reported to be one of the major causes of condemnation of fryer-roaster turkeys in the United States (5, 71). Air sac lesion rates of 10%–25% in first-run poults from MM-infected flocks over a season's production were reported under experimental and commercial conditions (47, 77, 95, 150).

Skeletal Abnormalities and Growth Performance

Five percent to 10% of the poults may show clinical signs, but on occasion the percentage may reach higher levels. Not all cases progress to an irreversible state (129). Incidence of the disease seems to increase with the age of the turkey breeders. Mortality is due primarily to cannibalism of affected birds. The problem is not associated with a particular strain of turkey, but males seem to be more susceptible.

Pathology

Gross

Although gross lesions, if any, in poults from infected dams at time of hatch are limited to the air sacs, the organism may be widely distributed in various tissues including feathers, skin, sinus, trachea, lungs, air sacs, cloacal bursa, intestine, cloaca (14, 101, 104), and hock joints (136). The air sac lesions are characterized by thickening of the air sac walls with adherence of a yellow exudate to the tissue and, occasionally, presence of variously sized flecks of caseous material free in the lumen (4). Extension of such lesions to the abdominal air sacs is a common occurrence by 3–4 weeks of age. It is also possible for the organism to be present in air sacs of day-old poults exhibiting no lesions; in such cases, air sac lesions may develop in 3–5 weeks (13). Lesions produced by MM when not mixed with *M. iowae* are not as extensive or fulminating as those described for *M. gallisepticum*. Figure 21.12 shows a poult, hatched from an MM-infected egg, with caseopurulent airsacculitis.

Skeletal lesions, when present, may be associated with severe airsacculitis (96). Sternal bursitis, synovitis (109), and ascites (130) are additional lesions observed in experimental infections. The sinusitis produced by MM

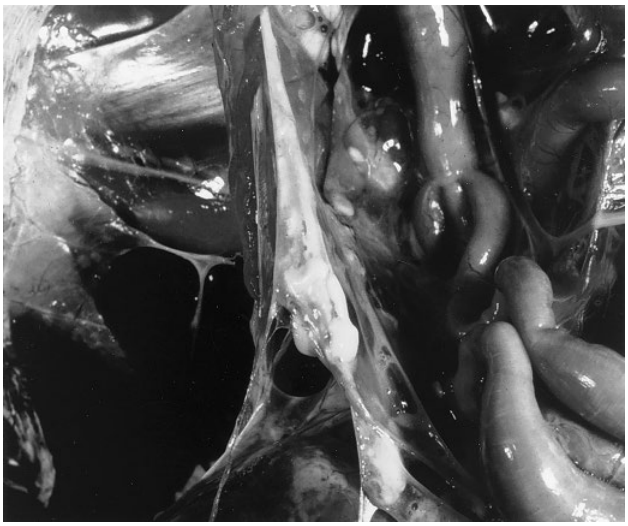


Figure 21.12 Airsacculitis in a 4-week-old turkey caused by egg-borne *Mycoplasma meleagridis* infection. (R. Yamamoto)



Figure 21.13 Bowing of tarsometatarsal bones of a 3-week-old turkey hatched from a *Mycoplasma meleagridis*-infected egg. (Y. Ghazikhanian)

and *M. synoviae* in mixed infections contains clear mucous to caseous exudate (107). Leg abnormalities in poults hatched from MM-infected eggs are shown in Figure 21.13.

Microscopic

In embryonic infection with MM, exudative airsacculitis and pneumonia were the only inflammatory lesions seen. Lesions that developed at 25–28 days of age were related to maturation of the inflammatory reaction. Air sac lesions consisted predominantly of heterophils with some mononuclear cells, including lymphocytes and varying amounts of fibrin and cellular debris. Epithelial necrosis was seen in severely affected air sacs. Mononuclear cells and fibrin were the prominent features of lung lesions (53, 104). Significant or marked microscopic changes in other organs in embryos or poults were not observed despite invasion of the organism into many of these sites (53).

In 7-week-old poults infected with MM by the air sac route, lymphocytic perivascular infiltration and fibrinoheterophilic exudate were observed in 2 days. Some areas of the air sac epithelium became hyperplastic and others underwent necrosis at about 4–8 days. Lymphoid follicles were observed in 16 days. When examined by electron microscopy, the follicles were found to be surrounded by encapsulated collagen bundles and composed of hemocytoblasts of bursal origin presumed to be involved in antibody formation (103). Others observed similar sequential changes in poults infected as embryos or between 1 and 3 days of age (6, 53, 94).

Wise et al. (129) indicated that gross and microscopic long-bone lesions of TS-65 were similar to those observed in perosis of dietetic origin. The main lesions were seen in proximal ends of the long bones. Cartilage farthest from blood vessels descending into the proliferative zone from the cartilaginous epiphysis lacked cell density and contained abnormal-appearing chondrocytes. In long-standing cases of 6–8 weeks or longer, growth plates often were normal, suggestive of repair, even though the bones were grossly deformed. These cellular changes in the proliferative zone of the growth plates were seen in all long bones examined, suggesting a generalized response. It was postulated that MM causes a secondary block of nutrients to the growth plates.

A secondary lesion in the medial side of the proximal end of the tarsometatarsal bone of chronic cases with varus deformity was described as a dyschondroplasia or chondrodystrophy resulting from partial failure of the metaphyseal blood supply at the growth plates (129).

Mild mononuclear cell infiltration was observed in the periarticular region of the hock joint in 2-week-old poults inoculated intravenously with MM (100).

The most prominent lesion in hens infected by the vaginal route was focal encapsulated accumulation of lymphocytes present most frequently in the fimbria, uterus, and vagina. Plasma cells and heterophils also were present in significant numbers in the lamina propria of the reproductive tract. The encapsulated follicles were believed to be active in antibody formation (105). Similar lesions in the reproductive tract of turkeys infected with MM were described by Ball et al. (7).

Gerlach et al. (51) examined histologically the phallus and accessory structures of males experimentally infected with MM. There was lymphofollicular formation in the region of the mucous-type glands in the submucosa of the lymph fold.

Immunity

Turkeys inoculated intravenously or by the respiratory route with MM were resistant to reinfection when challenged by the same routes 21 weeks later. There was no correlation, however, between antibody titer and resistance (90). However, repeated injections of 20-week-old hens with live organisms failed to induce protective immunity or reduce egg transmission (127). Studies indicate that an active immune mechanism can function to eliminate MM in infected birds after removal of the source of infection (e.g., contaminated semen). Persistence of infection in the latter study may be an expression of immune tolerance in hens infected by egg transmission (27, 37, 68, 72).

Maternal antibodies (agglutinins) may be detected in a high percentage of poults from infected dams and persist for approximately 2 weeks posthatching. Such antibodies do not protect against the development of air sac lesions in infected embryos (90, 141). Conversely, purified IgM

and IgG antibodies, when injected into the yolk sac of infected embryos, significantly reduced embryo mortality and the incidence of leg deformities in hatched poults, but they did not reduce air sac lesions or isolation rates when compared with the controls (17).

Diagnosis

Bacterial Isolation and Identification

Mycoplasma meleagridis may be isolated readily on several commercially available and laboratory-prepared media. For culture media and isolation methods, see Introduction and Ferguson-Noel et al. (44). Polymyxin B (100 units/mL) may be added to the broth portion of the overlay to facilitate isolation of MM from highly contaminated sources such as the cloaca and phallus. Mycostatin (50 units/mL) may be added to the agar and broth to inhibit fungi (92). *M. meleagridis* may be selectively isolated from specimens containing mixed cultures by adding to the medium immune serum against the undesired *Mycoplasma* (21).

At necropsy, the organism may be isolated from various sites of the respiratory (including sinus) and reproductive systems. The organism may be isolated from the vitelline membrane, air sacs, intestine, and many other sites of infected embryos (see Pathology: Gross). It may also be isolated from the kidneys of poults infected by the air sac route (130). *M. meleagridis* may be differentiated from other chicken and turkey mycoplasmas by the direct (28) and indirect (21) fluorescent antibody tests. In addition, an antigen-capture ELISA has been developed for detection of *Mycoplasma* antigen directly in broth culture (1).

DNA-based tests have been developed for the direct detection of the organism in clinical specimens (18, 43, 48, 75, 87, 88, 98, 146, 147). The main advantages of PCR-based methods are their rapidity and their ability to detect MM within a high background flora as in the case of cloacal swabs or from samples collected on antibiotic-treated birds.

Serology

Rapid plate (RP) as well as hemagglutination-inhibition (HI) tests are useful for detecting antibodies to MM infections (27, 66, 67). The micro-HI test has been used to identify false-positive RP reactions in flocks recently vaccinated with *Erysipelothrix* vaccine (136). Other tests developed for mass screening are the microagglutination indirect or blocking ELISA (35), recombinant antigen-based ELISA (9), and avidin-biotin enhanced dot-immunobinding assay (29).

Intervention Strategies

Although early studies placed much emphasis on the control of MM infections in turkeys by use of various antibiotic treatment regimens, the goal of primary breeder organizations was to eradicate the agent from their stocks. Because virtually all breeding stocks were infected, a program of test and slaughter – which had been effective in the control of *M. gallisepticum* – was not a practical approach for eradicating MM at that time (27). Experimental studies demonstrated that the administration of antibiotics into eggs either by dipping or by inoculation were useful methods to reduce the egg-transmission rate (40, 86). Antibiotic treatment was also effective in eliminating MM from turkey semen (27). Several antimicrobials have *in vitro* activity against MM; antibiotics have been administered therapeutically to affected flocks in drinking water to reduce mortality and to attempt to reduce egg transmission (27). Dipping hatching eggs in antibiotic solution significantly reduced the incidence of air sac infection (12, 71, 96, 110) concomitant with improved hatchability (71, 110), improved performance (12), reduced incidence of skeletal deformities (96), and reduced condemnation at processing (71). During the late 1960s to early 1980s, before MM-free eggs and poults were available, it was a common practice for multiplier breeders to dip their eggs in antibiotic solution. Tylosin (3,000 ppm) or gentamicin (500 ppm), along with a disinfectant such as quaternary ammonium compound (250 ppm), was used in dip solutions. However, repeated hatching-egg dipping in antibiotics to reduce the incidence of MM infection could lead to induction of antibiotic-resistant organisms. Eradication of MM from major primary breeding companies has reduced the practice of hatching-egg dipping in antibiotics by the commercial industry. Vaccines are not available for prevention of MM infection in turkeys.

Mycoplasma imitans

Mycoplasma imitans is of interest primarily because of its close relationship to *M. gallisepticum*. It has been isolated from ducks and geese in France and from a partridge in England (19). *M. imitans* strains share many phenotypic properties with *M. gallisepticum*, including biochemical reactions, hemadsorption, and hemagglutination. The original isolates were initially identified as *M. gallisepticum* on the basis of immunofluorescence and growth-inhibition tests. Further serologic studies indicated only a partial relationship to *M. gallisepticum*, and DNA hybridization studies with the type strains of *M. gallisepticum* showed a DNA homology of 40%–46% (19, 36). *M. imitans* contains a gene family closely related to the *pMGA* (now *vlhA*) family of *M. gallisepticum* (83), and shares epitopes with *M. gallisepticum* hemagglutinin *vlhA*, pyruvate dehydrogenase *pdhA*, lactate dehydrogenase, and elongation factor *Tu* (76).

PCR procedures for *M. gallisepticum* which are based on the *16S rRNA* gene (50) do not differentiate between *M. gallisepticum* and *M. imitans*. However, PCR reactions based on other targets such as mgc2, gapA, and MGA_0319 (49) do differentiate between the 2 species.

Mycoplasma imitans causes ciliostasis in chicken and duck tracheal organ cultures and has an attachment organelle and gliding motility similar to that seen in *M. gallisepticum* (2, 111). It reproduced respiratory disease similar to but somewhat milder than *M. gallisepticum* in red-legged partridges (45). An isolate of *M. imitans* gained virulence on back-passage in turkeys, and reproduced a respiratory disease which was more severe when it was present in a dual infection with rhinotracheitis virus (47). *M. imitans* did not produce signs or lesions when inoculated into chickens, but in a dual infection with infectious bronchitis virus a synergistic effect was seen (46).

Although *M. imitans* has not yet been reported in the United States, and it has not been found in commercial poultry flocks, there is concern about possible misidentification of isolates as *M. gallisepticum* and possible serologic cross-reactions in testing of field flocks.

Mycoplasma gallinarum

Mycoplasma gallinarum has not been considered to be one of the pathogenic avian *Mycoplasma* species, although there is 1 report of isolations associated with airsacculitis in a series of broiler flocks and the induction of airsacculitis with concurrent Newcastle disease-infectious bronchitis vaccine administration (65). It has also been suggested that *M. gallinarum* infection delays the onset of fatty liver syndrome in commercial layers (23). *M. gallinarum* and *M. gallinaceum* are often isolated as contaminants during attempts to isolate pathogenic avian mycoplasmas.

Originally classified as avian serotype B (31) it was named *Mycoplasma gallinarum* (38). It grows well on all commonly used avian *Mycoplasma* media, and has characteristics common to all mycoplasmas, including cell and colony morphology, absence of a cell wall, and a requirement for cholesterol. It does not ferment glucose, but reduces tetrazolium, is positive for arginine decarboxylase, and exhibits film and spots (8). The complete genome sequence of an *M. gallinarum* strain has been published (133). There is genetic heterogeneity among various strains (33) as measured by RFLP analysis of genomic DNA.

Mycoplasma gallinarum is ordinarily isolated primarily from chickens, but it may also be found in turkeys (10, 59). It has been isolated from jungle fowl (112), ducks (39), pigeons (99), and a Eurasian griffon (79). It is considered to be worldwide in distribution. *M. gallinarum* is commonly isolated as a contaminant during attempts to isolate *M. gallisepticum* or *M. synoviae*, especially from adult chickens. Isolation of *M. gallinarum* from chicken embryos (10) and demonstration of the organism in oviducts (30, 128) suggest the possibility of egg transmission. It is readily identified by immunofluorescence of colonies on agar (123). No serologic test is available.

Mycoplasma pullorum

Mycoplasma pullorum was classified as avian serotype C (31) and was later named *Mycoplasma pullorum* (60). It has been isolated from chickens, quail, partridge, pheasants, and turkeys (10, 22, 89). *M. pullorum* has been isolated from turkey embryos from flocks in France which were experiencing low hatchability and was shown to be pathogenic for chicken and turkey embryos (89). Like other mycoplasmas, *M. pullorum* isolates demonstrate genetic heterogeneity (78).

Avian Ureaplasmas

Ureaplasmas differ from mycoplasmas primarily in their ability to hydrolyze urea (69). There are several reports of isolation of avian ureaplasmas (57, 70). These organisms subsequently received the name *Ureaplasma gallorale* (69). There are no reports of avian *Ureaplasma* isolation in North America.

Very little is known about their pathogenicity. Artificial challenge of chickens produced no clinical signs or macroscopic lesions (70). Turkeys and chickens challenged with a turkey *Ureaplasma* isolated in Hungary developed fibrinous airsacculitis and serologic responses (118). Ureaplasmas were also isolated in Eastern Europe from turkeys that were experiencing problems with reduced fertility (115).

Mycoplasma Infections of Geese

Three serologically and biochemically distinct *Mycoplasma* species were isolated from geese in Europe (121). One of these has been further characterized and named *Mycoplasma anseris* (20); it has been associated with airsacculitis, peritonitis, and embryo mortality (117). Another was subsequently identified as *Mycoplasma cloacale* (120), and the third was designated strain 1220. Two other isolates, strains 1223 and 1225, also represent 2 additional species isolated from geese (126).

Clinically, strain 1220 has been associated with reductions in egg production and egg transmission, embryo

mortality, infertility, inflammation of the cloaca and phallus, salpingitis and lack of weight gain in hatched goslings (32, 114, 120, 122), but proof of etiology is unclear because mixed mycoplasma species were isolated. Strain 1220, on experimental inoculation of goose embryos and day-old goslings, resulted in embryo mortality and reduced growth of young goslings (122). Strain 1220 has also been implicated in a field syndrome of goslings with respiratory and nervous signs (116). A novel *Mycoplasma* species has also been associated with phallus disease in geese (25). Several *Mycoplasma* and *Acholeplasma* species are often associated with *Mycoplasma* disease in ducks and geese (119) and more work needs to be done to clarify the role of these mycoplasmas in the field syndromes described.

Mycoplasma Infections of Pigeons

There are 3 species of *Mycoplasma* primarily associated with pigeons: *M. columbinasale* (60), *M. columborale*, and *M. columbinum* (113). The genome sequence of *Mycoplasma columbinum* has been published (55). One or more of these *Mycoplasma* species have been isolated from normal birds (10, 61, 95), as well as birds showing signs of respiratory disease (62, 80, 81, 99, 125). An isolate of *M. columborale* reproduced airsacculitis in chickens (81). Medication of pigeons infected with *M. columborale* with tylosin elicited a favorable response (81, 99). Even though there has been isolation of these organisms from birds showing respiratory signs, and there have been favorable responses to medication, there is no conclusive proof that pigeon mycoplasmas are etiologically involved in naturally occurring respiratory disease of pigeons.

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22

Clostridial Diseases

Introduction

Martine Boulianne

Clostridia are among the most fascinating bacteria. The genus *Clostridium* consists of a diverse group of Gram-positive spore-forming anaerobic rods which are distributed worldwide. Most of the species are harmless nonpathogenic bacteria living in a wide range of environments, including wetlands and soils, marine and freshwater sediments, on plants, on the skin, and in the gastrointestinal tract of humans and animals. One of these commensals, *C. butyricum*, produces butyric acid, and is used as a probiotic in humans. It has even shown potential as an alternative to antibiotics in broilers exposed to pathogens such as *Salmonella* Enteritidis and *Escherichia coli* (16, 17). However, some of these species contaminate and cause food spoilage, and others are pathogenic for both humans and animals.

Clostridia lack much of the genetic machinery required to produce essential amino acids; they obtain them *in vivo* via an armada of exotoxins and exoenzymes, literally feeding on host tissues. When the environmental conditions are suboptimal for their survival, they can also go into hiding and produce metabolically dormant spores resistant to heat and dessication. Only under proper conditions will these spores go back to an active form and subsequently produce toxins.

Pathogenic clostridia species produce several toxins responsible for lesions and clinical signs hence the categorization of clostridial diseases into 3 major groups based on toxin activity: (1) those interfering with neurotransmitters, such as *C. botulinum* and *C. tetani*, (2) clostridial strains proliferating in the intestines and causing enteritis and toxemic shock such as *C. perfringens* type A–G and *C. difficile*, and finally (3) clostridium localized in liver and muscle – the histotoxic clostridia: *C. chauvoei*, *C. septicum*, *C. novyi* type A, *C. novyi* type B, *C. perfringens* type A, *C. sordelli*, and *C. haemolyticum* (11).

Most commonly encountered clostridial-caused diseases in poultry are described in this chapter: ulcerative enteritis, necrotic enteritis, botulism, and gangrenous dermatitis respectively caused by *C. colinum*, *C. perfringens*, *C. botulinum,* and *C. septicum* (in association or not with *C. perfringens* and/or *Staphylococcus aureus*). Clostridia have also been isolated from other conditions. For example, beta2-positive *C. perfringens* and *C. colinum* have been both associated with focal duodenal necrosis, a disease observed in egg layers (1). Some unusual clostridial species have also been associated with other avian diseases. These include outbreaks of necrotic enteritis-like disease diagnosed in commercial broiler chicken flocks and associated with *C. sordelii* (10), *C. chauvoei* causing lesions on the combs and liver of birds (9), and infection of the intestine and livers of ostriches resulting in neuroparalysis (7). *C. difficile* and *C. sordelli* associated disease has also been reported in ostriches (2, 8, 13). *C. perfringens* has been linked with hepatitis in birds (5, 6, 12) and *C. difficile* has caused mortality in ostriches; it is also associated with poultry and may pose a health risk to humans through contaminated meat and poultry products (3, 4). Other strains of *C. perfringens* that cause disease in birds also have been linked with enterotoxin production and foodborne illness in humans (15).

Given the ubiquitous nature of clostridia and the presence of other pathogens causing similar lesions to necrotic enteritis for example (14), confirmation of the diagnosis of these diseases requires identification of the etiological agents by morphological, cultural, and/or molecular methods.

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Ulcerative Enteritis

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Summary

Agent, Infection, and Disease. Ulcerative enteritis (UE) is a disease caused by *Clostridium colinum,* a Gram-positive, sporulated, anaerobic rod. The disease is most commonly recognized in young quail, although cases also occur in chickens, turkeys, and several other avian species. It is transmitted by the fecal–oral route and produces acute, sub-acute, or chronic UE and rarely colitis.

Diagnosis. A presumptive diagnosis of UE is usually based on gross and microscopic lesions, but final diagnosis must be based on detection of *C. colinum* by culture or polymerase chain reaction in intestine, liver, or spleen.

Intervention. Litter should be removed and clean litter introduced for each brood after thorough disinfection. Coccidiosis control, prevention of stress and immunosuppression, in-feed enzymes, and prophylactic antibiotics are the most important management procedures to prevent UE. No vaccines are available for the prevention of UE.

Introduction

Ulcerative enteritis (UE), a bacterial infection, was first described in quail, hence the name "quail disease." UE also occurs in chickens, turkeys, and other birds (12). The disease occurs worldwide, but it is important in some concentrated poultry-raising areas (10, 12), and is a threat to game birds in confinement and in the wild. Infection of humans has not been reported.

History

Quail disease was first reported in the United States in 1907 (29). Scattered outbreaks in quail and grouse (2, 16, 26–28, 33) occurred during the following 2 decades. Subsequently, UE was discovered in wild and domestic turkeys (3, 37) as well as in other avian species (12, 33).

Etiology

Classification

Clostridium colinum was initially classified as *Corynebacterium perdicum* (4). Peckham (31, 32) reproduced UE with this organism and fulfilled Koch's postulates. 16S rRNA sequence analysis places *C. colinum* in subcluster XIV-b with 6 other *Clostridium* spp. It is most closely related to *C. piliforme*, the agent of Tyzzer's disease (11, 35).

Morphology and Staining

Clostridium colinum is a Gram-positive rod, occurring singly as straight or slightly-curved rods, 3–4 μm by 1 μm, with rounded ends (12). Sporulation is rare on artificial media, but if present, spores are oval and subterminal. Sporogenic cells are much longer and thicker than nonsporing cells.

Growth Requirements

Clostridium colinum is fastidious in its growth requirements. It needs an enriched medium and anaerobic environment. The best isolation medium is tryptose-phosphate agar (Difco) with 0.2% glucose, 0.5% yeast extract, and 8% horse plasma. Pre-reduced plates are inoculated with material from liver, intestinal, or splenic lesions and incubated anaerobically for 24–48 hours at 35 °C–42 °C (12, 15). The resulting colonies are 1–2 mm in diameter, white, circular, convex, and semi-translucent, with filamentous margins. The organism may also grow on pre-reduced blood agar incubated anaerobically (39, 41). Growth in liquid medium can be detected as early as 12–16 hours postinoculation. Actively growing cultures produce gas, but for no longer than 6– 8 hours, after which growth settles to the bottom of the tube (6). Subcultures should be made from actively growing broth cultures still producing gas. *C. colinum* commonly shows subterminal enlargements but these are not usually obvious spores. Identification can be made by conventional biochemical tests, MALDI-TOF or by specific 16srRNA sequence-based polymerase chain reaction (PCR) (39).

Biochemical Characteristics

Glucose, mannose, raffinose, sucrose, trehalose, fructose, and maltose are fermented, although the latter two are fermented only weakly. Mannitol is fermented by some strains, one of which is the type strain, ATCC 27770. Arabinose, cellobiose, erythritol, glycogen, inositol, lactose, melezitose, melibiose, rhamnose, sorbitol, and xylose are not fermented. Esculin hydrolysis is common, but most strains, including the type strain, do not hydrolyze starch. Nitrate is not reduced and no indole is produced. Milk remains unchanged and casein is not digested. Pyruvate and lactate are not utilized, and

gelatin is not liquefied. Catalase, urease, lipase, and lecithinase are not produced (39). *C. colinum* resembles *C. difficile* in culture, but these 2 species can be differentiated biochemically; *C. difficile* hydrolyzes gelatin and does not ferment raffinose (15).

Susceptibility to Chemical and Physical Agents

Clostridium colinum is, by its production of spores, highly resistant to chemical agents and physical insults. Yolk cultures may remain viable for at least 16 years at –20°C, and they survive heating at 70°C for 3 hours, 80°C for 1 hour, and 100°C for 3 minutes (32).

Pathogenesis and Epidemiology

Very little is known about the mechanism of virulence of *C. colinum*; its genome has not been characterized and the basis for its remarkable virulence is unknown (35).

Incidence and Distribution

Ulcerative enteritis is most commonly recognized in quail, but other avian species are also affected (12). The disease occurs most frequently in young birds (15) of all species, between 4 and 12 weeks of age, although cases have been described, albeit unfrequently, in adults. Outbreaks may last 3 weeks, peaking 5–14 days after initial infection. UE occurs worldwide, including, but not limited to, the United States (37), England (19), Japan (25), Canada (30), Germany (37), and India (20, 38, 39).

Natural and Experimental Hosts

Ulcerative enteritis is found in a wide range of avian hosts, but quails are among the most susceptible species. Natural infections have been found in bobwhite quail (*Colinus virginianus*), California quail (*Lophortyx california*), Gambel's quail (*L. gambelii*), mountain quail (*Oreortyx picta*), scaled quail (*Callipepla squamata*), and sharp-tailed grouse (*Pedioecetes phasianellus*) (12, 29, 35). Most cases are reported in captive quail populations (as opposed to wild quail), suggesting that management plays a role in the incidence of UE (12, 42).

Ruffed grouse (*Bonasa umbellus*) (26, 27), domestic turkeys (*Meleagris gallopavo*) and chickens (*Gallus gallus*) (14, 37), European partridge (*Perdix perdix*), wild turkeys (*M. gallopavo*) (14), chukar partridge (*Alectoris graeca*) (35), pigeons (*Columba livia*) (17), pheasants (*Phasianus colchicus*), blue grouse (*Dendragapus obscurus*) (10), crested quail (*L.c. californicus*) (19), robins (*Turdus migratorius*), lories (*Trichoglossus* spp.), and red lories (*Eos* spp.) (33) are also affected.

Quail and chickens are often predisposed to infection by prior infection with coccidia. Chickens are also predisposed by immunosuppressive infections such as infectious bursal disease or infectious anemia (12, 20, 35). Natural infection does occur in chickens, but experimental infections with *C. colinum* alone have only been readily produced in quail (3, 13). UE occurs most frequently in young individuals of all species (chickens, 4–12 weeks; turkeys, 3–8 weeks; quail, 4–12 weeks); cases in adult quail are rarely observed (20).

Transmission

Clostridium colinum is transmitted by ingestion of feed, water, litter or other materials contaminated with feces. When cases of UE occur, the spores of *C. colinum* contaminate the premises which, after an outbreak of the disease, are assumed to remain contaminated for many months (3, 12, 34). Both birds with active infection and birds that recover from the disease become carriers and shed the organism in their feces. Anecdotal evidence suggests that they are amongst the most important factors in perpetuating the disease (12). It has also been suggested that flies feeding on contaminated feces may introduce infection in the premises (3).

Incubation Period

After experimental inoculation, the acute disease develops, accompanied by death of quail, in 1–3 days. The course of the disease in a flock is generally about 3 weeks, with peak mortality occurring 5–14 days after the initial case (12).

Pathogenesis

After oral infection, *C. colinum* adheres to the intestinal epithelium, producing the characteristic lesions in the small intestine and, occasionally, proximal colon. The organism then may migrate to the liver via portal circulation, producing the foci of hepatic necrosis frequently seen in cases of UE (12). Very little is known about the basis of virulence of *C. colinum* (34). The role of a toxin in the pathogenesis of UE has been suggested (12, 41), but not demonstrated. The genome of *C. colinum* has not been characterized (34).

Immunity

Immunity develops in birds recovering from naturally occurring infections. When survivors of a UE outbreak were subsequently challenged with *C. colinum*, no noticeable effect was seen (21), whereas 85% of similarly challenged susceptible controls died. It has

been observed, however, that those surviving because of antimicrobial therapy remain highly susceptible to infection (22, 23).

Clinical Signs

The hallmark of UE is diarrhea, which is initially watery but may become hemorrhagic (34). As UE progresses, infected birds become listless and humped up, with eyes partly closed and feathers dull and ruffled. Notable emaciation, with atrophy of pectoral muscles, is seen in birds affected for a week or longer. Birds may die from acute disease with no premonitory signs. Young quail may be subject to 100% mortality in a few days. The mortality rate in chickens typically ranges from 2% to 10% (12).

Pathology

Gross

Acute lesions in quail are characterized by severe ulcerative and hemorrhagic enteritis (Figure 22.1A,B). Variable size mucosal ulcers surrounded by a hemorrhagic halo may be visible from the mucosal and serosal side of the intestine (Figure 22.1A,B). Ulcers may be deep and involve the whole thickness of the intestinal wall, causing perforation and subsequent peritonitis (12, 34, 39).

Subacute or chronic lesions can be observed in birds surviving for several days. They consist of multiple large, roundish yellow ulcers surrounded by hemorrhages, in any part of the small or large intestine and ceca. These ulcers later coalesce to form larger lesions and may be covered by diphtheritic membranes (Figure 22.1C). Blood is commonly found in the gut. As ulcers increase in size, the hemorrhagic border disappears and it is replaced by a pale halo. Ulcers may be deep in the mucosa, but in older lesions, they are often superficial with raised edges. Ulcers in ceca may have a central depression filled with firmly attached, dark-staining, soft material. As in acute cases, perforation of ulcers frequently occurs, resulting in peritonitis and intestinal adhesions (12, 13, 34). Macroscopic lesions in chickens and other avian species are similar to those described in quail (12).

Liver lesions are not always evident. When present, they vary from light yellow mottling to multiple large, irregular, gray, or yellow circumscribed foci (Figure 22.1D), sometimes surrounded by a pale-yellow halo. The spleen may be congested, enlarged, and hemorrhagic, with or without multifocal necrotic areas (12). Gross lesions are usually absent from other organs, although peritonitis or polyserositis can be observed when intestinal perforation occurs (12, 13).

Microscopic

Acute cases reveal erosion and/or ulceration of small intestinal mucosa, and edema, congestion and heterophilic infiltration of the mucosa and, occasionally, the submucosa and other layers of the intestinal wall (Figure 22.1E–G). The intestinal lumen contains desquamated epithelial cells, erythrocytes, heterophils, cell debris, fibrin, and Gram-positive rods (12) (Figure 22.1E,G).

As the lesions progress, mucosal ulcers involving villi and extending into the submucosa grow deeper. Most ulcers are covered by thick pseudomembranes composed of desquamated epithelial cells, fibrin, cell debris, mixed inflammatory cells, and Gram-positive bacilli. A rim of heterophils, lymphocytes, plasma cells, and macrophages surrounds the ulcers. Variable size clumps of Gram-positive rods are often present deep in the mucosa, and in necrotic tissue and the lumen (Figure 22.1F,G). At later stages, transmural necrosis and inflammation can be observed (Figure 22.1G) (12).

Vascular thrombosis, including a large number of Gram-positive intravascular rods in the mucosa is an almost constant finding in both the acute and chronic lesions of UE.

Liver lesions, when present, are poorly demarcated foci of coagulative necrosis, with minimal inflammatory reaction and occasional intralesional clumps of Gram-positive rods, randomly scattered throughout the parenchyma (Figure 22.1H) (12, 18).

Diagnosis

A presumptive diagnosis of UE can be made based on gross and microscopic lesions. The observation of large, Gram-positive rods usually with subterminal spores, and a few free spores on liver smears stained with Gram, adds certainty to this presumptive diagnosis (12, 39, 41). Final diagnosis, however, should be based on detection of *C. colinum* by culture or PCR in intestine, liver, or spleen (1, 12, 36). Because the organism is frequently present in the liver in more or less pure form, isolation from liver rather than from intestine is preferable (7–9, 12, 15, 39). A small proportion of *C. colinum* is Gram-positive in stained smears of the culture.

Differential Diagnosis

Similar diseases that must be differentiated from UE include mainly coccidiosis, necrotic enteritis by *C. perfringens* and histomoniasis (40). Furthermore, some of these diseases may occur simultaneously, necessitating use of 2 different treatments.



Figure 22.1 Ulcerative enteritis lesions in quail. (A) Ulcers visible from serosal surface in small intestine. Note hyperemia and hemorrhage around ulcers. A few pin point lesions are also visible on the surface of the liver. (T. Abdul-Aziz) (B) Acute ulcers on the mucosal surface of the small intestine; some of them hemorrhagic and/or surrounded by a hemorrhagic rim. Reproduced with permission from Cooper et al. 2013. *J Vet Diagn Invest.* 25:314–327. (C) Chronic ulcers seen on the mucosal surface of the small intestine; notice diphtheritic membranes covering the ulcers. Reproduced with permission from Cooper et al. 2013. *J Vet Diagn Invest.* 25:314–327. (D) Multifocal areas of necrosis in the liver. (T. Abdul-Aziz) (E) Acute lesions affecting mostly the mucosa of the small intestine. (F) Mucosal and submucosal ulcers showing numerous bacterial colonies typical of *Clostridium colinum*. (G) Deep lesions affecting all layers of the small intestine. (H) Microscopic appearance of liver lesion. Note the multifocal areas of necrosis without distinct separation from normal tissue, minimal inflammatory response and bacterial colonies. Reproduced with permission from Cooper et al. 2013. *J Vet Diagn Invest.* 25:314–327.

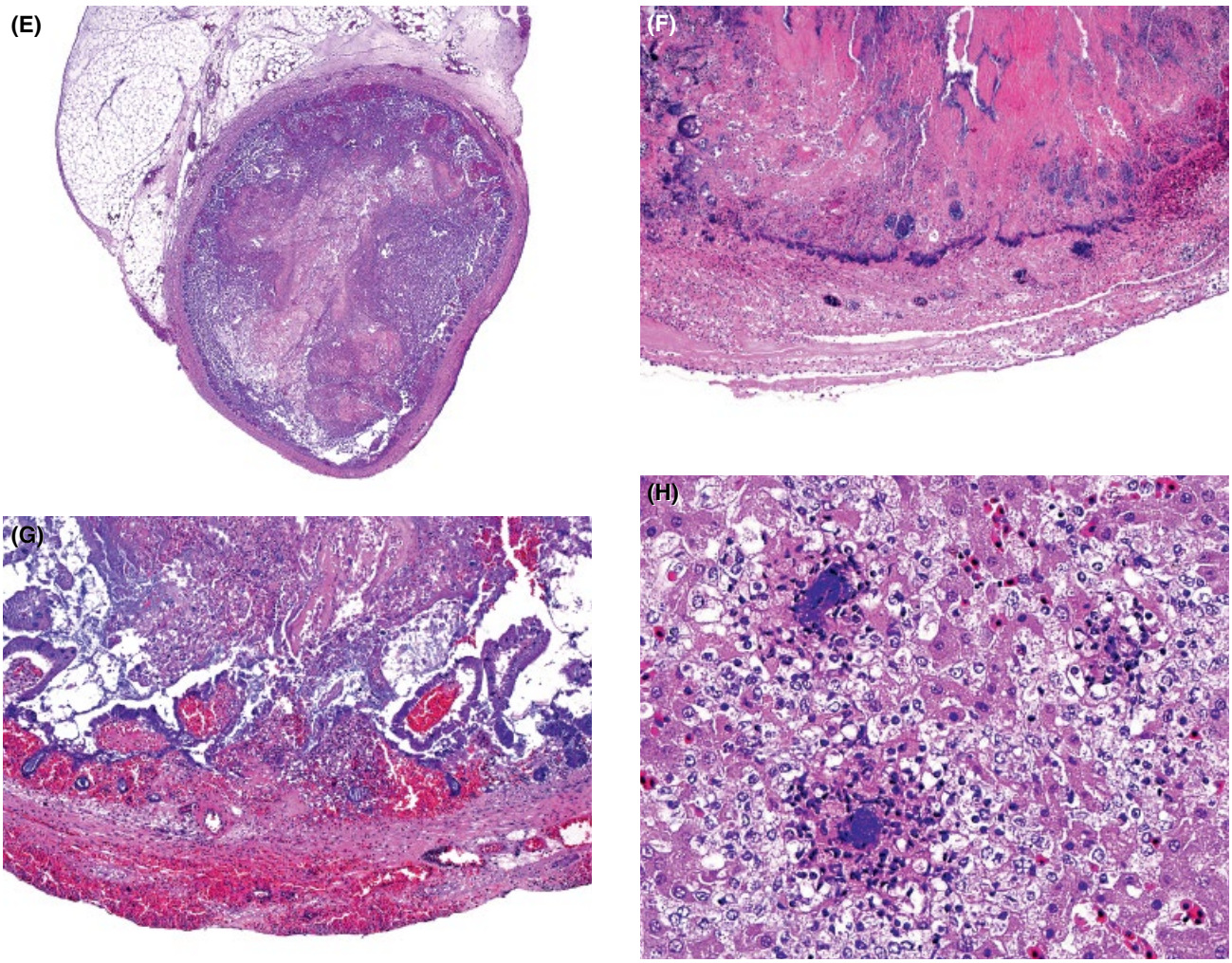


Figure 22.1 (Continued)

Intervention Strategies

Management Procedures

Clostridium colinum is shed in feces and remains viable indefinitely in litter. Thus, on problem farms, contaminated litter should be removed and clean litter introduced for each brood after thorough disinfection. Coccidiosis control, prevention of stress and immunosuppression, in-feed enzymes, and prophylactic antibiotics are the most important management procedures to prevent UE. Bobwhite quail do not adapt easily to cages, which can be quite stressful to them and may result in mortality associated with UE. Therefore, rearing systems in captivity that do not require cages are favorable for livability and a reduced incidence of UE (G. Zavala, personal communication). Game farm managers should exercise caution to avoid overgrazing ranges and overcrowding birds. Survivors of an outbreak may be carriers and should not be mixed with unexposed birds. No vaccines are available for the prevention of UE.

Treatment

Ulcerative enteritis can be prevented and/or controlled through medication of either drinking water or feed. Drug use to control and/or treat this disease should be in compliance with veterinary feed directives. Clinically affected flocks can be treated successfully with bacitracin, lincomycin or penicillin where legal. Streptomycin administered by injection or in feed or water has prophylactic and therapeutic value against the disease in quail. Streptomycin at a level of 60 g/ton of feed or 1 g/gal of water gives complete protection when administered prophylactically (20–23, 32). Addition of 100 g bacitracin/ton of feed provides protection (32). Bacitracin methylene disalicylate can be used at 200 g/ton for control of UE in quail. Other chemotherapeutics

reported to have efficacy for control of UE in quail include furazolidone, chlortetracycline (32), penicillin, ampicillin (24), and tylosin (24). In cases of coinfection with coccidia, treatment/prevention of the latter usually prevents outbreaks of UE. In a persistent outbreak of UE, prevention of coccidiosis with monensin and salinomycin, combined with tylosin, was the only treatment effective to control the outbreak (5).

Necrotic Enteritis

Kenneth Opengart and Martine Boulianne

Summary

Agent, Infection, and Disease. Necrotic enteritis is a well-described disease of poultry caused by the toxins produced by pathogenic strains of *Clostridium perfringens* type A, type C and type G. A sudden increase in mortality along with distension of the small intestines and necrosis of their epithelium is observed. Necrotic enteritis is associated with predisposing factors such as coccidiosis, inferior feed quality, or feed ingredients and immunosuppressive diseases that allow for the proliferation of a pathogenic *C. perfringens* strain within the intestinal lumen.

Diagnosis. Diagnosis is based on clinical signs, macroscopic lesions, and the presence of numerous Gram-positive rods at the lesion site.

Intervention. Affected birds may respond very well to the administration of antibiotics such as penicillin or bacitracin, but the disease is better prevented by adequate control of predisposing factors, especially coccidiosis and addition of in-feed antimicrobials when permitted.

Introduction

Traditionally, necrotic enteritis (NE) has been largely prevented and controlled with the use of antimicrobial feed additives. As the use of these compounds has lost favor, the incidence of NE is once again a re-emerging concern. The focus today is on identifying novel ways to manage the disease effectively. Because much of the basic information about NE has been covered in past editions of *Diseases of Poultry*, this subchapter will focus primarily on the latest findings and alternative methods of prevention and control.

Definition and Synonyms

Clinical NE can be defined as a disease of primarily young chickens, caused by infection with, and toxin production by, *Clostridium perfringens* type A, type C and type G. The clinical infection is characterized by sudden onset, high mortality, and necrosis of the small intestine mucosa. The disease is also known as clostridial enteritis, enterotoxemia, and rot gut.

Economic Significance

Necrotic enteritis is a major cause of loss of efficiency of growth and increased mortality in poultry, both of which significantly negatively impact profitability (19, 48, 59). Losses because of NE vary widely and are estimated to cost the poultry industry billions of dollars annually. The economic consequence of subclinical NE, although difficult to quantify, is growing in importance as the use of antimicrobials to prevent and control bacterial enteridities declines.

Public Health Significance

Clostridium perfringens type A and type C, in addition to producing toxins which can induce NE in poultry, also produce enterotoxins at the moment of sporulation which can produce foodborne illness in humans. Two distinct diseases are induced by these subtypes: type A *C. perfringens* produces diarrhea and type C *C. perfringens* produces necrotic enteritis in humans (69). High percentages of *C. perfringens*-positive carcasses have been reported following processing (13, 52) and outbreaks of type A food poisoning traced to consumption of chicken have been reported (23, 61).

History and Etiology

Information on the history of NE and the conventional morphologic description and biochemical characteristics of *C. perfringens* can be found in earlier editions of *Diseases of Poultry*.

Strain Classification and Toxins

Clostridium perfringens produces 17 or more toxic exoproteins (6) and a new classification into 7 toxigenic types A–G based on the combination of 6 exotoxins produced by the bacteria (α, β, ε, ι, CPE and Netβ) has been recently suggested (59a)). NE is commonly caused by *C. perfringens* types A (α or CPA toxin), C (β or CPB, and α

Table 22.1 Selected key *Clostridium perfringens* toxins: gene, location, and biological activity.

	Selected key *Clostridium perfringens* toxins commonly used for toxinotyping			
Toxin	**Gene**	**Location**	**Biological Activity(ies)**	**Action(s)**
CPA	*cpa*	Chromosomal	Necrotizing, hemolytic, smooth muscle contraction	Phospholipase C/ sphingomyelinase
CPB	*cpb1*	Plasmid	Dermonecrosis, edema, enterotoxic	Pore former
CPB2	*cbp2*	Plasmid	Dermonecrosis, edema, enterotoxic	Unknown
Enterotoxin CPE	*cpe*	Chromosomal/ plasmid	Erythema, enterotoxic	Pore former
NetB	*netB*	Plasmid	Hemolytic	Pore former
TpeL	*tpel*	Plasmid	Unknown	Glucosylating cytotoxin

toxins) and G (Netβ toxin)(28, 59a, 65) (Table 22.1). Besides expressing typing toxins, some *C. perfringens* strains produce additional toxins, such as *C. perfringens* enterotoxin (CPE), or *C. perfringens* large cytotoxin (TpeL) (43). Many of the genes associated with these toxins are located on large plasmids which allows for the horizontal transfer of toxin-associated genes among *C. perfringens* strains (3).

Pathobiology and Epizootiology

The epizootiology of NE in poultry has been well described in detail in earlier editions of *Diseases of Poultry*.

Clinical Signs

Birds with NE are depressed, with ruffled feathers, diarrhea, anorexia, and dehydration (44, 69). Clinical illness is very short; birds can be found dead without clinical signs of disease but with very characteristic intestinal lesions upon necropsy. Mortality rates may be as high as 50% (4, 14).

Pathology

The small intestine is distended and friable (44, 69). Focal-to-confluent necrotic ulcers progress to a pseudomembrane covering the intestinal mucosa (Figure 22.2A,C).

Microscopically, there is extensive villous necrosis (44) and cellular degeneration may reach the submucosa or even the muscularis mucosa (Figure 22.2B). Coagulation necrosis is common at villous apices and the demarcation between necrotic and normal tissue is defined by accumulation of mononuclear cells at the junction (44). Large, Gram-positive rods will attach to the villi tips early in the infectious process (56) and are later associated with areas of necrosis (Figure 22.2D).

Clostridium perfringens may gain access to portal circulation and then the biliary ducts, leading to the development of cholangiohepatitis (45). Pale, focal liver lesions (69) examined microscopically reveal extensive periportal coagulative necrosis or granulomas with Gram-positive rods located centrally and bile duct proliferation (45).

Pathogenesis of the Infectious Process

The pathogenesis of NE is still not fully understood. For years, scientists strictly focused on the role of toxins. CPA toxin, a phospholipase C/sphingomyelinase (20, 44, 55, 67), was thought to be mainly responsible for the observed intestinal damage, causing hydrolysis of the phospholipids, damaging the enterocyte wall and hence leading to cell death. The role of CPA in the development of NE was, however, revisited when CPA-deleted mutants retained full virulence *in vivo* (32) which led to the discovery of the NetB toxin. Indeed, *netB*-positive *C. perfringens* strains were able to reproduce NE lesions, whereas *netB*-negative strains did not induce lesions (29). The gene encoding NetB resides on a large pathogenicity locus (NELoc-1) and like many *C. perfringens* toxins, is plasmid-borne (41). NetB induces the formation of pores within the enteric cellular membrane causing ionic influx which results in osmotic cellular lysis (60). Although some epidemiological studies have shown a high percentage (70% and 95%) of NE field isolates positive for the *netB* gene (29, 56), others showed that this gene is not always present in strains recovered from NE positive farms with low *netB* prevalence (52% and 58%) (1, 50). Interestingly, whereas the presence of NetB is perceived by many as essential to the pathogenicity of *C. perfringens*, NE-like lesions were reproduced with a *netB*-negative strain in an intestinal ligated loop model (56). Thus, there may be other factors, in addition to NetB, involved in the pathogenesis of the condition.

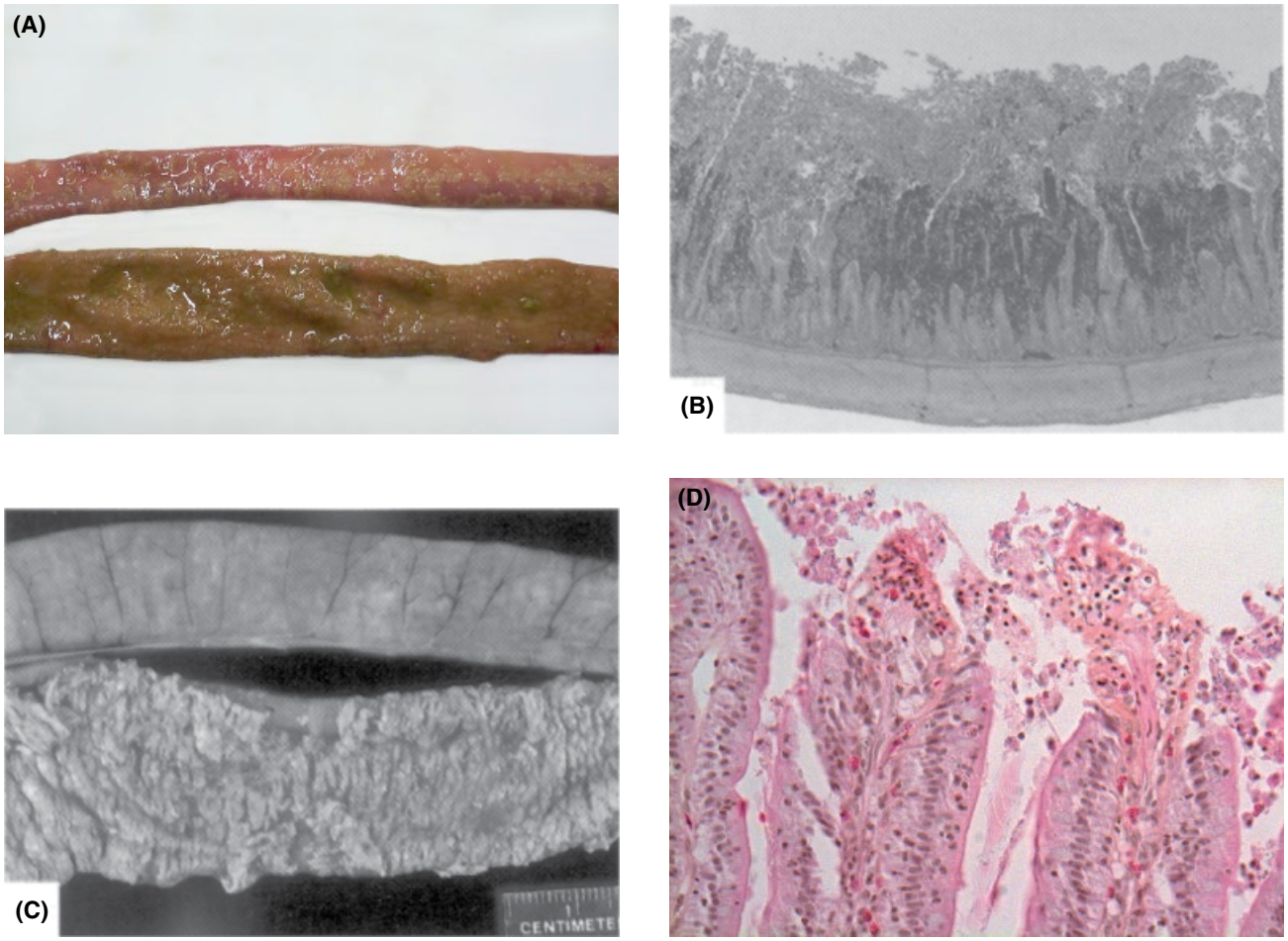


Figure 22.2 (A) Mild to moderate necrotic enteritis in a 5-week-old broiler chicken with concurrent coccidiosis. Note the hyperemia and diffuse necrosis of the mucosa with multifocal ulcerations. (M. Boulianne) (B) Intestine of a turkey showing uniform diffuse coagulation necrosis of mucosa. Deeper viable mucosal tissue is demarcated from necrotic luminal mucosal tissue by a zone of intense hyperemia, hemorrhage, and inflammation. (H.J. Barnes) (C) Severe necrotic enteritis in a commercial broiler. Note the "Turkish towel" appearance to the necrotic pseudomembrane covering the intestinal mucosa. (C. Hofacre) (D) Large, Gram-positive rods will attach to the villi tips early in the infectious process and are later associated with areas of necrosis. (E. Parent and M. Boulianne)

Conjugative plasmid transfer has been demonstrated *in vivo* and *in vitro* in chickens (38). This finding is quite significant epidemiologically because it demonstrates that both virulence and antibiotic resistance plasmids can be transferred to nonpathogenic and pathogenic strains resulting in the formation of new virulent strains.

The *C. perfringens* population is normally less than 10^2–10^4 cfu/g of the intestinal contents in the small intestine of healthy chickens compared with 10^7–10^9 cfu/g in diseased birds (34). Numerous risk factors have been associated with necrotic enteritis outbreaks, all of which will favor the rapid colonization of *C. perfringens*. For example, coccidiosis is a major predisposing factor. *Eimeria* species infection results in enterocyte damage with secondary release of essential amino acids to a bacterium unable to synthesize many of them on their own. High protein or poorly digestible diets likely have the same effect, whereas certain cereals increase ingesta viscosity, slowing peristalsis and favoring bacterial adherence to the enterocytes.

Creating an optimal intestinal environment is key to the proliferation of *C. perfringens,* because it must also compete against an established bacterial community in order to colonize the small intestine. For example, *C. perfringens* is able to produce antimicrobial peptides, called bacteriocins, which will inhibit the growth of closely related strains. An example of one of these bacteriocins, named perfrin, is produced by NE-associated *netB*-positive *C. perfringens* strains. It has been suggested that other similar peptides can be produced by a single clostridial strain, not necessarily simultaneously, and with a different inhibitory spectra (66). This might explain why during an outbreak a single pathogenic strain will become dominant (56).

Bacterial quorum sensing (QS) is a cell–cell communication process by which the bacteria can respond to perceived bacterial cell population density and adjust gene

expression accordingly, including the production of virulence factors. It is known that *C. perfringens* encodes at least 2 QS systems: the LuxS and the Agr-like system (74). Whereas the LuxS has not been associated with virulence, the Agr-like QS system has been shown to play a key role in regulating the expression of *C. perfringens* virulence-related proteins including CPA, CPB, CPB2, CPE, and NetB (53, 54, 74). Indeed, the Agr-like QS would turn on the VirS/VirR regulatory system, initiating the CPA and NetB toxin production (10, 43). Even more fascinating is the role of Agr-like QS in regulating the expression of various genes which are likely involved in adherence, located on the chromosomal locus VR-10B (40).

Although adherence is recognized as an initial step in the establishment of a bacterial infection, few researchers have investigated this path to understand further the pathogenesis of NE. Severity of histopathological NE lesions was associated with the number of Gram-positive rods initially lining the villi tip, suggesting that intestinal mucosa adherence and attachment by *C. perfringens* plays a role in the development of NE (56). A chromosomal locus VR-10B found predominantly in NE-causing *C. perfringens* strains encodes 7 genes, most of which are predicted to encode cell surface proteins; one was identified as a putative collagen adhesion gene (40). It was later confirmed that not only the adhering capability of pathogenic *C. perfringens* strains to collagen was correlated with the presence of one of these genes (*cnaA*) (71), but that a deleted *cnaA* mutant could no longer cause NE (70). However, whereas that deletion abolished virulence in one strain, it only reduced it in another. Furthermore, some *cnaA*-negative strains can still cause NE.

The disease process is extremely rapid and NE-like lesions have been observed as early as 7 hours postinoculation in an intestinal ligated loop model (56). These recent findings demonstrate that the onset of NE is a complex dynamic process involving multiple *C. perfringens* virulence factors related amongst others, to the acquisition of nutrients, competition, colonization and adhesion.

Immunity

Chicks mature immunologically at about 3–4 weeks of age (37), whereas maternal anti-CPA antibodies persist for approximately 3 weeks (22). These factors suggest a possible explanation for the fact that most NE outbreaks in broiler chickens occur later than 2–3 weeks of age (46). Vaccination of hens with vaccines containing *C. perfringens* type A alpha-toxoid or recombinant NetB toxin (30) has been shown to provide partial protection to *C. perfringens*-challenged progeny. Similarly, a mucosal IgA response against alpha toxin, NetB, and other immunogenic proteins only partially protected vaccinated chickens against NE (17, 26, 31).

Protection against subclinical NE and hepatitis was greater in chicks vaccinated with the type C toxoid despite the fact that the anti-CPA immunologic response was higher in type A vaccinates (46).

Chicks inoculated with a fully virulent NE strain of *C. perfringens* (daily for 5 days) and then treated with bacitracin were protected upon re-challenge. Immunization of birds with recombinant CPA toxoids provides partial (12) or complete (35) protection against challenge. The incidence of NE in vaccinated birds was significantly less than in nonvaccinated birds. Inoculation with a CPA-deficient mutant also has been shown to protect birds (65).

Vaccination of birds with supernatant from *netB*-positive strains of *C. perfringens* or recombinant NetB toxin have been shown to be partially protective against NE challenge (31, 39).

Oral immunization with an avirulent *Salmonella enterica* serovar Typhimurium vaccine vector carrying genes encoding FBA or HP also was significantly protective against challenge (36, 62, 75). Additionally, administration of a live attenuated recombinant *Salmonella* vaccine which produced 2 distinct proteins, the C-terminal portion of the alpha toxin and NetB toxin, resulted in protective immunity in *C. perfringens*-challenged birds (27).

Diagnosis

The diagnosis of NE in poultry has been well described in detail in earlier editions of *Diseases of Poultry.* Isolation and proper identification of the causal agent remains of the utmost importance in the diagnostic workup (68).

Intervention Strategies

Necrotic enteritis can be controlled or prevented by reducing exposure to risk factors (4). The use of organic acids and formaldehyde has been shown to reduce levels of clostridial contamination in plant and animal feed ingredients (7).

Coccidiostatic drugs have been the method of choice for the prevention of coccidiosis in broiler chickens for the past 50 years (16, 72). Ionophores have been most popular and these drugs are serendipitously anticlostridial (2). Anticoccidial poultry vaccines use wild-type and attenuated strains of *Eimeria* spp. and live, multivalent anticoccidial vaccines are now registered around the world (72). Ionophores have been used in combination with coccidial vaccines to both promote the development of coccidial immunity and limit the potential for controlled coccidial exposure to induce NE (42, 72). The live parasites in these coccidial vaccines cause intestinal damage (49, 69, 72) allowing *C. perfringens* to proliferate extensively in recently immunized birds. Thus, anticoccidial vaccination increases the risk of developing NE

(72) and untimely withdrawal of ionophores from feed may contribute to the development of the disease.

The incidence of NE and the subsequent use of therapeutic antibiotics to treat the disease have increased in areas of the world where the use of in-feed antibiotics for the purpose of growth promotion has been curtailed (8, 69). NE has been treated with lincomycin, bacitracin, and tylosin in water, or bacitracin, lincomycin, virginiamycin, and avoparcin, in feed (5, 11, 21, 57, 58). As the overall use of antimicrobials for enteric health has decreased, the evaluation of use of other products (prebiotics, probiotics, phytogenic compounds, etc.) has increased in an attempt to mitigate the increasing risk of NE.

Prebiotics

The use of prebiotics such as yeast wall extracts, which stimulate growth of beneficial intestinal flora, have produced inconsistent results (24, 69). Others, however, have shown protective effects with the use of these products (18, 47).

Probiotics

Probiotics have been shown to lessen the impact of NE in laboratory challenge. Competitive exclusion products have been shown to decrease the incidence and severity of NE in experimental challenge (14, 15). *Bacillus subtilis* spores competitively exclude *C. perfringens* from broiler chicks (37) and *Bacillus*-colonized chicks have increased body weight, feed efficiency, and intestinal integrity compared with controls (25, 64). Other direct-fed microbials, including *Lactobacillus fermentum*, *L. acidophilus*, *Enterococcus faecium* and *B. licheniformis* (33, 51, 63, 69) have been shown to produce similar effects in NE-challenge models.

Phytogenic Compounds

Phytogenic compounds have been evaluated for their efficacy in reducing the incidence and severity of NE. Anise oil (9), citral (73), and essential oil blends (51) have all been shown to be effective in providing protection from *C. perfringens* challenge.

Vaccination

Immunization with single proteins does not appear to protect against severe challenge and combinations of different antigens are needed. Most published studies have used multiple dosage vaccination protocols that are not practical to use in the commercial broiler industry, and single vaccination protocols for day-old chicks appear nonprotective. Future vaccine development should also include optimization of vaccine delivery to day-old chicks.

The Future

Reduction in the use of antimicrobials that have been used to prevent and control NE has led to greater incidences of subclinical and clinical NE. This trend has created welfare concerns and negative economic implications in affected flocks. Consequently, understanding the pathogenesis, prevention, and control of NE will certainly continue to be a focus of research in the coming years.

Botulism

Martine Boulianne and Francisco A. Uzal

Summary

Agent, Infection, and Disease. Botulism is a disease of humans and other animals caused by clostridia-produced botulinum neurotoxins (BoNTs). Outbreaks of avian botulism are reported in waterfowl, game birds, and poultry. Most avian cases are caused by *Clostridium botulinum* type C or mosaic type C/D. The disease includes high mortality and flaccid paralysis which progresses cranially and eventually leads to death by cardiac and respiratory failure.

Diagnosis. A definitive diagnosis is usually based on the demonstration of the BoNTs in specimens of affected birds and the identification of the toxin type.

Intervention. Management of the disease primarily consists of carcass collection during outbreaks.

Introduction

Avian botulism is a neurotoxic disease of birds resulting mostly from the ingestion of toxin produced by botulinum neurotoxins (BoNTs)-producing clostridia. The disease occurs worldwide. It is sporadic in poultry but has caused massive mortality in waterfowl.

Definition

Botulism is a flaccid paralytic disease caused by intoxication with BoNTs produced mainly by *Clostridium botulinum*.

Economic Significance

Avian botulism is a significant cause of morbidity and mortality in free-living wild waterfowl, game birds, and

poultry. Worldwide, avian botulism probably represents the most important cause of death in migratory birds, with a single outbreak killing tens of thousands of birds (34). Botulism occurs sporadically in commercial poultry flocks although it is considered an emerging disease in Europe (22) with the number of outbreaks having increased over the last decade (40).

Public Health Significance

The public health significance of avian type C outbreaks is considered minimal. There are no recorded occurrences of poisoning of people caused by type C BoNT, although several other types of BoNTs are highly poisonous to humans (18). Nonhuman primates, however, have succumbed to type C botulism (44).

History

Botulism was first reported in chickens in 1917 (6). For additional historic information, see previous editions of *Diseases of Poultry*.

Etiology

Several species of clostridia may be involved in botulism. It is therefore more accurate to describe this disease as caused by toxins from BoNT-producing clostridia. The most commonly reported neurotoxin is secreted by *C. botulinum*, a strictly anaerobic, spore-forming Gram-positive bacterium commonly found in the soil and feces of many animals.

Classification

Clostridium botulinum and some strains of *C. baratii* and *C. butyricum* are currently classified as BoNT-producing clostridia.

Morphology and Staining

Clostridium botulinum is a Gram-positive rod that measures 1–1.2 μm by 4–7 μm (38). The microorganism is motile and it often occurs singly or in short chains (28). Subterminal or occasional terminal endospores are present in aging cultures (7).

Growth Requirements

Clostridium botulinum can be cultivated anaerobically at 30 °C–42 °C in cooked-meat medium or trypticase-peptone-glucose-yeast medium (40). *C. botulinum* strains responsible for avian botulism are nonproteolytic or weakly proteolytic and show varying lecithinase and lipase activity (28, 38). The organism can be isolated through repeated culturing in broth and on agar plates.

Colony Morphology

Colonies have an irregular morphology: they can be flat or raised, and present round or irregular edges. Lipase- and lecithinase-positive colonies can be distinguished by an iridiscent sheen and an opaque precipitation formed on egg yolk agar (40).

Biochemical Properties

Because the organisms are very diverse there is no simple biochemical test(s) that will identify bacteria as *C. botulinum*.

Susceptibility to Chemical and Physical Agents

Hydrogen peroxide vapor is effective at deactivating spores of *C. botulinum* (19). Sodium hypochlorite (0.1%) inactivates spores within 5 minutes (29).

Strain Classification

Based on their genotypic, phenotypic, and biochemical characteristics, BoNT-producing clostridial strains are divided into 6 groups: *C. botulinum* (groups I-IV), *C. butyricum and C. baratii;* the latter sometimes also referred to as Groups V and VI, respectively (32). The BoNTs have also been traditionally classified into 6 serotypes distinguishable with animal antisera and designated from A to G (31). Recent molecular genetic analysis has led to the discovery of genes encoding for many novel BoNTs, such as type H (3). They still can be grouped within an existing serotype but are characterized by different sequences, which led to the apparition of subtypes.

Human disease has primarily been associated with groups I and II (types A, B, E, and F), whereas animal botulism is usually caused by strains belonging to group III (types C and D and their mosaic C/D and D/C toxins) (46–48). Cases of avian botulism have primarily been caused by *C. botulinum* type C, although type A has also caused disease in poultry, and type E has caused disease in fish-eating birds (11, 34). Further analyses of samples from avian botulism outbreaks often report neutralization by both C and D antitoxins (12, 22, 48), which correlates with genetic studies demonstrating a chimeric neurotoxin gene coding for 2 parts type C toxin and 1 part type D toxin, also called a mosaic toxin (25, 40, 48).

Pathogenicity

Different strains of *C. botulinum* group III have different pathogenic capabilities, depending on whether or not they harbor the phage carrying the neurotoxin gene. Indeed, *C.*

botulinum is considered by many as a saprophytic bacterium that uses the neurotoxin to kill a host and create a source of nutrients. It has also been proposed that strains can be transformed into various pathogenic variants by exchanging plasmids containing other toxin genes (42).

Virulence Factors

Botulinum neurotoxins are among the most potent toxins known. The virulence factors so far investigated from group III *C. botulinum* strains are the BoNTs (referred to as C1 and D1) and 2 ribosylating toxins (C2 and C3), all located on extrachromosomal elements (42). The completed genome of a type C/D strain also revealed genes coding for other plasmid-encoded toxins (39) with functions yet to be defined.

BoNTs cause flaccid paralysis by inhibiting neurotransmitter release mainly at peripheral cholinergic nerve terminals of the skeletal and autonomic nervous system. For detailed description of the molecular mechanism of nerve terminal paralysis, please consult the 13th edition of *Diseases of Poultry*.

Pathobiology and Epizootiology

Incidence and Distribution

The disease has affected waterfowl and poultry worldwide (18). Many severe cases have been reported in confined and free-range broiler chickens, turkeys and laying hens flocks, and in wild birds (18, 27, 43). Botulism in waterfowl, broiler chickens, and pheasants occurs most frequently and with greater severity during warmer months.

Natural and Experimental Hosts

Type C botulism has occurred in many species of birds including broiler chickens, laying hens, turkeys, ducks, gulls, pheasants, and ostriches (1, 20, 27, 41, 43, 46, 49). Other animal species affected by type C toxin include mink, ferrets, rodents, cattle, pigs, dogs, horses, fish, and a variety of zoo mammals (23).

Age of Host Commonly Affected

Mature hens might be less susceptible to BoNTs because they start showing symptoms of paralysis at a later stage of the disease (41), but otherwise all ages are susceptible.

Transmission, Carriers, and Vectors

The spread of avian botulism in waterfowl via the bird carcass–maggot cycle is well-known. Invertebrates, notably fly maggots, are vehicles of BoNTs during outbreaks in waterfowl (14, 49). Birds are intoxicated after ingesting an invertebrate carrying the toxin. Low water level in ponds and other water reservoirs is considered a predisposing factor for waterfowl botulism, because it allows birds to feed at the bottom, where rotten vegetation and other sediment generates an anaerobic environment that promotes *C. botulinum* growth and toxin production (21).

Clostridium botulinum type C is distributed worldwide and is considered as a normal bacterium of marshland, soils, and sediments, although with limited prevalence (49). Type C spores can survive for decades in the environment and botulism can be recurrent in affected farms (30). Numerous conditions must, however, be present for an outbreak to occur.

First, BoNTs are produced only after the spores germinate, when the bacteria is actively growing and multiplying, that is, when temperature, moisture, and anaerobic conditions are ideal. Indeed, temperature plays a critical role in the multiplication of *C. botulinum*, with optimal growth in the laboratory occurring between 30 °C and 42 °C. This explains partly why most botulism outbreaks take place during the summer and fall when ambient temperatures are high.

Second, *C. botulinum* requires an energy source for growth and multiplication. Because it lacks the ability to synthesize certain essential amino acids, it requires a high protein substrate and is essentially a "meat lover." Decaying carcasses, both vertebrate and invertebrate, support toxin production and also offer a means of toxin transfer to birds when they feed on carcasses.

Incubation Period

The incubation period of avian botulism is usually short, varying from a few hours to 13 days, depending on the amount of preformed toxin or type C spores ingested. With high levels of neurotoxin, disease appears within hours. With low toxin doses, onset of paralysis occurs within 1–2 days (10). Chickens orally administered with 10^2 or more type C spores developed illness in 6–13 days (24). In another experiment, symptoms and death occurred on day 3 when 10^7 viable spores were administered (15).

Clinical Signs

Avian botulism affects the peripheral nerve endings and results in paralysis of muscles. Clinical signs of botulism in chickens, turkeys, pheasants, and ducks are similar. In chickens, flaccid paralysis of legs, wings, neck, and eyelids are predominant features of the disease (Figure 22.3A,B.). Initially, affected birds are found sitting and are reluctant to move. If coaxed to walk, they appear lame. Paralysis progresses cranially from the legs, affecting wings, inner eyelid or nictitating membrane, and neck muscles; the latter resulting in inability to hold

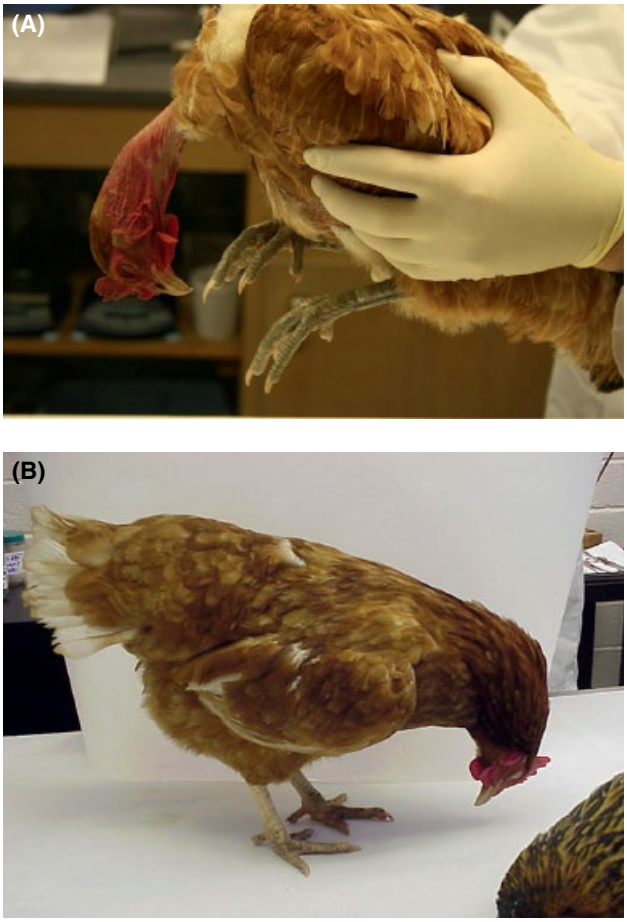


Figure 22.3 (A) and (B) Botulism in chickens showing paralysis of wing and lower eyelid, difficulty breathing caused by partial paralysis of respiratory muscles, and ruffled hackle feathers. (Gabriel Senties-Cue)

the head erect. Gasping can be observed in some birds shortly before death, which results from cardiac and respiratory failure. Affected chickens have ruffled feathers, which may fall out with handling. Quivering of certain feather tracts has been observed. Broiler chickens showing signs of botulism may have diarrhea with excess urates in the loose droppings.

Morbidity and Mortality

Morbidity and mortality are related to the levels of intoxication. In severe cases, up to 40% mortality has been observed in broiler flocks (33). Millions of wild waterfowl have died from type C botulism throughout the world, with single mortality episodes of above 100,000 birds (18).

Pathology

Gross, Microscopic, and Ultrastructural

Birds with type C botulism lack characteristic gross, microscopic, and ultrastructural lesions.

Pathogenesis of the Infectious Process

Two pathways have been described to induce poultry botulism outbreaks: (1) ingestion of preformed toxins by feeding on carcasses; and (2) toxico-infection, associated with the cecal colonization of *C. botulinum* and *in situ* production of BoNT. It is possible that these 2 pathways coexist. More than 2,000 minimum lethal doses (MLD) of type C toxin/g have been found in carcasses of intoxicated birds (4). Fly-blown carcasses may have maggots containing as much as 10^5 mean lethal dose (LD_{50})/g neurotoxin (14). Ingestion of toxin-laden invertebrates has been proposed as the main cause of type C botulism in ducks (49) with small crustaceans and insect larvae containing *C. botulinum* in their gut.

The term toxico-infection has been adapted to describe the form of botulism caused by intraintestinally produced toxin in broiler chickens. The toxico-infectious form of type C botulism has been shown experimentally in birds fed *C. botulinum* spores (20, 24).

Experimental treatment of chickens with the immunosuppressive drug cyclophosphamide indicates that stress and concurrent infections can predispose chickens to the disease (30).

Immunity

Because the toxigenic dose is lower than the immunogenic dose, chickens and ducks recovering from botulism do not develop immunity (10).

Diagnosis

Although clinical signs are often strongly indicative of the disease, they are not specific. A definitive diagnosis is usually based on the demonstration of the BoNT in specimens of affected birds and the identification of the toxin type.

Isolation and Identification of Causative Agent

Although isolation of BoNT-producing clostridia is of little help in diagnosis (17), its detection in feed or environmental samples may be useful in epidemiologic studies. Another problem isolating toxigenic strains is the unstable lysogeny of the phage carrying the neurotoxin gene (36). To reduce the use of animals in identifying and typing *C. botulinum* strains, several polymerase chain reaction-based methods have been developed for the detection of the BoNT gene of types A–F (2, 8, 22). Knowledge of toxin subtype is valuable, not only for epidemiologic reasons, but also because the mosaic toxins have shown a higher toxic activity in animals compared with nonmosaic toxins (26).

Fingerprinting methods such as pulsed-field gel electrophoresis (PFGE) and randomly amplified polymorphic DNA analysis (RAPD) have been applied to group I–III strains (13, 16, 26, 40).

Toxin Detection

The gold standard for confirmation of botulism remains the demonstration of BoNTs in serum, crop, gastrointestinal content, or liver from morbid birds by the mouse bioassay. If available, serum is the preferred specimen because it has showed the highest sensitivity.

However, the mouse bioassay is a time-consuming and expensive method requiring use of laboratory animals. Mass spectrometry (Endopep) has been used to detect active toxin and is anticipated to be an attractive alternative to the mouse bioassay (12). Other immunoassays for types C and D toxin also have been developed (5, 9, 35).

Differential Diagnosis

The differential diagnosis of botulism is based on clinical signs and lack of gross or microscopic lesions. In advanced stages of the disease, clinical signs of flaccid paralysis are obvious; during mild intoxications, only leg paralysis may be observed. The mild form of the disease must be differentiated from the transient paralysis syndrome of Marek's disease, Newcastle disease, avian encephalomyelitis, fowl cholera, drug and chemical toxicity, and appendicular skeletal problems. Lead poisoning of waterfowl is commonly confused with botulism (34).

Intervention Strategies

Management Procedures

Management practices should emphasize removal of potential sources of the organism and its toxin from the environment. Prompt disposal of dead birds and culling of sick birds is very important in prevention and control. Fly control may be another means of reducing the risk of toxic maggots in the environment (37). Removal of contaminated litter and thorough disinfection using calcium hypochlorite or formalin may help reduce spore numbers in the environment in problem areas. Disinfection of areas around poultry houses has been recommended because spores may be located in the soil outside of the poultry facility and can be transported back into houses.

Vaccination

Field Vaccination Protocols and Regimes

Active immunization with inactivated toxin has been successfully used in pheasant operations and to protect chickens and ducks from experimental botulism (20, 47). During the last decades recombinant subunit vaccines have been developed to prevent botulism (45). However, the practical usefulness of vaccination of large numbers of birds has so far not been evaluated.

Treatment

Many sick birds, if isolated and provided with water and feed, will recover. Treatment of large numbers of morbid birds is difficult and various protocols have been used but not verified experimentally. The patterns of disease in untreated broiler houses can rise and fall during a given outbreak and therefore it is difficult to know whether a particular treatment is effective (7). However, several treatments have been reported to be of benefit.

Commercial broiler chickens in outbreaks of botulism have been successfully treated with several antibiotics including bacitracin, streptomycin, tylosin, amoxicillin, penicillin, and chlortetracycline (33). Inoculation with specific antitoxin neutralizes only free and extracellular bound toxin and might be considered for treating valuable birds in zoologic collections. However, this is impractical in commercial poultry, duck, or pheasant outbreaks.

Gangrenous Dermatitis

Kenneth Opengart

Summary

Agent, Infection, and Disease. Gangrenous dermatitis (GD) is primarily a clostridial disease of chickens and turkeys caused by *Clostridium septicum* and *C. perfringens* type A. Other bacteria (*Staphylococcus aureus* and *Escherichia coli*) have been implicated as causative or contributing agents. GD is characterized by a sharp increase in mortality and discolored, dry to weepy cutaneous lesions of the wings, thighs, breast, and abdomen, and inflammation and necrosis of the underlying subcutaneous tissue and muscle.

Diagnosis. Characteristics of GD that typically aid in diagnosis are a rapid increase in mortality within a flock and distinctive lesion appearance and location. Confirmatory diagnosis is made through histopathology and bacterial culture.

Intervention. Active cases of GD have been treated with antibiotics or water acidification with limited success. Environmental management and vaccination against immunosuppressive agents may reduce or eliminate the incidence of the disease.

Introduction

Definition

Clostridium septicum, C. perfringens type A, and *Staphylococcus aureus* are the primary causative agents of GD, a disease of chickens and turkeys. Characteristic serosanguinous lesions may occur on the wing, thigh, breast, and abdomen and will appear as dark reddish-purple to green, weepy areas of the skin. Subcutaneous emphysema and crepitus may or may not be present. GD is also characterized by a sudden onset of acute mortality within a flock.

Economic Significance

The primary economic impact of GD is associated with the mortality that accompanies the disease. Losses are associated with any lost investment in production costs (chick/poult cost and feed consumed) and the resulting loss of income related to the reduction in marketable pounds. Commercial turkey flocks with GD had an estimated increased cost of production of 0.031–5.5 cents per kg (25).

Public Health Significance

Since nearly all affected birds succumb quickly to GD and do not make it to processing age, the public health significance of this disease is thought to be minimal.

History

Gangrenous dermatitis was first reported in a diagnostic case in which *C. perfringens*, *C. septicum*, and *C. novyi* were isolated from chickens (2) and as severe necrosis of muscle and subcutaneous tissue following intramuscular inoculation of *C. welchii* (*C. perfringens*) (32). It has since been reported in many countries around the world (33).

Etiology

Classification

Names and Synonyms

Gangrenous dermatitis is also referred to as clostridial dermatitis, necrotic dermatitis, gangrenous dermatomyositis, gangrenous cellulitis, gas edema disease, avian malignant edema, wing rot, and, in some instances, blue wing disease – a component of chicken infectious anemia virus infection (3, 34, 44).

Morphology and Staining

Clostridia are short, thick, Gram-positive, anaerobic, spore-forming bacillus. Clostridial spores, when present, are oval and located subterminally.

Growth Requirements

Because clostridia are anaerobic organisms, diagnostic samples collected are best transported in some type of anaerobic transport system. Culture for *C. septicum* and *C. perfringens* should be carried out anaerobically on blood agar and PEA (phenylethanol alcohol agar) agar plates incubated for 1–2 days at 37 °C (38). Growth characteristics as well as morphological and biochemical properties of *S. aureus* have been described in Chapter 23 (Staphylococcosis).

Colony Morphology

Clostridium perfringens colonies appear smooth and circular (2–4 mm) and are surrounded by an inner β-hemolytic and outer α-hemolytic zone. On prereduced blood agar plates, *C. septicum* colonies appear glossy, gray, and β-hemolytic, and are circular (1–5 mm) with slightly raised irregular margins. *C. septicum* swarms on blood agar after 24 hours of incubation whereas *C. perfringens* tends to form distinct colonies. PEA will reduce swarming as well as prevent overgrowth of contaminants. Individual colonies can be used in enzyme identification systems for rapid isolate identification and confirmation (38).

Biochemical Properties

Clostridium septicum ferments glucose, maltose, lactose, and salicin but not sucrose or mannitol. Principal products of fermentation are acetic, butyric, and formic acids. Growth on McClung–Toabe egg yolk agar demonstrates an absence of lecithinase and lipase production. The use of the same agar with *C. perfringens* cultures, however, can be used to detect the presence of lecithinase and the absence of lipase production. *C. perfringens* produces large amounts of acetic and butyric acids with lesser amounts of propionic and formic acids (38).

Antigenic Structure and Toxins

Clostridium septicum produces 4 exotoxins (alpha, beta, delta, and gamma) which have distinct functions that collectively lead to the pathogenicity of the organism

(31). The specific role of each of these toxins in the production of GD is unclear at this time. Antibodies to alpha and NetB toxins of *Clostridium perfringens* type A were found to be significantly higher in healthy chickens compared with birds with GD. It was speculated, therefore, that these antitoxin antibodies could have a protective role in GD infection (22).

Strain Classification

Genetic or Molecular

A quantitative real-time polymerase chain reaction (PCR) test has been reported which targets the *C. septicum*-specific gene *csa*, thus providing a mechanism for accurately quantifying the levels of *C. septicum* (30). PCR has also been used to determine toxin types of *C. perfringens* (27). Multilocus sequence typing (MLST) has been used to determine the degree of relatedness among populations of both *C. perfringens* (18) and *C. septicum* (31) recovered from GD.

Pathogenicity

The prevalence of *C. septicum* and *C. perfringens* toxin genes has been investigated using MLST (18, 31) and suggests that, as has been reported in more recent field cases (23, 25, 30, 31, 39), *C. septicum* is the more prominent etiologic agent of GD. There is great genetic diversity among strains of clostridia and their ability to produce toxins, which partially accounts for the varied degree of pathogenicity, especially among strains of *C. perfringens* (18).

Pathobiology and Epizootiology

Incidence and Distribution

Gangrenous dermatitis has been reported throughout the world in chickens and turkeys.

Natural and Experimental Hosts

Natural outbreaks of GD have been described most commonly in broiler flocks 4–8 weeks of age (10, 11, 37), commercial layer (11, 37) and broiler breeder flocks (12), and 13–18-week commercial turkey flocks (4, 6, 9, 25, 40). Male broilers and turkeys have a higher incidence of GD than their female counterparts (25, 33). Experimental reproduction of GD in chickens (17, 37) and turkeys (37) following intramuscular or subcutaneous inoculation of *C. septicum*, *C. perfringens* type A, or *S. aureus* has been reported to cause mortality and lesions similar to those that occur in naturally occurring outbreaks.

Transmission, Carriers, and Vectors

Clostridia are normal inhabitants of the avian intestinal tract and are ubiquitous in the poultry house environment. They can be easily isolated from feces, soil, contaminated litter, dust or feed, and intestinal contents. Staphylococci are common inhabitants of the skin of poultry and are also present in environments where poultry are hatched, reared, and processed. Despite the ubiquitous nature of clostridia and staphylococci, the isolation of these organisms from the environment does not necessarily indicate that they have a role in GD pathogenesis. Other contributing factors are thought to play a part in the development of clinical disease within a flock (see Pathogenesis of the Infectious Process).

Incubation Period

Mortality associated with GD is often the first sign that a flock has a GD problem. Birds that succumb to the infection are often well-fleshed and may still have feed in their crop indicating peracute to acute mortality. Birds that are found alive with characteristic lesions are often recumbent, depressed, and near death. The incubation period and course of disease, therefore, are relatively short (12–24 hours).

Clinical Signs

The clinical signs associated with GD include depression, inappetence, leg weakness, ataxia, and pyrexia (10, 11, 37).

Morbidity and Mortality

The onset of GD is acute and morbidity may, therefore, appear low. The earliest indicator of infection may be a greatly elevated rate of mortality with the presence of characteristic lesions. Mortality from GD infection can be quite variable and may range from 1%–60% (10). Commercial broiler and turkey flocks experiencing GD typically have 1%–5% higher mortality than unaffected flocks. The severity of the lesions and mortality depend upon the bacterial strains involved in the infection and the specific toxins they produce (18, 45).

Pathology

Gross

Gross lesions of GD in turkeys and chickens appear as dark reddish-purple to green, weepy areas of the skin. Affected areas usually include breast, abdomen, wings, thighs and legs (6, 10, 11, 37). Extensive serosanguinous edema, with or without gas (emphysema), is present in the subcutaneous tissue below the affected skin lesions. (Figures 22.4 and 22.5A–C). Underlying musculature is



Figure 22.4 Characteristic subcutaneous congestion and emphysema associated with gangrenous dermatitis in a chicken. (D. Ritter)

often discolored gray or tan and may contain edema and gas between muscle bundles. Affected birds may also have enlarged, darkened visceral organs indicative of the septicemia and toxemia characteristic of the infection. Lesions associated with clostridial dermatitis in turkeys also may occur around the tail head. Vesicle-like lesions and tissue edema are present laterally and ventrally around the tail. Tail feather shafts may be soft, blood-filled, and broken (4, 6).

Microscopic

Microscopic changes are characterized by edema and emphysema (Figure 22.5D) with numerous large, basophilic bacilli or small cocci within subcutaneous tissues. Severe congestion, hemorrhage, and necrosis of underlying skeletal muscle are often present. Liver, if affected, contains small, randomly scattered, discrete areas of coagulation necrosis with intralesional bacteria. Bursal changes, in cases suspected to have concurrent infectious

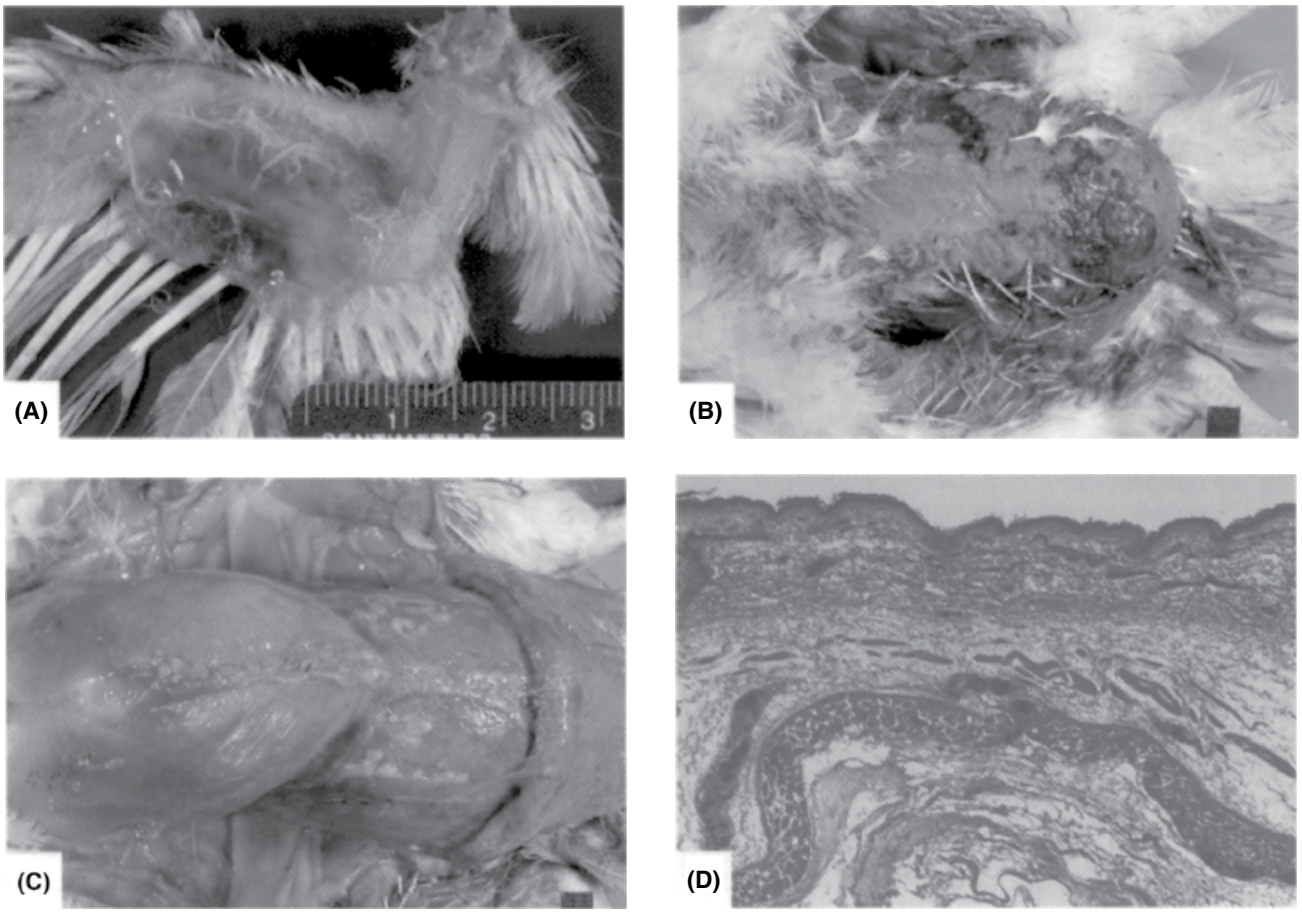


Figure 22.5 (A) Gangrenous dermatitis affecting the wing of a 12-day-old broiler. Spontaneous separation of epidermis revealing edematous, hyperemia in the dermis. (L. Munger and H.J. Barnes) (B) Broiler, 6-weeks-old, with gangrenous dermatitis. Extensive discolored patches of necrotic skin are present on the abdomen. (H.J. Barnes) (C) Same bird as in (B). Skin is reflected to show discolored muscle and serosanguinous fluid expanding underlying dermis. (H.J. Barnes) (D) Skin from a turkey with gangrenous dermatitis. Dermis beneath a normal epidermis is markedly expanded by fluid and gas. Cutaneous muscle is undergoing rhadomyolysis. Cellular changes are minimal to absent. (H.J. Barnes)

bursal disease (IBD), are characterized by extensive follicular necrosis and atrophy (5, 37).

Pathogenesis of the Infectious Process

The specific mechanism through which clostridia or other bacteria arrive at the site of infection has still not been fully elucidated. One proposed mechanism describes the translocation of clostridia across the intestinal mucosa and into the bloodstream for distribution throughout the body. The clostridial spores remain dormant in these areas until conditions favor germination and proliferation of the organism are present. Exotoxin production by the organism produces local tissue necrosis which further fuels clostridial growth and the distribution of exotoxins throughout the body (24). Others have speculated that the source of infection enters through scratches in the skin which then sets up a local infection from which exotoxins are produced (6, 40).

Contributing Factors

Environmental and management factors may predispose flocks to GD. Factors which increase litter moisture such as poor ventilation, poor drinker management, or poor litter quality may lead to GD within a flock. Other management practices which may cause scratching and skin damage such as overcrowding, feed outages, meal time feeding, and bird migration in tunnel-ventilated houses, can increase GD incidence (45). Failure to remove moribund or dead birds in a timely manner may predispose a flock to GD because these birds can serve as a source of clostridia for other birds. In the absence of other predisposing factors such as immunosuppressive infectious agents and management factors, GD tends to be associated with season of the year with peak occurrence in the spring, flocks that perform above production standards, certain strains or breeds and males more often than females. Affected farms tend to have repeat outbreaks because clostridial spores may seed down the environment and are quite resistant to environmental factors and chemical disinfection (25, 33).

GD is believed to occur as a sequela to other disease agents which produce immunosuppressive effects such as IBD virus, chicken infection anemia virus (CIAV), reticuloendotheliosis virus, reovirus, and avian adenovirus, including inclusion body hepatitis virus (3, 5, 7, 8, 10, 15, 19, 26, 29, 34, 36, 44). Outbreaks of GD in broilers have been reported to be breeder flock-associated (i.e., progeny from specific breeder flocks consistently develop GD) (13, 36) suggesting that either vertical transfer of immunosuppressive viruses to progeny or a lack of protective maternal antibody transfer to these agents increases susceptibility to GD.

Diagnosis

Field cases of GD in broilers and turkeys are often diagnosed without laboratory support based on the acute onset of elevated mortality with very low morbidity and characteristic skin lesions. Laboratory confirmation may be obtained with the isolation of the causative agent from the lesions. Staphylococci and clostridia have been isolated from exudates of skin and subcutaneous tissue or underlying muscle (5, 11, 37).

Isolation and Identification of Causative Agent

Swabs or samples of tissues or exudates from gross lesions should be submitted for bacterial culture as soon as possible after collection. If clostridial infection is suspected, anaerobic transport media is helpful and will improve the success of isolation.

Differential Diagnosis

Coliform cellulitis, a condition in market-age broilers caused by *Escherichia coli* involving the subcutaneous tissues of the abdomen, thigh, and leg, can cause the dermis to appear reddened and edematous (14). This condition, however, typically does not have emphysematous lesions or mortality associated with it and is generally only a problem observed at the processing plant. A variety of other skin conditions must be differentiated from GD including contact or ulcerative dermatitis ("breast burn") (16), scabby hip dermatitis of broiler chickens (17, 35), and avian pox.

Intervention Strategies

Management Procedures

Because infected birds serve as a primary source of infection for others, frequent removal of GD-affected birds may help to limit the disease within a house. Thorough cleaning and disinfection of the poultry house and floor reduces or eliminates GD infection on farms with historical problems. In these cases, large amounts of water mixed with a phenolic disinfectant (1,500 gallons/20,000 feet^2) have been used to achieve a saturation depth of 3–4 inches of the dirt floor pad. Treating the dirt floor with salt at 60–100 pounds/1,000 feet^2 prior to placement of bedding material has also been shown to decrease the incidence of GD on problem farms. Generally, management procedures to improve litter condition, reduce litter moisture, acidify litter pH, reduce bacterial levels in the environment, and minimize trauma are useful adjuncts to other prevention and control methods (33).

Vaccination

Experimentally, administration of a mixed clostridial bacterin at 1 day of age has been shown to reduce losses in flocks caused by GD (13). Similar results have been reported in 5-week-old chickens vaccinated with a mixed *E. coli*, *S. aureus*, and *C. perfringens* bacterin following bacterial challenge with live cultures of the same organisms (20). A recombinant noncytolytic α toxin peptide was shown to provide partial protection against *C. septicum* challenge in turkeys (21). *C. septicum* and *C. perfringens/C. septicum* toxoids have been shown to reduce GD in commercial turkeys following a single subcutaneous administration at 6 weeks of age (41, 42). There are currently no commercial vaccines available for the control of GD.

In the face of underlying and predisposing viral infections, modification of vaccine programs directed against immunosuppressive agents such as IBD and CIAV may sometimes be used to combat widespread GD problems.

Treatment

Historically, antibiotic therapy in either the feed or drinking water has been used to effectively manage GD (33). In many cases and more recently, however, antibiotics have proven to be of limited use in treatment of the disease (10, 13).

Water treatment with copper sulfate (1) or drinking water acidification with citric or propionic acid have been used to reduce, but not eliminate, GD-associated mortality in flocks where the rate of mortality does not justify the use of an antibiotic or where antibiotics have been demonstrated to no longer be effective.

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23

Other Bacterial Diseases

Introduction

Catherine M. Logue

Bacterial diseases of poultry tend to cause significant losses to the poultry industry worldwide annually. This chapter focuses on some of the sporadic bacterial diseases that are not covered in specific chapters but yet have a significant impact on poultry. The less common pathogens included in this chapter are *Staphylococcus*, *Streptococcus*, *Erysipelas*, spirochetes, and tuberculosis. The section on enterococci and its association with disease is included with the streptococci. These organisms have an increased influence in poultry production due to their ability to cause local and systemic disease (10, 13) and in light of the limited use of growth promoters (1, 2, 5, 14).

This chapter also includes a section devoted to miscellaneous organisms that have caused disease in poultry or are a public health concern. In most cases the disease strain has caused an unusual outbreak in production or exotic birds. In some instances, reproduction of the disease using bird models was not possible. Earlier editions of *Diseases of Poultry* have specific chapters for organisms such as *Bacillus anthracis*, *Brucella*, *Coxiella*, and *Franciscella* (Tularemia).

New taxonomic classification using genomic-based approaches has reclassified some bacteria (e.g., *Pasteurella anatis* is now classified in the genus *Gallibacterium*) (3), or created new genera or species for previously unnamed organisms (e.g., *Coenonia*, *Pelistega*, and *Suttonella*) (7, 15, 16).

Public health concerns from pathogens such as *Campylobacter* (Chapter 17), *Arcobacter*, *Helicobacter* (12), *Listeria* (4, 8), methicillin-resistant *Staphylococcus aureus* (MRSA) (6, 9), and mycobacteria (11) have gained increased attention because of the risk of poultry as a source of these pathogens for human illness. In each case, their potential for causing illness in humans appears to be greater than the risk for disease in birds where the organism has a commensal relationship with its host.

Disease syndromes also included in the final section of this chapter include beak necrosis, venereal disease of geese, and liver granulomas, but are not identified to a specific organism responsible because of the multifactorial nature of the disease.

Unusual pathogens identified in disease include *Neisseria, Candida*, yeasts, eubacterium, and *Enterococcus*.

Often, bacteria are identified as the primary pathogen or cause of disease in birds but more often it is likely that the organism is an opportunist or is a co-pathogen with another agent. Efforts to reproduce the disease often fail because all of the conditions of the disease may not be reproducible or there are too many factors to reproduce such conditions.

Staphylococcosis

Claire B. Andreasen

Summary

Agent, Infection, and Disease. *Staphylococcus* spp. are ubiquitous in the environment and infections are common in poultry. In poultry, *Staphylococcus aureus* infections often become systemic and disseminate to involve bones, joints, and tendons. Poultry morbidity and mortality often are due to decreased mobility and the inability to access food and water.

Diagnosis. Bacterial culture is the primary confirmatory test.

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Intervention. There is currently no effective vaccine; therefore, management for disease prevention is critical. Recommendations include prevention of other immunosuppressive diseases, and a clean environment to decrease bacterial entry, for example, clean bedding and the absence of sharp objects that can result in penetrating wounds.

Introduction

Definition and Synonyms

Staphylococcus infections are common in poultry. These infections are primarily caused by *Staphylococcus aureus*, although other species are occasionally involved (3, 4, 10, 22, 83), the clinical signs vary with the site of entry, and affected sites often are the bones, tendon sheaths, and joints, especially the tibiotarsal and stifle joints (Table 23.1). Staphylococcal infections occur less frequently in other locations including skin, sternal bursa, yolk sac, vertebrae, eyelid, testis (4), heart (15, 83), and granulomas in the liver and lungs (4). Infections are usually characterized by increased heterophil counts and marked heterophilic infiltration of tendons, synovial membranes, and other affected organs (6). Staphylococcal septicemia, causing sudden deaths in laying birds (14), seems to be most prevalent in hot weather and resembles fowl cholera. The route of entry, pathogenesis, and host response are not completely defined. Staphylococcal disease is usually chronic and responds poorly to antimicrobial therapy or immunization.

Economic Significance

Staphylococcal infections are a worldwide problem in chickens and turkeys, and cause economic losses due to decreased weight gain, decreased egg production, lameness and bird losses from osteomyelitis and septicemia, and condemnation of carcasses at slaughter (58, 65). During the processing of turkeys, a high correlation between green-discolored livers and *S. aureus* has been made and termed "green-liver osteomyelitis complex" (12, 23). Although *S. aureus* is the most commonly isolated pathogen in this condition, *Escherichia coli* and numerous opportunistic bacteria also have been isolated from affected turkeys (12).

Public Health Significance

In addition to being a major disease-producing organism for poultry, approximately 50% of typical and atypical *S. aureus* strains produce enterotoxins that can cause food poisoning in humans (4, 41). Poultry-associated food poisoning can occur due to the contamination of carcasses with these strains at processing. *S. aureus* strains from processed poultry are thought to be endemic to the processing plant or transmitted from the hands of workers in the plant (2, 72, 86). The literature varies as to the origin of processing plant strains with biotyping indicating the passage of human staphylococcal strains to poultry in processing plants; and plasmid profiling indicating that endemic strains in the processing plant are primarily introduced by incoming birds (30).

Methicillin-resistant *Staphylococcus aureus* (MRSA), which has emerged as an important human pathogen, is also a concern, particularly for people who are in contact with colonized poultry on farms or during slaughter (4, 26, 33, 36, 37, 48, 56, 62, 64, 73–75, 89, 90, 92). *MecA*-bearing methicillin-resistant strains of *S. aureus* are resistant to beta-lactam antibiotics including the semisynthetic penicillins. Many isolates also are resistant to numerous other antibiotics including the fluoroquinolones. *MecA*-MRSA has been detected in chickens, turkeys, and other poultry in a number of countries, with the majority of these reports from Europe (27, 53, 69, 87, 88, 92). Many of these organisms belong to the livestock-associated clonal complex CC398 (36, 53, 62, 64, 69, 73, 75, 89, 90), but other clonal complexes including animal-associated MRSA CC9, as well as MRSA normally carried by humans, have also been detected in some flocks (29, 62, 64, 69, 90).

In Europe, some studies indicate that the overall prevalence of MRSA seems to be relatively low in

Table 23.1 Staphylococcal-related infections in poultry.

Location	Age	Lesion	Usual Outcome
Bone	Any, usually older	Osteomyelitis	Lameness
Joint	Any, usually older	Arthritis/synovitis	Lameness
Yolk sac	Chicks, poults	Omphalitis	Death
Blood	Any	Generalized necrosis	Death
Skin	Young	Gangrenous dermatitis	Death
Feet	Mature	Bumblefoot	Lameness

poultry, especially when compared with other animals such as pigs or veal calves (27, 37, 53, 69); however, there are also reports where this organism occurred in 20% or more of the birds sampled, and/or large numbers of birds were colonized in infected flocks (36, 75). A number of the latter reports involved turkeys (36, 62, 75). Animal-associated MRSA can also contaminate raw poultry products at slaughter (17, 29, 33, 53, 63, 76, 90). Although there is relatively little information about MRSA in North American poultry flocks, these organisms seem to be either absent or present only at low levels on chicken meat, and the isolates on meat generally seem to be MRSA clones carried by humans (1, 20, 40, 51). Such isolates are likely to be introduced by people during meat processing. There are currently no published reports of poultry with *mecC*-bearing MRSA, which occurs in other livestock and may have a different antibiotic susceptibility profile than *mecA*-MRSA (11).

Methicillin-resistant *Staphylococcus aureus* clones carried by poultry can be transferred to humans who handle the birds or are exposed to their environments. Colonization by MRSA CC398 has been documented in poultry farmers (37, 48, 75, 87–89, 92, 93), and poultry slaughterhouse workers (63, 64), with higher rates of carriage among people who handle live birds than dead birds (64). MRSA CC398 has also been found on family members who live on poultry farms but do not directly handle the birds, albeit at significantly lower levels than in farmers (89). Humans can carry MRSA CC398 asymptomatically; however, this organism can also cause opportunistic infections, some serious (13). The significance of MRSA on poultry meat is still unclear, although some authors have suggested that it might contribute to colonization in people who handle contaminated meat (46).

It is theoretically possible for the *mecA* and *mecC* genes, which are responsible for methicillin resistance, to be transmitted between species of staphylococci found in animals and staphylococci found in humans (4). For this reason, poultry-associated methicillin-resistant species other than *S. aureus* can also be a concern for human health. Some of the methicillin-resistant coagulase-negative staphylococci that have been detected in healthy or sick chickens include *S. lentus*, *S. sciuri, S. epidermidis, S. saprophyticus, S. hyicus, S. intermedius, S. epidemidis*, and *S. haemolyticus* (47, 75).

History

Staphylococcosis in poultry and other avian species has been recognized for more than 100 years; most early reports describe arthritis and synovitis (4).

Etiology

Classification

The genus *Staphylococcus* contains approximately 45 species and 24 subspecies (32). It is the most important genus in the family Staphylococcaceae. The term staphylococcus refers to the morphology of these microorganisms; in stained smears, they often resemble clusters of grapes. Other genera in the family include *Gemella*, *Macrococcus*, and *Salinicoccus* (32). *Macrococcus* and *Salinicoccus* are considered to be nonpathogenic. *Gemella* spp. have, in rare cases, been involved in human disease (4).

A number of *Staphylococcus* species have been isolated from the skin and nares of healthy poultry, including *S. aureus*, *S. epidermidis, S. xylosus*, *S. cohnii*, *S. lentus*, *S. saprophyticus*, *S. sciuri*, and *S. gallinarum* (4). *S. aureus* is the most common species isolated from poultry that are ill (4, 14). Other species are also found occasionally and may in some cases be opportunists. In 1 outbreak of systemic illness, the major staphylococcal species isolated from the liver, blood, and hock of sick 6-week-old chickens included *S. lentus*, *S. simulans*, *S. cohnii*, *S. gallinarum*, and *S. capitis* (10). In this study, *S. aureus* was uncommon. *S. hyicus* has been associated with fibrinoheterophilic blepharitis in chickens and turkeys and was isolated from 5 of 9 tibiotarsal growth plates of turkeys with stifle joint osteoarthritis (4). This species was also linked to chronic folliculitis and epidermitis with acantholysis in layer hens of the Leghorn breed (22). *S. sciuri*, *S. simulans*, *S. epidermidis*, *S. lentus*, *S. warneri*, *S. cohnii*, and *S. intermedius* have been reported from scabby hip lesions in broiler chickens (4), and *S. simulans* has been linked rarely to endocarditis (83). *S. agnetis* was isolated from outbreaks of bacterial chondronecrosis with osteomyelitis (femoral head necrosis) in chickens on wire flooring and was also found in 1 case of bacteremia (3). Other staphylococci found in humans and domestic animals are not known to be important pathogens in poultry.

Morphology and Staining

Typical staphylococci are Gram-positive, coccoid in shape, and found in clusters when grown on solid media. In liquid media, they may occur in short chains. Older cultures (more than 24 hours) may stain Gram-negative.

Growth Requirements

Staphylococci are readily isolated on 5% blood agar with growth evident within 24 hours.

Colony Morphology

Staphylococcus aureus is considered to be the most pathogenic staphylococcal species in poultry, and is isolated

from the majority of clinical infections. Within 24 hours, aerobic growth of *S. aureus* results in circular, smooth, ß-hemolytic colonies, 1–3 mm in diameter, which are often pigmented white to orange. Colonies of coagulase-negative staphylococci are similar but are often gray to cream or white, and nonhemolytic.

Biochemical Properties

Staphylococcus aureus is aerobic, facultatively anaerobic, ß-hemolytic, usually coagulase positive, catalase-positive, fermentative for glucose and mannitol, and gelatinase-positive. *S. hyicus* is similar biochemically to *S. aureus* but some strains have a delayed positive coagulase reaction. Most other staphylococci found in poultry are coagulase negative. Coagulase-negative staphylococci can be identified to the species level using panels of biochemical tests (4), automated systems (4), or genetic testing (4).

Susceptibility to Chemical and Physical Agents

Staphylococci are extremely hardy and remain viable for long periods on solid media or in exudates. Some strains are heat and disinfectant resistant (59). A resistance feature used to isolate *S. aureus* from heavily contaminated clinical material is its tolerance to high (7.5%) concentrations of sodium chloride (4).

Antigenic Structure and Toxins

The antigenic nature of *S. aureus* is often complex. Strains have a capsule which can consist of glucosaminouronic acid, manosaminouronic acid, lysine, glutamic acid, glycine, alanine, or glucosamine; polysaccharide A consisting of linear ribitol teichoic acid, *N*-acetylglucosamine, and D-alanine; and protein-A, a cell-wall component that interacts nonspecifically with the Fc portion of immunoglobulin and may be a virulence factor. A variety of enzymes and toxins including hyaluronidase (spreading factor), deoxyribonuclease, fibrinolysin, lipase, protease, hemolysins, leukocidin, dermonecrotic toxin, hemolysins, exfoliative toxins, and enterotoxins also can contribute to a strain's pathogenicity and virulence (4, 8, 45). Toxic shock syndrome toxin-1 (TSST-1) also has been found in *S. aureus* isolated from chickens, but there is currently no evidence that this toxin is directly linked to disease in poultry (68).

Strain Classification

Antigenicity

Strains have been classified using plasmid profiles, and serotyping based on capsular polysaccharides (28). Chicken capsular types were type 5 (91%) and type 8 (9%), and turkey capsule types were type 5 (33%), type 8 (38%), and nontypeable (29%) (28).

Immunogenicity or Protective Characteristics

Phenotyping techniques, such as biotyping and phage typing, as well as genetic techniques, have been used to classify poultry *S. aureus*. Biotyping can determine the origin and epidemiological "connections" of *S. aureus* isolates as host-specific (human or domestic animal) ecovars (4) or nonhost-specific biotypes (81). Phage typing has been used for poultry and historically for human *S. aureus* strains (4, 81). In poultry, phage typing has been used to determine the association between country of origin (Europe, Australia, Argentina, Japan) and pathogenic and nonpathogenic strains (4, 81) but 2.2–25.8% of chicken *S. aureus* remain nontypeable by this method (81). Phages tend to be specific for *S. aureus* of poultry origin and cannot be used to type strains from other species (80).

Genetic or Molecular

Genomic fingerprinting by pulsed-field gel electrophoresis (PFGE) is also a useful method for discriminating poultry *S. aureus* strains and for subtyping strains of avian phage groups or poultry-specific ecovars (19, 44, 81). This technique was able to type all chicken *S. aureus* strains, including those that were not phage typeable (81). *S. aureus* can also be genetically typed by multilocus sequence typing (MLST) and spa (staphylococcal surface protein A) typing (85). MLST can be used to group MRSA into clonal complexes, such as CC398, which contain genetically related sequence types (85). Spa typing can distinguish strains that cannot be typed by PFGE or MLST. The MLST type ST5 (CC5) is reported to be particularly common among poultry-associated *S. aureus*, although other isolates including CC398 have also been found (21, 42, 57).

Pathogenicity

A variety of enzymes and toxins listed previously under Antigenic Structures and Toxins, including enterotoxins, can contribute to a strain's pathogenicity and virulence (4, 8, 45). Also, antibiotic susceptibility or resistance can contribute to pathogenicity and strains have been classified according to these characteristics (11, 32, 51).

Virulence Factors

Coagulase-positive isolates of *S. aureus* are considered to be pathogenic for poultry. Coagulase-negative strains are often nonpathogenic in poultry but can be pathogenic in some species. The specific staphylococcal genes involved in virulence are still poorly understood for poultry; however, some putative virulence factors have been described (16, 54, 57, 67, 84, 95).

Pathobiology and Epizootiology

Incidence and Distribution

Staphylococcus spp. are ubiquitous, normal inhabitants of skin and mucous membranes and are common environmental organisms where poultry are hatched, reared, or processed. Most staphylococcal species are considered to be normal flora, which suppress other potential pathogens through interference or competitive exclusion. Some have the potential to be pathogenic and produce disease if allowed entry through the skin or mucous membranes.

Staphylococcus spp. and staphylococcosis have been associated with poultry throughout the world (4). According to 1 recent genetic study, many *S. aureus* in broiler chickens may have descended from a single MLST clone (ST5) that was acquired from humans approximately 30–60 years ago and became adapted to chickens and disseminated widely (57).

Natural and Experimental Hosts

All avian species are susceptible to staphylococcal infections.

Transmission, Carriers, and Vectors

The pathogenesis of *S. aureus* infections is not completely defined, but for infection to occur, a breakdown in the natural defense mechanisms of the host must occur (8, 45). In most cases, this would involve damage to an environmental barrier, such as a skin wound or inflamed mucous membrane, and hematogenous dissemination where a locus of osteomyelitis is established, usually in the metaphyseal joint (4). The open navel of newly hatched chicks leading to omphalitis, minor surgical procedures, and parenteral vaccinations may offer additional means of entry for staphylococci.

Another type of host defense impairment occurs following infectious bursal disease (77), chicken infectious anemia, or possibly Marek's disease, in which the bursa of Fabricius or thymus is damaged and the immune system is compromised. Under these conditions, septicemic staphylococcal infections can cause sudden death.

Escherichia coli was discovered to be the predominant bacterial organism in the livers of turkeys immediately following challenge with virulent hemorrhagic enteritis virus (HEV). However, when livers of survivors were cultured 2 weeks postexposure, *Staphylococcus* spp. were the predominant bacteria (70). This suggests HEV, and possibly other similar viral intestinal infections, may create portals of entry and provide the underlying basis for subsequent staphylococcal problems associated with older, commercial turkeys.

Susceptibility to staphylococcal infections also may be genetically influenced. Two related lines of New Hampshire chickens had significant differences in mortality following experimental infection (25). A syndrome of acantholytic folliculitis and epidermitis associated with *S. hyicus* also appears to be genetically influenced and was restricted to hens in 1 line of Leghorn chickens (22). The avian major histocompatibility complex influences the susceptibility to staphylococcal skeletal disease in chickens (50).

Incubation Period

The incubation period is short. In experimental infections of chickens, clinical signs were evident 48–72 hours following intravenous inoculation, but the severity of lesions was dose dependent (6). Experimentally, chickens can be readily infected by the intravenous route, but not as well by the intratracheal or aerosol routes (49). The ability to produce experimental disease consistently is dependent on the number of intravenously administered bacteria (6), needing at least 10^5 organisms/kg body weight (65, 66).

Clinical Signs

Early clinical signs include ruffled feathers, lameness in 1 or both legs, drooping of 1 or both wings, reluctance to walk, and fever (65). This can be followed by severe depression and death. Birds surviving the acute disease have swollen joints, sit on their hocks and keel bone, and are reluctant or unable to stand (65). Clinical signs of septicemic staphylococcal infection and gangrenous dermatitis can occur in birds in good condition and may be evident only because of increased mortality in the flock (14).

Staphylococcal-related hatchery infections are common and can cause increased mortality within the first few days after hatching. Chicks have wet navel areas and deteriorate rapidly. Internally, the yolk sacs are enlarged with abnormal color and consistency.

Morbidity and Mortality

Morbidity and mortality due to staphylococcosis is usually low, even in the face of septicemia, unless chicks have been massively contaminated from exposure to unusually high numbers of bacteria. This may occur in the hatchery environment or through vaccination or service procedures. Reluctance to walk to feeders and waters can lead to debilitation and death; therefore, nonambulatory birds should be euthanized to prevent this occurrence. Several reports from diagnostic laboratories have indicated that *S. aureus* is the most common bacterial agent isolated from infected legs and joints (52). The

number of chickens that develop gangrenous dermatitis is low, but usually all chickens that develop lesions succumb to the infection (4). In some geographic areas, *S. aureus* is an important cause of mortality in broiler breeder females and males.

Pathology

Gross

Gross lesions of osteomyelitis in bone consist of focal yellow areas of caseous exudate or lytic areas (Figure 23.1A), which cause affected bones to be fragile. Sites most frequently involved are the proximal tibiotarsus and proximal femur, and less commonly, the proximal tarsometatarsus, distal femur, distal tibiotarsus, proximal humerus, ribs, or vertebrae. In affected birds, the femoral head often separates from the shaft by a fracture through the neck when the coxofemoral joint is disarticulated (femoral head necrosis) (Figure 23.1C) (65). An unusual outbreak, described in 3 successive turkey flocks on 1 farm, affected the skull bones and mandibula (24), and had associated sinusitis and blepharitis.

Arthritis, periarthritis, and synovitis are common. Affected joints are swollen and filled with inflammatory exudate as the osteomyelitis extends from nearby metaphyseal areas (Figure 23.1D) (61). Spondylitis involving articulating thoracolumbar vertebrae may cause lameness indirectly because of the impingement on the spinal cord.

Gross lesions of septicemic staphylococcal infection consist of necrosis and vascular congestion in many internal organs including the liver (Figure 23.1E), spleen, kidneys, and lungs (14). Dark, moist areas under the skin with crepitation are seen in gangrenous dermatitis (14).

Plantar abscess ("bumblefoot") is not common in current modern production facilities, but when seen in mature chickens leads to massive swelling of the foot and lameness. Partially, or less commonly, entirely green-discolored livers (Figure 23.1F) have been associated with osteomyelitis and/or associated soft tissue lesions, such as arthritis, periarthritis, or tenosynovitis in commercial turkeys at processing, and is known as green-liver osteomyelitis complex. Carcasses with lesions from which staphylococci or other bacteria are isolated also have liver discoloration, but frequently turkeys with liver discoloration do not have demonstrable osteomyelitis or associated lesions, or bacteria cannot be isolated from the lesions (12, 23).

Liver spots are another common cause of condemnation in commercial turkeys, but usually are not culture positive for aerobic or facultative anaerobic bacteria. In 1 study, *S. cohnii* and other staphylococci were isolated most frequently from the few culture-positive livers in 2 flocks with histories of high liver condemnation (71). Ascarid larval migration appeared to be primary cause of the liver lesions (71).

Microscopic

Histologically, staphylococcal lesions consist of necrosis; bacterial colonies are composed of large numbers of Gram-positive, coccoid bacteria and heterophils (Figure 23.1B) (8, 65). Supernatants from pathogenic *S. aureus* resulted in increased chemotaxis of heterophils compared with supernatants from nonpathogenic *S. xylosus*; this appears to correlate with the heterophilic infiltrate seen in staphylococcal lesions (7). Long-standing lesions are primarily granulomatous.

Immunity

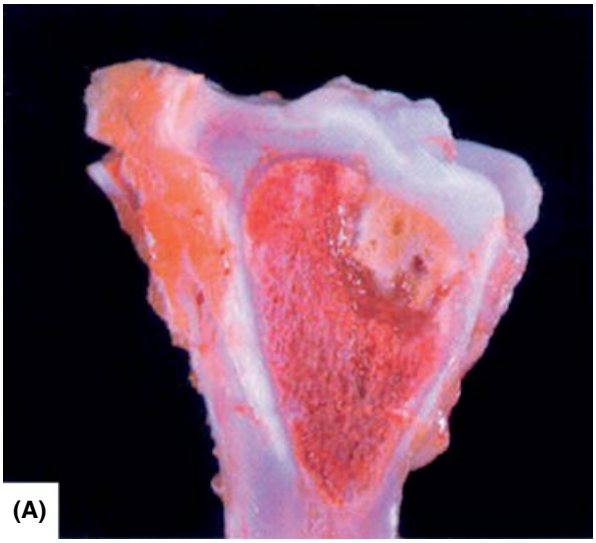
Neither active nor passive immunity appears to be effective in preventing *S. aureus* infections in poultry. It has been implied that a specific antibody to *S. aureus* may promote the development of *S. aureus*-related infections in chickens (34, 38). Additionally, antistaphylococcal antibodies may not significantly increase the opsonization and phagocytosis of *S. aureus* compared with the naturally exposed complement-activating cell wall materials during infection (5). However, egg yolk antibodies from immunized hens can inhibit *in vitro* growth of *S. aureus* (39, 96). Staphylococcal toxoids have not proven to be effective in inducing immunity in other species (8, 45).

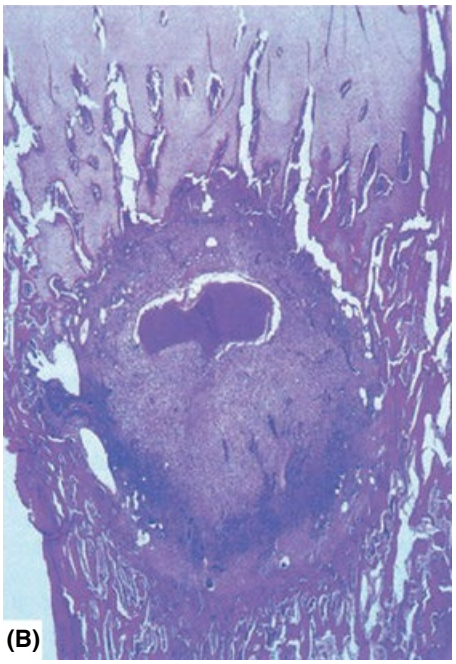
Diagnosis

Isolation and Identification of Causative Agent

Staphylococcosis is diagnosed by culturing suspected clinical material including exudate from joints, yolk material, and stab swabs of internal organs. The basic medium for growing staphylococci is blood agar (preferably sheep or bovine). Organisms grow well with colonies 1–3 mm in diameter within 18–24 hours. Most *S. aureus* strains are ß-hemolytic; other staphylococci are usually nonhemolytic. Heavily contaminated material should be streaked onto a selective medium inhibitory for Gram-negative bacteria, such as mannitol-salt or phenylethyl-alcohol agar (4).

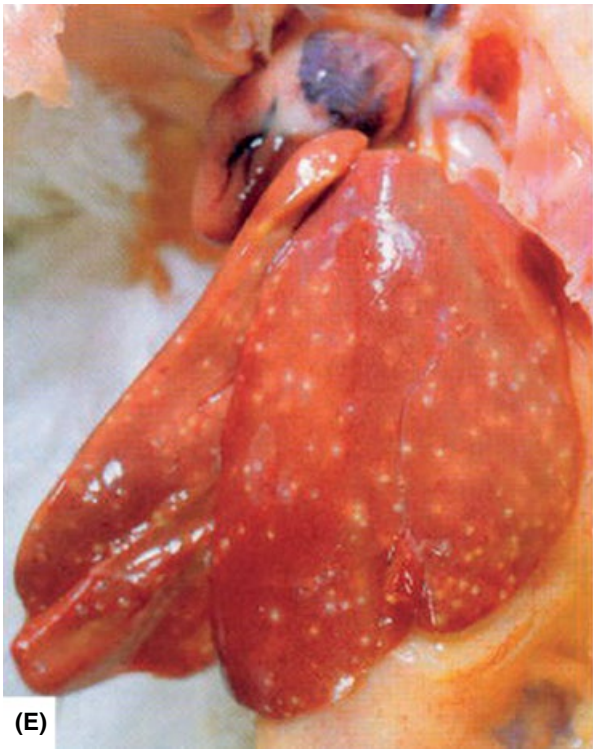
Most *S. aureus* colonies will be pigmented, while most other staphylococcal colonies are gray to white. Colonies should be selected and Gram stained, and will be seen as Gram-positive cocci. Biochemical tests such as the catalase test can differentiate staphylococci from other Gram-positive organisms such as *Streptococcus*. Coagulase and mannitol fermentation tests are useful in the presumptive identification of *S. aureus*. The coagulase test is commonly used to differentiate *S. aureus* from











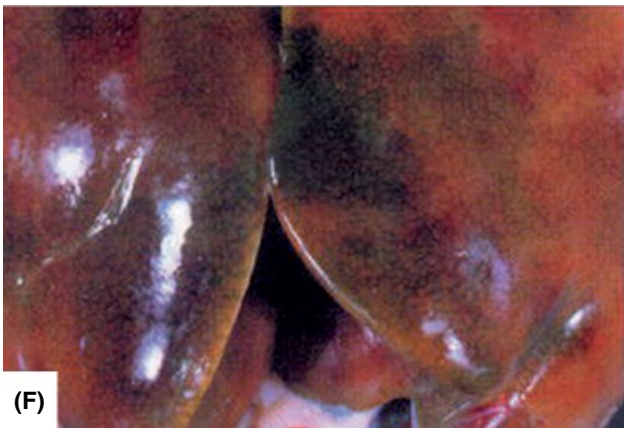


Figure 23.1 Lesions of staphylococcosis. (A) Osteomyelitis of proximal tibiotarsus (×5). (H.J. Barnes) (B) Focal osteomyelitis subjacent of physis of proximal tibiotarus. (H.J. Barnes) (C) Bilateral osteomyelitis of femoral head due to *Staphylococcus aureus* infection in a 2-week-old turkey. Note the extension through the joint into the body cavity. (D) Three-week-old turkey. Swollen hock joint with extension of inflammatory exudates along tendon sheaths. (L. Munger) (E) Leghorn, 20 weeks old. Multiple foci of necrosis in liver following septicemic staph infection. (L. Munger) (F) Green liver discoloration seen in turkeys with osteomyelitis. (H.J. Barnes) (*For color detail, please see the color section*.)

Table 23.2 Differentiation of *Staphylococcus aureus* and *S. epidermidis*.

Characteristic	S. aureus	S. epidermidis
Colony pigment	+	–
Hemolysis	+	–
Coagulase	+/–	
D. mannitol fermentation	+	–

coagulase-negative staphylococci such as *S. epidermidis* (Table 23.2). A few other staphylococcal species including *S. pseudintermedius, S. intermedius*, *S. hyicus*, *S. lutrae*, *S. lugdunensis*, *S. schleiferi* subsp. *coagulans*, and *S. delphini* may also be coagulase positive (4) but none of these species are commonly associated with clinical disease in chickens. Unlike most other staphylococci, *S. aureus* also ferments mannitol. Panels of biochemical tests (4) and genetic testing (4) can be used for the definitive identification of staphylococci to the species level; however, this is rarely done in clinical laboratories. Commercially available systems can also be used, but these systems may have difficulty identifying some species from veterinary specimens.

Serology

Serology is not generally used for the diagnosis of staphylococcosis, but a microtiter plate agglutination assay (5, 34) and an indirect immunofluorescent antibody titer assay have been described (5). Both have primarily been used in research.

Differential Diagnosis

Staphylococcosis can resemble infection with *E. coli, Pasteurella multocida, Salmonella gallinarum, Mycoplasma synoviae*, reoviruses, or any other infection of bones or joints that is hatchery-related, associated with mechanical trauma, or causes septicemia.

Intervention Strategies

Management Procedures

Any management procedure reducing damage to host defense mechanisms will help prevent staphylococcosis, including wounds, stress, and other disease agents. Because wounds are a portal of entry for *S. aureus* into the body, management that decreases the risk of injury is important in preventing the risk of infection. Sharp objects that can cut or puncture the feet should be eliminated from areas where poultry are reared. Maintenance of good litter quality will reduce foot pad ulceration. Particular attention should be given to hatchery management and sanitation. *S. aureus* is ubiquitous, and conditions in incubators and hatchers are ideal for bacterial growth. Recently hatched and hatching chicks with open navels and immature immune systems can be easily infected, leading to mortality and chronic infections shortly after hatching. Prevention of early infections with infectious bursal disease virus and chicken infectious anemia virus also will help prevent staphylococcosis (77).

Poultry under mild stress may be more resistant to experimental staphylococcosis (43, 55, 65), but excess stress should be avoided (e.g., heat). Some mild stress-related resistance is attributed to an increase in heterophil numbers, which can occur in birds under stress. The heterophil is thought to be the most important cell in controlling bacterial infections, particularly *S. aureus* (6, 66).

Vaccination

There is currently not an effective vaccine for *S. aureus*. Vaccines with various *S. aureus* virulence factors and surface antigen components have had variable results (18). Components have included cell wall components, such as peptidoglycan and teichoic acid; capsular polysaccharides; and cell wall-anchored proteins, such as the hemoglobin receptor IsdB, to induce active immunity (5, 18, 35, 78, 79, 82). Some approaches appear to show promise, but effective staphylococcal vaccines for some clinically important conditions in mammals remain elusive, perhaps due to the ability of staphylococci to evade immune responses and persist as part of the normal flora (18, 35, 78, 79).

Also, staphylococcal bacterins have been ineffective in preventing infections in poultry (8, 45), but the use of live, avirulent vaccines based on the principle of bacterial interference has shown some promise. Using the principle of bacterial interference, a live, avirulent vaccine for the prevention of staphylococcosis in turkeys was developed. A naturally occurring, coagulase-negative, avirulent *S. epidermidis* isolate, designated strain 115, that colonizes cells and tissues in the respiratory tract and prevents adherence of virulent strains of *S. aureus* was used (60). In addition to interfering with the colonization of virulent *S. aureus, S. epidermidis* 115 secretes a stable, antibiotic-like bacteriocin capable of inhibiting and killing virulent *S. aureus*. The vaccine is administered by aerosol at 1–10 days and again at 4–6 weeks of age. Use of strain 115 in commercial flocks has reduced the number of turkeys with staphylococcosis and improved overall health and survival (60). Similar results were found when strain 115 was used in chickens (4, 49).

Treatment

Staphylococcus aureus infection sometimes can be treated successfully, but sensitivity tests should always be

performed, because antibiotic resistance is common (4, 94). MRSA has been reported in clinical cases (9), although there is no indication that these organisms are commonly involved. Drugs that have been used for treatment include penicillins, streptomycin, tetracyclines, erythromycin, novobiocin, sulfonamides, lincomycin, and spectinomycin. Treatment of some localized lesions is often difficult, due to poor penetration by orally administered antibiotics (31).

Competitive gut exclusion using *Lactobacillus acidophilus* was attempted to exclude *S. aureus* from experimentally infected, germ-free chickens. The treatment was effective in reducing *S. aureus* counts in crop contents, but counts in the ceca and rectum were unaffected (91).

Streptococcus and *Enterococcus*

Luke B. Borst

Summary

Agent, Infection, and Disease. While streptococcal infections continue as uncommon and sporadic poultry health challenges, enterococcal infections are becoming increasingly recognized as an important cause of morbidity and mortality in poultry. Diseases caused by bacteria in both of these genera typically present within a flock in the early-life period as a septic phase with accompanying clinical morbidity and highly variable mortality (~0.5%–50%). Following the septic phase, these agents often cause chronic infections at various sites (e.g., myositis, osteomyelitis, endocarditis, etc.) resulting in clinical signs referable to the site of infection leading to additional morbidity and mortality.

Diagnosis. During the septic phase, gross lesions are indistinguishable from those caused by Gram-negative organisms like *Escherichia coli*. In subacute or chronic infection, some species in this group cause characteristic clinical signs and gross lesions (e.g., symmetrical paralysis due to infection of the free thoracic vertebra with pathogenic *Enterococcus cecorum*). Culture of lesions or spleen on agar that includes 5% sheep blood, incubated in the presence of CO_2, is generally sufficient to cultivate all species.

Intervention. No efficacious vaccines exist for these diseases; however, penicillin given in the acute phase may decrease morbidity and mortality within a flock. There is no efficacious treatment for these diseases during the chronic phase of infection.

Streptococcus

Introduction

The genus *Streptococcus* contains Gram-positive cocci, which divide in a single plane resulting in chains of bacteria instead of clusters like staphylococci. Streptococci are ubiquitous in nature as intestinal commensals but can be pathogens of birds and a variety of mammals including humans. In domestic and free-living avian species, they comprise a large proportion of normal intestinal and mucosal flora (8, 34). Streptococci and enterococci were once considered within a single genus with enterococci forming the bulk of Lancefield group D streptococci ("fecal streps"). Enterococci have been reclassified as a unique genus based on DNA-DNA and DNA-rRNA hybridization (41, 66, 70).

Streptococcosis is defined as any disease caused by streptococci. The disease is infrequent but can cause economic losses particularly in the turkey and broiler industries. The etiologic agent is usually *S. gallolyticus* subsp. *gallolyticus* (formerly *S. bovis* biotype I). Infection of humans with *S. gallolyticus* can result in septicemia and meningitis in infants as well as meningitis, endocarditis, and spondylitis in adults. An association between sepsis and colon cancer in people has also been identified (14, 56). Similarly, *S. zooepidemicus* is an uncommon zoonosis that can affect people and broilers who come into contact with infected horses (12).

History

Historical perspectives of streptococcal taxonomy have been reviewed (41, 75, 84). Streptococcal infections causing sepsis in poultry were first described in chickens in 1902 (71) and 1908 (65). Streptococcosis in turkeys was reported as early as 1932 (93). Bacterial or vegetative endocarditis associated with streptococci was first reported in 1927 (70) and again in 1947 (49).

Etiology

Medically important streptococci in birds mostly fall within Lancefield groups C and D. They grow best on blood agar incubated in an atmosphere enriched with 5% CO_2. On blood agar, streptococci frequently exhibit partial or complete hemolysis, a feature that can be useful for identification. Colonies with partial (α) hemolysis appear dark green while colonies with complete (β) hemolysis are surrounded by a clear zone in the agar. They are cata-

lase negative and ferment a variety of sugars, another feature useful for identification. Although streptococci are often susceptible to several classes of antimicrobials including penicillins, macrolides, lincomycin, tetracyclines, chloramphenicol, and nitrofurans (27), antimicrobial resistance in field isolates can vary and susceptibility testing should be performed to guide therapy. In general, streptococci do not survive well in the environment and are generally susceptible to most disinfectants.

Streptococci most commonly associated with disease in poultry include *S. gallolyticus* and *S. zooepidemicus*. *S. gallolyticus* is generally α-hemolytic and can be further differentiated into at least 2 subspecies: *S. gallolyticus* subsp. *gallolyticus* (formerly *S. bovis* biotype I) and *S. gallolyticus* subsp. *pasteurianus* (formerly *S. bovis* biotype II/2), both of which can infect birds. *S. zooepidemicus*, a β-hemolytic streptococcus that typically affects laying hens, has seen a recent resurgence due to free-range rearing of chickens (12, 79). *S. dysgalactiae, S. pleomorphus*, and *S. mutans* are rare causes of disease in poultry (9, 21).

Pathobiology and Epidemiology

Streptococcus gallolyticus subsp. *gallolyticus* is an important cause of septicemia in young and adult pigeons despite the fact that 40% of pigeons carry the bacterium in their intestinal tracts (29). This bacterial species also can infect young layers, broilers, and turkeys; however, pigeons and turkey poults are most frequently affected. *S. gallolyticus* subsp. *pasteurianus* similarly infects turkey poults and causes meningitis and sepsis in goslings and ducks (11, 40, 63, 83, 85, 86). *S. zooepidemicus* occurs almost exclusively in mature laying chickens but has caused mortality in wild birds (49, 79). *S. zooepidemicus* has largely been eliminated from commercial broiler systems; however, it remains a problem in free-range flocks, particularly those in which the chickens have contact with horses (12). *S. zooepidemicus* isolates with identical genotypes have been recovered from diseased humans, horses, and chickens on the same farm (12).

Transmission of streptococci occurs primarily via oral and aerosol routes but can occur through skin lesions in caged layers. Aerosol transmission of *S. zooepidemicus* results in acute septicemia in chickens. Incubation periods range from 1 day to several weeks, with 5–21 days being most common. Endocarditis and other localized infections can occur when septicemic infections progress to subacute and chronic stages (50).

Clinical Signs

Streptococcal infection in birds begins as an acute septicemia that is often characterized by rapidly increasing mortality in the flock without clinical signs. When signs are present, they include lassitude, yellow or slimy green droppings, dehydration, cyanosis, and pallor of the comb and wattles (75). Some birds may have blood-stained feathers around the mouth and head with blood coming from the mouth (64, 75). Goslings and turkey poults with meningitis are obtunded (11, 63, 83).

Birds that survive the acute phase of streptococcal infection often develop localized chronic infections including heart valves, liver, spleen, and joints. Clinical signs of lameness and musculoskeletal infections are seen. Pigeons infected with *S. gallolyticus* are lame, anorexic, have diarrhea, and cannot fly, likely because of pectoral myositis (26). Egg production drops of up to 15% can occur in broiler breeders and layers.

Mortality from streptococcal infections is variable. Mortality in turkey poults with *S. gallolyticus* subsp. *pasteurianus* infection was reported as 18%. In pigeons, natural and experimental *S. gallolyticus* subsp. *gallolyticus* infection results in high morbidity which approaches 90% (26, 28). Mortality in broilers infected with *S. zooepidemicus* can reach 50%–80% (12, 75)

Pathology

Gross

Gross lesions of acute streptococcal infection are indistinguishable from other bacterial causes of sepsis. In birds succumbing to acute infection, the only lesions observed are hepatomegaly, splenomegaly, and hyperemia of other tissues. Some birds may show fibrinous exudate on the serosal surfaces of the liver and heart (perihepatitis, pericarditis). On close inspection, minute pale tan foci can occasionally be observed in enlarged livers and spleens. These areas correspond histologically to areas of necrosis with intralesional colonies of cocci. Liver, spleen, and heart may have 3–10 mm infarcts that appear as firm, dry areas of pallor (Figure 23.2). They extend from the surface into the organ on cut section (50). In pigeons, congestion of the spleen and liver with excess fluid around pectoral muscles is seen (26).

Chronic lesions include fibrinous arthritis and/or tenosynovitis, osteomyelitis, salpingitis, fibrinous pericarditis and perihepatitis (Figure 23.3), myocardial necrosis, and valvular endocarditis (75). In pigeons, a striking focal area of pale green pectoral myositis occurs in chronic *S. gallolyticus* infections. Vegetative valvular lesions in the heart are usually yellow, white, or tan and are most often found on the mitral valve in the left heart.

Microscopic

Histologic evaluation of liver and spleen reveals congestion or hyperemia with multifocal areas of necrosis containing fibrin and heterophils (Figure 23.4). In cases with liver infarcts, thrombosis of portal or centrilobular veins can been seen adjacent to areas of widespread necrosis.

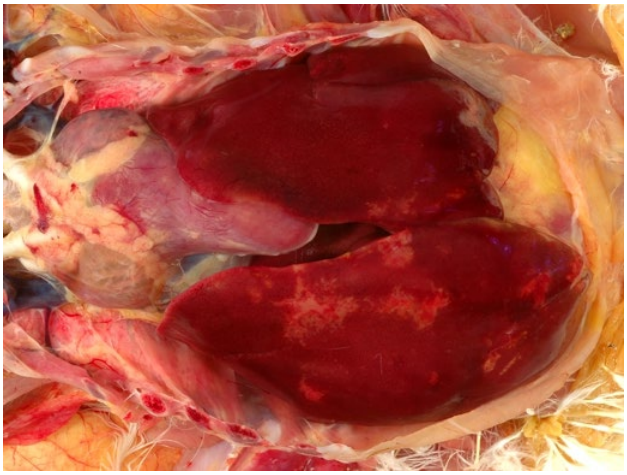


Figure 23.2 Bacterial endocarditis, showing infarcts of liver and myocardium.



Figure 23.3 *Streptococcus zooepidemicus* infection showing perihepatitis and peritonitis. (M.C. Peckham)

In areas of necrosis, intracellular and extracellular cocci can be identified on routine hematoxylin and eosin stains and confirmed as Gram-positive using a tissue Gram stain. Bacteria are also frequently observed within fibrinoheterophilic exudate on the serosal surfaces of the heart and liver. In the heart, valvular lesions consist primarily of laminations of fibrin with large colonies of bacteria mixed with thrombocytes, heterophils, macrophages, and fibroblasts (50). Microscopic lesions secondary to endocarditis include cerebral vasculitis and infarction, leptomeningitis, glomerulonephritis, and thrombosed pulmonary vessels (50).

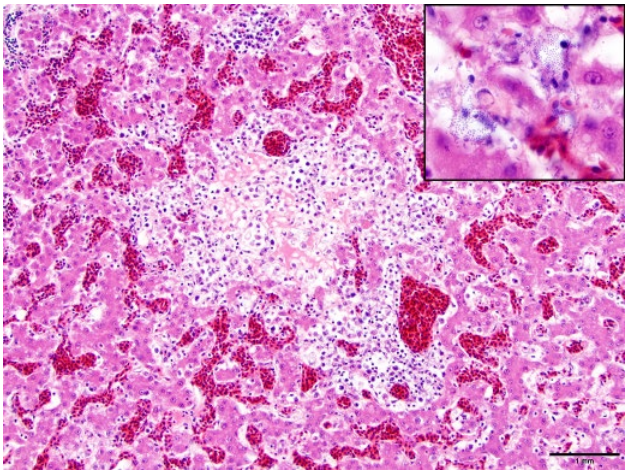


Figure 23.4 Liver with necrosis, fibrin and intralesional *Streptococcus gallolyticus* (insert) in a turkey poult.

Diagnosis

Isolation and Identification of Causative Agent

Clinical signs of acute streptococcal infection are not specific. Bacterial isolation, identification, and susceptibility testing are necessary to provide an accurate diagnosis and guide antimicrobial selection. Demonstration of chains of cocci in blood films or impression smears of affected heart valves can provide a presumptive diagnosis of streptococcal endocarditis.

Streptococci are readily isolated from liver, spleen, blood or pericardial swab inoculated onto blood agar and incubated in 5% CO_2. Identification of streptococci using standard microbiologic methods relies on the ability of streptococci to ferment a variety of sugars in manual or automated systems (6). However, occasionally speciation of streptococci requires genomic analysis (e.g., polymerase chain reaction [PCR] and sequencing of the 16S ribosomal RNA gene) for definitive identification.

Differential Diagnosis

Differential diagnosis includes other bacterial septicemic diseases (e.g., staphylococcosis, colibacillosis, pasteurellosis, and erysipelas).

Treatment

Treatment is only effective in the acute phase of the disease. Streptococci are generally susceptible to β-lactam antibiotics such as penicillin; however, susceptibility to

erythromycin, novobiocin, oxytetracycline, chlortetracycline, and tetracycline can vary. *In vitro*, *S. gallolyticus* from pigeons is susceptible to penicillins, macrolides, lincomycin, tetracyclines, chloramphenicol, and nitrofurans (27). Treatment in the chronic phase of the disease is generally ineffective. Culling should be considered for clinically affected birds.

Prevention and control require reducing stress and preventing immunosuppressive diseases and conditions. Proper cleaning and disinfection can reduce environmental streptococci to minimize external exposure. Use of formaldehyde reduces the total count of *Streptococcus* spp. in hatchers by as much as 85.7% (95). Contact between free-ranging poultry and horses needs to be prevented.

Enterococcus

Introduction

Enterococci play a complicated role in health and disease. They are early colonizers of the avian intestinal tract and become a dominant member of the gut microbiota of adult birds (34). Given their participation in establishing and maintaining the gut flora, enterococci are often regarded as beneficial and are included in probiotics or other supplements, which have a demonstrated beneficial effect on growth and feed efficiency (73). Despite their beneficial role, enterococcal-associated diseases of poultry are becoming increasingly recognized as important causes of morbidity and mortality, particularly in ducks, turkeys, and broiler chickens.

History

The history of *Enterococcus* spp. infections is sparse due to the recent segregation of this genus from the Lancefield group D streptococci. "Fecal strep" infections in poultry were reported as early as 1947 (76) and again in 1956 (2), 1962 (44), and 1971 (50). These reports probably refer to enterococci when they were included in the genus *Streptococcus*, which makes interpretation of earlier reports difficult. In these reports, fecal streptococci were implicated as causing endocarditis, hepatic granulomas, and occasionally acute septicemia.

Since 2002, reports from Europe, the United Kingdom (UK), North America, Africa, and the Middle East have tracked the global emergence of pathogenic strains of *E. cecorum* (4, 5, 7, 15, 25, 32, 53, 55, 65, 78, 89, 91, 98). These emerging pathogenic strains of *E. cecorum* have become established in commercial broiler systems as an important cause of lameness and mortality.

Etiology

The genus *Enterococcus* is composed of Gram-positive cocci that occur in singles, pairs, and short chains. Enterococci are catalase negative and considered lactic acid bacteria because they produce acid when they ferment sugars. Common avian isolates can be separated by their differential ability to ferment mannitol, sorbitol, L-arabinose, sucrose, and raffinose. Like streptococci, they grow best on blood agar incubated under increased CO_2. In contrast to streptococci, most enterococcal species can grow in high sodium chloride concentrations and in the presence of bile (oxgall). Selective media for isolating enterococci based on these characteristics exist; however, an important *Enterococcus* species, *E. cecorum*, does not grow on these media (33, 35, 90).

Enterococci have high levels of naturally occurring and acquired antimicrobial resistance. Enterococci from avian species are likely important in the maintenance and spread of antimicrobial resistance. This high level of antimicrobial resistance complicates treatment in birds and people. *E. faecalis* and *E. faecium* are leading causes of hospital acquired infections in people and *E. cecorum* has occasionally been associated with sepsis in people with serious concurrent diseases (3, 24, 30, 43, 46, 74, 94, 97).

Enterococcus spp. most frequently associated with disease in avian species includes *E. faecalis, E. faecium*, and *E. cecorum*. *E. faecalis* and *E. faecium* have been associated with omphalitis/yolksacculitis, both alone and in combination with *E. coli*, in broiler chicks and turkey poults in the first few weeks of life. *E. cecorum* is the etiologic agent of enterococcal spondylitis ('kinky back'), an emerging disease in poultry, especially broiler chickens and broiler breeders (16). Sporadically occurring valvular endocarditis has been reported to be caused by *E. faecalis* (10, 22, 58, 81, 87), *E. faecium* (73, 82), *E. durans* (1, 18), and *E. hirae* (20, 31, 42, 57). Enterococci also cause infectious encephalomalacia and have been associated with amyloid arthropathy (1, 18, 31, 60, 61).

Pathobiology and Epidemiology

Enterococcus faecalis affects birds of all ages. It produces serious disease in embryos and young chicks that hatch from eggs contaminated with feces (37, 59). Transmission or fecal contamination of hatching eggs with enterococci may result in late embryo mortality and an increased number of chicks or poults unable to "pip" or penetrate through the shell at hatch. Overall bacterial contamination, including *Enterococcus* spp., at the time of hatching can contribute to mortality of chicks early in life (80). The contribution of *E. faecalis* and *E. faecium* to first week mortality in chickens and turkeys is likely underestimated. Enterococci are frequently isolated from birds

succumbing to omphalitis/yolksacculitis and sepsis both in combination with *E. coli*, and less frequently as single agents. *E. faecium* also causes mortality in ducklings (82).

Pathogenic strains of *E. cecorum* cause outbreaks of mortality and lameness in commercial broilers and broiler breeders worldwide. Fatal septicemia also occurs in infected ducks and pigeons (51, 54). Sepsis has also occurred in experimentally infected broilers (16). Route of transmission is likely a combination of vertical and horizontal transmission. However, several researchers have attempted and failed to confirm vertical transmission from breeder flock or hatchery to broiler farm (13, 15, 55, 65, 78, 96). Evidence for vertical transmission includes the ability to isolate identical genotypes of pathogenic *E. cecorum* from outbreaks on geographically separated broiler farms that received chicks from the same hatchery and breeder flocks (15, 78). A biologic vector can be considered, as it is possible to culture pathogenic strains of *E. cecorum* from flies in rooms with experimentally infected birds (unpublished data). Regardless of source, once a farm experiences an outbreak of pathogenic *E. cecorum*, that farm can have repeated outbreaks in subsequent flocks. Isolates from the flocks often have identical genotypes suggesting the bacterium remains on the farm. However, enterococci do not survive well in the environment and pathogenic *E. cecorum* is not readily recovered from litter, feed, water, or air vents following removal of affected birds. Additionally, some farms with several affected flocks in a row will suddenly stop experiencing outbreaks. It is not clear what factors impact the likelihood of repeated outbreaks on a broiler facility.

Horizontal transmission occurs within a flock and is detected by increasing prevalence of gut colonization and sepsis within the first 3 weeks (16). The route of infection is likely fecal–oral; however, possible aerosol exposure cannot be ruled out. In natural infection, intestinal colonization by pathogenic *E. cecorum* occurs during the first week of life (16). In contrast, commensal strains of *E. cecorum* typically do not colonize the intestinal tract before week 3 (16). Clinical signs begin during week 4 and peak during weeks 5–6. Birds infected with pathogenic *E. cecorum* develop the characteristic spinal lesion, which gives the disease its common name 'kinky-back'. For spondylitis lesions to develop in the free thoracic vertebra, lesions of *osteochondrosis dissecans* (OCD) in the articulating spinal cartilages are necessary (16, 34).

Despite recent advances in the understanding of the pathogenesis of *E. cecorum* infection, the epidemiology of how these pathogenic strains spread among flocks remains unclear.

Less common enterococcal species causing infection in birds include *E. durans* and *E. hirae*. *E. durans* and *E. hirae* have been associated with focal brain necrosis and encephalomalacia in young chickens (1, 18, 31). *E. hirae* causes increased mortality in broiler flocks due to septicemia and endocarditis (20, 69). Sepsis and endocarditis has been experimentally reproduced in 4-week-old chickens with both *S. gallinaceus* and *E. hirae* (19). Osteomyelitis occurred in the proximal femur of 3-week-old broilers that also had endocarditis and liver necrosis (57).

Clinical Signs

Like streptococci, enterococcal infections in poultry often have an acute septicemic phase. Birds that survive the acute phase frequently develop chronic infection, particularly of the heart valve and skeletal system.

The acute phase of enterococcal infections (weeks 1–3) is often heralded by increased mortality without clinical signs. Clinical signs of acute infection, when present, are related to septicemia and include depression, lethargy, lassitude, ruffled feathers, diarrhea, and fine head tremors. Mortality in this period typically ranges from 1% to 3%. Broiler chicks and turkey poults can experience omphalitis/yolksacculitis and sepsis in the first week of life caused by *E. faecalis* and *E. faecium*, either alone or in combination with *E. coli*. Infection with pathogenic strains of *E. cecorum* can also result in clinical sepsis around day 14, which can result in mortality of 5%–10%. However, most birds that become systemically infected with pathogenic *E. cecorum* do not show clinical signs of sepsis (16).

In chronic enteroccocal infections, clinical signs are related to the site of infection and include depression, loss of body weight, and lameness. Birds surviving acute *E. faecalis* and *E. faecium* sepsis can develop and eventually succumb to vegetative valvular endocarditis. Chronic infection of the free thoracic vertebra with pathogenic *E. cecorum* results in a characteristic posture of symmetrical paralysis. In contrast with other musculoskeletal diseases, which are asymmetrical and result in a 'splayed-leg' stance, birds with spinal lesions of pathogenic *E. cecorum* sit back on their hocks with both legs and feet extended forward (Figure 23.5). Mortality due to lameness in affected flocks can reach 25% (67).

Pathology

Gross

Gross lesions in acute disease are characterized by splenomegaly, hepatomegaly, fibrinous pericarditis, and fibrinous peritonitis (perihepatitis). Chicks or poults infected at hatching have omphalitis and/or enlarged yolk sacs (81). Hepatomegaly, splenic necrosis, fibrinous pericarditis, perihepatitis, and airsacculitis occur in ducks infected with *E. faecium* (82).

Acute septicemia in chickens can be induced experimentally using aerosols of *E. faecalis* (2). Chickens



Figure 23.5 Five-week-old broiler with characteristic clinical stance of symmetrical paralysis from spine lesion of pathogenic *Enterococcus cecorum*.

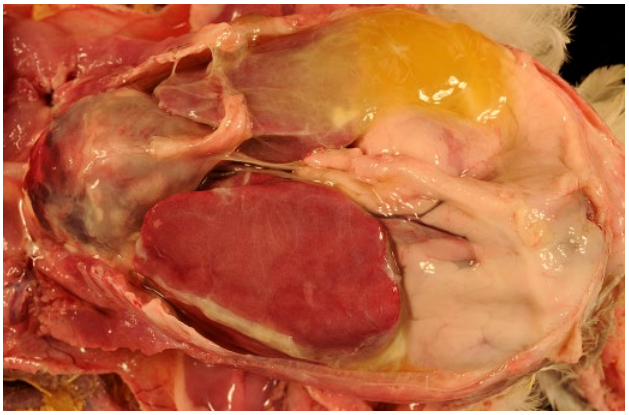


Figure 23.6 Fourteen-day-old broiler with fibrinous pericarditis and perihepatits due to *Enterococcus cecorum*.

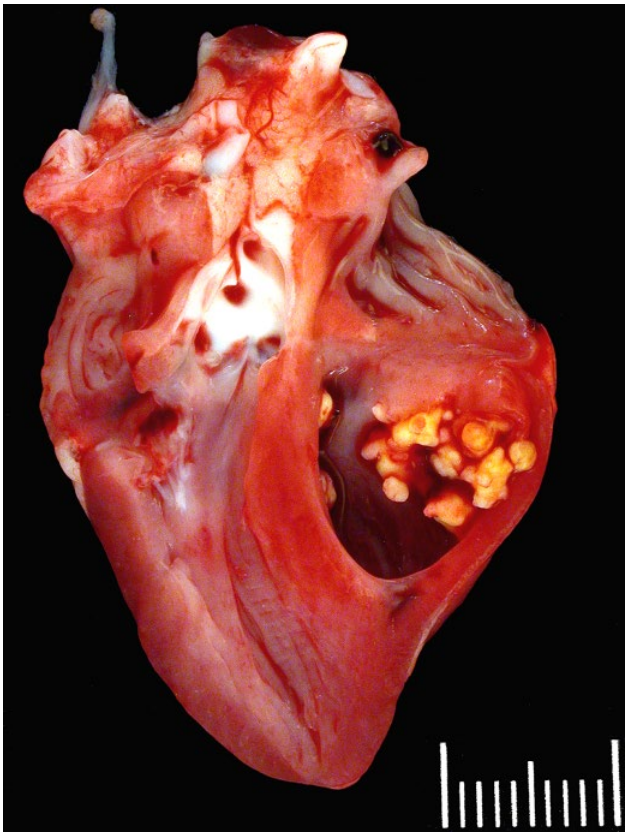


Figure 23.7 Bacterial endocarditis (*Enterococcus faecium*) showing vegetations of mitral valve.

experimentally inoculated intravenously with *E. faecalis* developed leukocytosis 2–3 days postinoculation; highest values occurred in birds that had endocarditis (44). High mortality from acute septicemia and liver granulomas occur after experimental oral inoculation with *E. faecalis* (40). *E. faecalis* has been incriminated as the cause of intestinal epithelium integrity loss, which allows bacteria such as *Bacteroides* spp., *Catenabacterium* spp., *Eubacterium* spp., and *Streptococcus* spp. to produce liver granulomas in turkeys (63).

Acute infection with pathogenic *E. cecorum* at a day of age results in clinical signs of sepsis and mortality 2–3 weeks postexposure with a peak around 14 days. Severity of sepsis varies with the strain of pathogenic *E. cecorum*. In ducks, septicemia at 2 weeks is the prominent feature of *E. cecorum* infection. In broilers, most outbreaks have a mild septic phase with 40%–80% of birds having no clinical signs.

Lesions of chronic enterococcal infections include fibrinous arthritis and/or tenosynovitis, osteomyelitis, fibrinous pericarditis and perihepatitis (Figure 23.6), necrotic myocarditis, and valvular endocarditis. Endocarditis can occur when a septicemic enterococcal infection progresses to a subacute or chronic stage. *E. faecalis* isolates from intestines of apparently normal birds can produce endocarditis (44, 50, 76, 77). Vegetative valvular lesions are usually yellow, white, or tan, are small, and have raised rough areas on the valvular surface (Figure 23.7). Valve lesions are found most consistently on the mitral valve, and less frequently on the aortic or right atrioventricular valves. Additional gross lesions associated with valvular endocarditis include: enlarged, pale, flaccid heart; pale to hemorrhagic areas in the myocardium, especially at the base of the valve, below the affected valve, or at the apex of the heart (50); infarcts in the liver, spleen, or heart; and, less commonly, infarcts in the lungs, kidneys, and brain. Infarcts can be light colored or hemorrhagic with sharp margins. In the liver, infarcts usually are located near the ventral and posterior margins and are well demarcated, extending beneath the capsule into the parenchyma (50). Lesions of longer duration tend to have a sharp, narrow, lighter colored band just inside the infarct margin (50). *E. faecalis* has also been reported to be a bacterial component of amyloid arthropathy in chickens (60, 61).

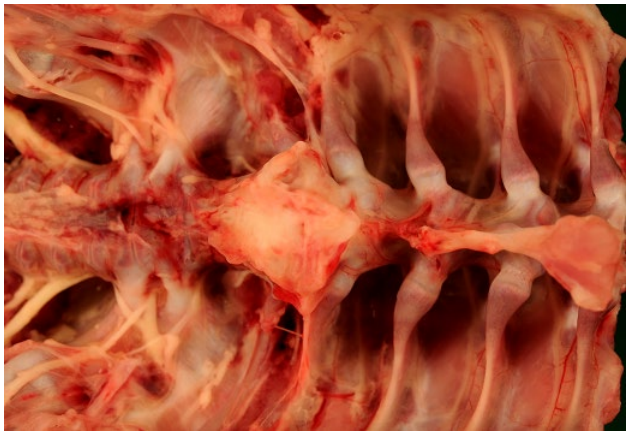


Figure 23.8 Spinal lesion of pathogenic *Enterococcus cecorum* in a 28-day-old broiler.

Chronic pathogenic *E. cecorum* infection causes chondritis and osteomyelitis of the hips and free thoracic vertebra (16, 32, 98). The vertebral lesion is always located in the cartilage articulations of the free thoracic vertebra; rarely, more than 1 vertebra adjacent to the free thoracic vertebra is affected (Figure 23.8). Spinal cord compression by the expanding inflammatory mass at the free thoracic vertebra is responsible for the clinical signs of paresis or paralysis.

Microscopic

Microscopic lesions of acute enterococcal infections, regardless of the underlying agent, are similar to those caused by streptococci. Sepsis results in focal areas of hepatic and splenic necrosis accompanied by variable amounts of fibrinoheterophilic exudate. Bacterial colonies are often located within splenic or hepatic lesions, fibrinous epicarditis, and myocarditis. Later, splenomegaly is due to congestion, hyperplasia of cells in the mononuclear phagocytic system, and lymphoid hyperplasia (45).

In chronic infections, valvular endocarditis lesions consist primarily of fibrin with bacteria, heterophils, macrophages, and fibroblasts (44, 50). Other microscopic lesions related to endocarditis include cerebral vasculitis and infarcts, leptomeningitis, glomerulonephritis, and thrombosed pulmonary vessels. Focal granulomas can be found in virtually any tissue because of septic emboli.

Infection of the free thoracic vertebra with pathogenic *E. cecorum* requires microscopic clefts of degenerative cartilage produced by concurrent OCD (16). OCD is a failure of endochondral ossification, which results in fragile acellular areas within articular cartilage (23, 62, 68, 72, 99). In chickens, OCD is common and affects primarily the hips and free thoracic vertebra (16, 23, 39). Affected cartilage at these weight-bearing joints can fragment and form clefts, which fill with hemorrhage and thrombocytes. Early in infection with pathogenic

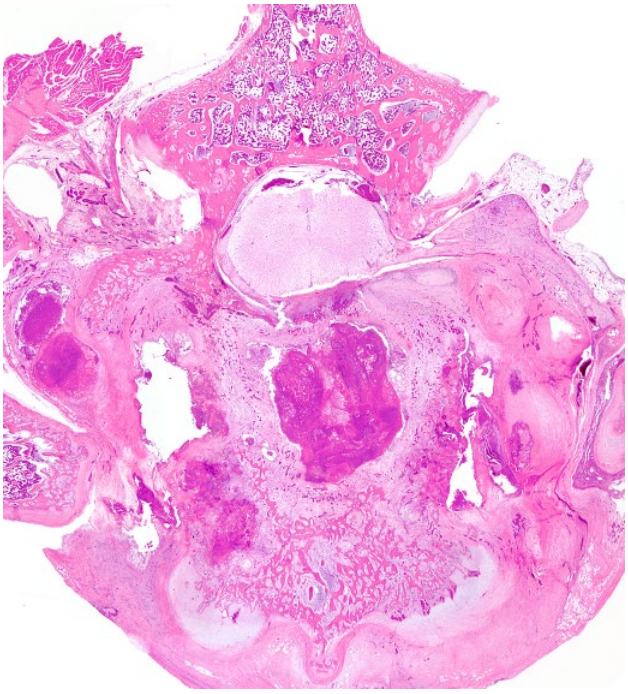


Figure 23.9 Chronic spinal lesion due to infection with pathogenic *Enterococcus cecorum* in a 35-day-old broiler (Poulet Rouge).

E. cecorum, large confluent bacterial colonies fill these clefts (16). In more advanced lesions, heterophils and macrophages infiltrate and destroy affected cartilage (Figure 23.9) (16, 89). However, phagocytes are unable to control the infection and lesions progress to a central area of fibrin, hemorrhage, necrosis, degenerative heterophils, multinucleated giant cells, cellular debris, and bacterial colonies surrounded by maturing fibrosis (16, 89). Woven bone adjacent to these masses typically has evidence of resorption, remodeling, and new bone formation. Proliferation of cartilage around the lesion resembles formation of a callus following a fracture. Changes in the white matter tracts of the spinal cord compressed by the vertebral lesion are consistent with Wallerian degeneration. Neuronal necrosis and hemorrhage in the spinal cord occur occasionally.

Diagnosis

Bacterial culture is required for diagnosis, as clinical signs and gross lesions of acute enterococcal infections overlap with other bacterial diseases of chickens. Preferred tissues for culture include spleen, blood, yolk, and swabs from the center of osseous lesions. When culturing spinal lesions for pathogenic *E. cecorum*, spraying the mass with 70% ethanol prior to incision with a sterile blade is recommended to decrease surface contamination.

Isolation and identification of *Enterococcus* spp. (without fecal contamination) from typical lesions in poultry with appropriate clinical signs will confirm enterococcosis.

Enterococci are readily isolated using blood agar incubated under 5% CO_2 (6, 38). Gram-positive selective media (e.g., Columbia blood agar with colistin and nalidixic acid), can be used to remove contaminants or separate enterococci from *E. coli* in polymicrobial infections. Enterococcal species exhibit variable α-hemolysis or no hemolysis (γ-hemolysis). Agar slants or plate media containing bile, esculin, and high sodium chloride concentrations can be used to differentiate streptococci from enterococci. Enteroccocal selective media (Enterococcosel broth and agar) exist; however, they select against pathogenic *E. cecorum*, diminishing their value for use in avian species (33, 90).

Further speciation can be accomplished using differential fermentation of mannitol, sorbitol, arabinose, sucrose, or raffinose; however, carbohydrate metabolism is variable among enterococcal strains even within a given species (15, 17, 35, 36). This feature of enterococci makes commercial identification systems for bacterial identification sometimes unreliable. Other methods may be required to identify enterococcal species including mass-spectrophotometry, multiplex PCR, or PCR and sequencing the 16S RNA gene (47, 52, 88, 92).

Differential diagnosis includes other bacterial septicemic diseases (e.g., staphylococcosis, colibacillosis, pasteurellosis, and erysipelas).

Intervention Strategies

Prevention and control require reducing stress and preventing immunosuppressive diseases and conditions. Proper cleaning and disinfection can reduce environmental enterococcal resident flora to minimize external exposure.

Treatment

Treatment can include the use of antibiotics such as penicillin, erythromycin, novobiocin, oxytetracycline, chlortetracycline, or tetracycline in acute and subacute infections. Clinically affected birds respond well early in the course of the disease. As the disease progresses within a flock, treatment efficacy decreases.

Novobiocin has been found to be efficacious in ducks with *E. faecium* infection (81). Bacitracin in feed decreases the incidence of some strains of enterococci in young chickens but is ineffective in *E. cecorum* infection (unpublished data) (10). Certain *Enterococcus* strains can develop resistance after exposure to antibiotics such as tylosin, but curtailing treatment with such antibiotics may not shift the overall number of resistant organisms (15). Pathogenic *E. cecorum* isolates are sensitive to penicillin, have high level resistance to macrolide and aminoglycoside antibiotics and are variably susceptible to tylosin and tetracyclines (15, 48, 90).

Erysipelas

Helena Eriksson

Summary

Agent, Infection, and Disease. Erysipelas, the disease caused by the bacterium *Erysipelothrix rhusiopathiae* affects most poultry species. Affected flocks may suffer from high mortality and egg production losses. Gross lesions in birds that have died during an outbreak display signs of septicaemia. *E. rhusiopathiae* is also a zoonotic agent.

Diagnosis. Necropsy followed by demonstration of *E. rhusiopathiae* in tissue samples either by culture or polymerase chain reaction is necessary for diagnosis.

Intervention. Prevention of erysipelas should be based on biosecurity measures. When an outbreak occurs, antibiotic treatment, vaccination alone or in combination, or euthanasia should be considered. Vaccination is recommended for subsequent flocks on farms with previous outbreaks or in areas with a history of erysipelas.

Introduction

Definition and Synonyms

Erysipelas is a septicaemic disease caused by the Gram-positive bacterium *Erysipelothrix rhusiopathiae*, which may affect a wide variety of avian species. The disease has a peracute to acute course in individual birds and affected flocks may suffer from high mortality and egg production losses.

Economic Significance

Economically significant outbreaks may occur worldwide and in most poultry species. Losses result from mortality and a decrease in egg production may also be seen. Additional costs during an outbreak are associated with treatment, pre-emptive culling of affected flocks, cleaning and disinfection, and vaccination of subsequent flocks. Costs for routine preventive vaccination may be relevant in some areas and/or populations.

Public Health Significance

Erysipelothrix rhusiopathiae infection in humans is considered an occupational disease. Here, the infection exists in different forms, most commonly as an acute phalangeal cellulitis (erysipeloid) of the upper limb. A diffuse cutaneous form, arthritis, endocarditis, and septicaemia, sometimes with a fatal outcome, may also occur (53). Suspected cases in personnel handling *E. rhusiopathiae*-infected poultry have occurred. The infection has occasionally been reported from patients lacking occupational exposure.

History

In 1876, Koch was the first to isolate the bacterium that a few years later was described as the causative agent of erysipelas in pigs (53). The first report of erysipelas in poultry was in 1904, in a turkey, followed by reports of the disease in other avian species (2).

Etiology

Classification

Name and Synonyms

Erysipelothrix rhusiopathiae belongs to the family *Erysipelotrichaceae* of the order *Erysipelotrichales*, class *Erysipelotrichia* within the phylum *Firmicutes*. The genus *Erysipelothrix* currently consists of 4 recognized species, *E. rhusiopathiae*, *E. tonsillarum*, *E. inopinata*, and *E. larvae*. Based on DNA-DNA hybridization studies, at least 2 additional species have been proposed (55, 56). Only *E. rhusiopathiae* causes erysipelas in avian species.

Morphology and Staining

Erysipelothrix rhusiopathiae is a Gram-positive straight or slightly curved, slender rod with rounded ends measuring 0.2–0.4 by 0.8–2.5 µm. The bacteria may arrange singly, in pairs at an angle that gives a V-form, as short chains or as long filaments that may reach more than 60 µm in length. The organism is nonmotile and not acid-fast. It does not form spores. Cells may sometimes appear Gram-negative as they readily decolorize with increasing age (52).

Growth Requirements

Erysipelothrix rhusiopathiae is relatively easy to isolate on routine media. The exact growth requirements have not been determined, but riboflavin, several amino acids and oleic acid have been reported as essential. Growth is enhanced by 5%–10% serum, 0.2%–0.5% glucose or tryptophan (52).

The bacterium is facultatively anaerobic. Reduced oxygen or increased carbon dioxide (5%–10%) enhances growth but is not necessary. The optimum temperature for growth *in vitro* is 30 °C–37 °C, but growth occurs between 5 °C and 42 °C. *Erysipelothrix rhusiopathiae* grows at a pH of 6.7–9.2 with an optimal pH of 7.2–7.6 (52).

Colony Morphology

Colonies of *E. rhusiopathiae* are small (0.3–1.5 mm) and on blood agar plates a narrow zone of green incomplete (alpha) haemolysis often appear. There are two distinctly different colony types, the smooth form (S-form) and the rough form (R-form), and there is also an intermediate colony type. Colonies of the S-form are pinpoint-sized and transparent with a smooth shiny surface and entire edges. The R-form colonies are larger and have a matte opaque surface and irregular edges. In gelatin stab cultures incubated at 22 °C, *E. rhusiopathiae* is characterized by faint growth after 24 hours, which later extends to resemble a "pipe cleaner" (lateral radiating projections along the stab line) (52).

Biochemical Properties

Erysipelothrix rhusiopathiae is catalase- and oxidase-negative. The carbohydrates dextrin, fructose, galactose, glucose, lactose, maltose, and *N*-acetylglucosamine are fermented without gas production. When grown in litmus milk, a weak acid or no change is seen. It does not produce indole or acetoin (Voges–Proskauer test) and does not reduce nitrates. Urea and esculin are not hydrolysed and gelatin is not liquefied (52). Most strains produce hydrogen sulfide (H_2S).

Erysipelothrix rhusiopathiae is distinguished from *E. tonsillarum* based on the ability of the latter to ferment sucrose (55). However, this has been questioned as a diagnostic criterion (12, 34).

Susceptibility to Chemical and Physical Agents

Most routine disinfectants are effective against *E. rhusiopathiae*. Chlorine, sodium hydroxide, and formalin also inactivate the organism.

The bacterium is resistant to various food-preservation methods such as salting, pickling, and smoking and may be viable in frozen or chilled meat for a long time while moist heat at 55 °C for 15 minutes is lethal (53).

Antigenic Structure and Toxins

The fatty acids in the cell wall of *E. rhusiopathiae* are primarily straight chain mono-unsaturated and saturated

fatty acids. A group B peptidoglycan based on lysine is present, but there are no mycolic acids (52). Both heat-labile species-specific and heat-stable type-specific antigens exist. The bacterium does not produce any toxins.

Strain Classification

Antigenicity

Erysipelothrix isolates have long been classified into serotypes based on the presence of heat-stable cell wall antigens. Today, 23 serotypes are known within the genus (52). In addition, within serotypes 1 and 2, subtypes designated by a lowercase letter after the number have been described.

Serotyping is becoming less used because it is labor intensive, time consuming, and requires use of laboratory animals (i.e., antisera that are produced in rabbits). Moreover, based on results using molecular techniques, serotyping is now considered an unreliable tool for epidemiological studies (10, 12, 15).

For more information on *Erysipelothrix* spp. serotypes and serotyping please refer to the 13th edition of *Diseases of Poultry*.

Immunogenicity or Protective Characteristics

Only a few protective antigens have been identified in *E. rhusiopathiae*. The surface protective antigen (Spa) protein is the most studied. Based on the amino acid sequences, 3 types of the Spa protein have been identified: SpaA, SpaB, and SpaC (28, 61). To and Nagai (61) found that the Spa proteins had a high amino acid sequence similarity within Spa-types and a low similarity between types. The N-terminal half of the protein, which is involved in immunoprotection, was the most diverse part. Furthermore, it has been reported that an isolate may possess more than one Spa-type and that the Spa-type may not be serotype-specific (21). So far, only SpaA has been found among isolates from poultry (22).

Shi et al. (46) identified 3 surface-exposed choline-binding proteins: CbpA, CbpB, and CbpC. Of these, recombinant CbpB was shown to induce protection against clinical disease in mice and pigs. Also, one of the two rhusiopathiae surface proteins, RspA induced partial protection in mice (51).

The role of the different surface proteins in the development of protective immunity in poultry has not been clarified.

Genetic or Molecular

Several molecular and genetic techniques have been applied to *Erysipelothrix* spp. strains. DNA-DNA hybridization was used for interspecific classification of a collection of isolates of all known serotypes of *Erysipelothrix* spp. and 3 putative unknown species were proposed (56).

Several molecular methods and types of variable markers have been proposed for epidemiologic studies: randomly amplified polymorphic DNA (RAPD) (34), automated ribotyping (35), restriction fragment length polymorphism (1), multilocus enzyme electrophoresis (10), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (3), pulsed-field gel electrophoresis (PFGE) (36) and multilocus sequence typing (MLST) (22).

In 2001, Okatani et al. (36) showed that PFGE performed with the enzyme *Sma*I was more discriminatory than RAPD and ribotyping.

More recently, MLST was applied to a large number of isolates from poultry and other animals together with PFGE and Spa-typing. The authors concluded that, although MLST distinguished the 165 investigated isolates into 72 sequence types, this method was better for portraying long-term epidemiology and evolutionary aspects than for investigating the epidemiology of ongoing outbreaks. For this latter purpose PFGE was more suitable (22).

Forde et al. (15) used whole genome sequencing when investigating a large number of isolates from a wide range of animal species including poultry. Isolates from various host species and different continents were distributed between 3 distinct clades and a difference in Spa-type between clades was detected. Whole genome sequencing of isolates provides opportunities for future phylogenetic, ecological, and epidemiological studies, including interlaboratory comparisons.

Pathogenicity

Only *E. rhusiopathiae* has been shown to be pathogenic for chickens (58). Different strains appear to vary in virulence but studies in mice have not detected any correlation between serotype and pathogenicity while different *spaA* gene variants have been reported to differ in pathogenicity in pigs (38, 62).

Virulence Factors

The factors responsible for *E. rhusiopathiae* virulence in poultry and other animals are not clearly defined. In 2000, a review of the pathogenicity and virulence factors of *E. rhusiopathiae* was published (48). When the first whole genome sequence of the bacterium was reported in 2011, more insights into putative virulence factors were gained (33). Recently, Janßen et al. (22) found that most of the investigated isolates from poultry and other hosts contained all 16 putative virulence genes. In addition, two of the genes were detected in *E. tonsillarum* while none of the genes were present in the *E. inopinata* type strain.

Capsule

A polysaccharide capsule with a reported virulence-associated function is present in *E. rhusiopathiae* (45, 48). Using noncapsular mutants, it was shown that

the capsule has a function related to partial resistance to phagocytosis by polymorphonuclear leucocytes. Moreover, the capsule is involved in intracellular survival within murine macrophages, mediated by a reduced production of reactive oxidative metabolites that are essential for the bactericidal activity of phagocytes (48).

Enzymes

Erysipelothrix rhusiopathiae produces the enzymes hyaluronidase and neuraminidase. Hyaluronidase appears to be surface-associated (33). For other pathogens, this enzyme facilitates the spread into host tissues, but for *E. rhusiopathiae* studies in nonavian species have not confirmed its role as a virulence factor (49). Neuraminidase mediates adhesion and tissue spread based on the ability of the enzyme to cleave sialic acids on host cell surfaces. Neuraminidase activity has been demonstrated in *E. rhusiopathiae* but not in *E. tonsillarum* (63).

Phages

Through whole genome sequencing of *E. rhusiopathiae* strains, Forde et al. (15) detected phage sequences in 55% of the investigated isolates, including those of poultry origin. However, no clear association between phage sequences and pathogenicity was observed.

Plasmids

Plasmids of unknown function have been detected in up to a third of investigated *E. rhusiopathiae* isolates from pigs. More recently, plasmid sequences were detected in only seven out of 86 investigated isolates, one of which was of poultry origin (15).

Other Factors

Additional putative virulence factors such as the Spa antigens (described previously) and RspA and RspB have been identified through whole genome sequencing (26, 33). The Rsp proteins bind to fibronectin and collagen I and II, and may have a role in biofilm formation and possibly also as protective factors against clinical disease (51). Recently, the first moonlighting protein in *E. rhusiopathiae* was detected as glyceraldehyde 3-phosphate dehydrogenase. It was shown to aid in the adhesion of *E. rhusiopathiae* to pig vascular endothelial cells and is a receptor in the recruitment of fibronectin and plasminogen (70).

Erysipelothrix rhusiopathiae also possesses several genes for antioxidant proteins that are of major importance for intracellular survival. Additional enzymes that may help the organism to survive inside phagocytic cells are phospholipases. Other putative virulence factors include adhesins, hemolysins, and other extracellular proteins and enzymes (26, 33).

Pathobiology and Epizootiology

Incidence and Distribution

Erysipelas occurs worldwide in poultry. Historically, erysipelas was considered to be of importance primarily in turkeys. However, following the ban of conventional battery cages for laying hens in Europe to improve bird welfare, there has been a significant increase in erysipelas outbreaks in several countries. These outbreaks are presumed to be associated with housing of hens indoors on litter and in particular in free-range and organic housing where hens are provided with outdoor access. In the United States the prevalence in laying hens is also increasing, with several cases documented in the south-eastern United States in flocks classified as cage-free sometimes with access to the outdoors. In the midwest United States, the disease is of moderate prevalence, primarily in breeding turkeys, whereas in Australia, and the south Pacific, although not common, erysipelas has occurred in turkeys and free-range laying hens.

Natural and Experimental Hosts

Erysipelothrix rhusiopathiae has been isolated from at least 50 mammalian species, including domestic as well as free-living and captive wild animals. Apart from causing infections with clinical symptoms, it has been isolated from clinically healthy mammals such as pigs and cattle, from the surface slime of fish, and from cephalopods and crustaceans.

The bacterium has been isolated from a wide range of avian species and erysipelas has been reported in most, if not all poultry species, such as turkeys, chickens, ducks, emus, geese, guinea fowl, partridges, pheasants and quail. Pigeons are also susceptible. For more information on affected species please refer to the 12th edition of *Diseases of Poultry*.

Chickens and turkeys are the two most studied avian species in challenge trials. In chickens, the bacteria were administered intramuscularly. However, in some studies difficulties in reproducing clinical signs and mortality have been encountered (8, 17, 47, 58). When the same dose of *E. rhusiopathiae* was administered both intramuscularly and orally to chickens of 3 different ages, no obvious difference in mortality rates and clinical signs between the two administration routes were noted in the two youngest groups. In the eldest groups of chickens, mortality rates differed significantly (29). In turkeys, septicaemia and mortality follow when birds are challenged subcutaneously, intramuscularly, intravenously, per os or through scarcification (4, 7). Intramuscular and intravenous administration of *E. rhusiopathiae* are also effective routes in turkey.

Age of Host Commonly Affected

Naturally occurring outbreaks of erysipelas in turkeys are most commonly diagnosed in older birds, but cases may occur at any age. One report describes erysipelas in 2-to-4 day-old turkey poults, following toe trimming (20).

Outbreaks in chickens very rarely occur in young birds such as broilers and pullets (23, 31). An age-related sensitivity in chickens has been suggested, as the mortality in hens inoculated intramuscularly increased with bird age (29). However, in the groups where hens were orally inoculated the age-related sensitivity was not as obvious. Experimentally, it has been possible to induce clinical signs and mortality in young chickens through intramuscular inoculation of *E. rhusiopathiae*.

Transmission, Carriers, and Vectors

Erysipelothrix rhusiopathiae was long believed to survive interminably, and even to multiply in soil. However, in experimental studies, a maximum survival time of 73 days in soil has been recorded (54, 67). Due to the diversity of hosts, many species might serve as reservoirs and potential sources of the infection. The distribution of *E. rhusiopathiae* in the environment may speculatively be caused by a combination of contamination by the wide range of host animals and the survival ability. The organism may survive for many months in animal carcasses and liquid pig manure. The survival time of *E. rhusiopathiae* in different matrices under various conditions have been reviewed and compiled by Mitscherlich and Marth (32).

Homogeneous PFGE-banding patterns observed during clinical outbreaks of erysipelas in laying hens would suggest that outbreaks are of a clonal nature (11). Spread of the infection within a flock is not fully understood but it has been suggested that *E. rhusiopathiae* may gain entry through broken skin and mucous membranes and that cannibalism, fighting, and feather pecking may favour transmission. Artificial insemination is a proven source of spread in turkey hens. Carcasses of septicaemic birds may serve as a source of infection for other birds in the flock if left in the barn.

Isolation of *E. rhusiopathiae* from jejunal contents and manure indicates a faecal–oral transmission route in housing systems where birds are exposed to faeces (11). The alleged increased risk for laying hen flocks in litter-based systems compared with flocks in cages may support this, as caged birds are less exposed to faeces than birds kept on litter (13). Vertical transmission of *E. rhusiopathiae* has not been demonstrated (30).

The possibility of a carrier state in avian species has not been fully investigated. However, *E. rhusiopathiae* isolates were obtained from the pharynx of healthy chickens, ducks, and geese, suggesting a potential carrier state (68).

The role of vectors in the transmission of *E. rhusiopathiae* have not been studied in detail but biting flies and mosquitoes were shown to transmit *E. rhusiopathiae* to pigeons (64). As the bacterium has been isolated both from the integument and the interior of the poultry red mite, *Dermanyssus gallinae*, this parasite may act as a vector and reservoir (9).

Incubation Period

The incubation period for outbreaks in flocks in the field cannot be ascertained. In experimental infection studies in chickens and turkeys, clinical signs and mortality occurred between 1 and 7 days postinfection (4, 29, 47).

Clinical Signs

Outbreaks of erysipelas in poultry flocks are characterized by sudden onset of mortality, often without accompanying clinical signs. Nonspecific signs include depression, ruffled feathers, diarrhoea and pale combs, and may be seen in some or several birds in the flock some hours prior to death. Additional signs may include dropped tails and wings. Death usually occurs 6–24 hours after onset of clinical signs. A drop in egg production may occur in laying birds. Swollen joints in quails and geese, conjunctival oedema in laying hens, and skin lesions, such as rashes and a swollen snood in turkeys, may also occur (41, 44).

Morbidity and Mortality

In the field, morbidity and mortality rates of *E. rhusiopathiae* in infected poultry flocks are usually similar, as most infected birds die. During an outbreak, mortality may increase rapidly, leading to high numbers of dead birds in a period of a few days. Mortality may start in one group of birds in a barn with subsequent spread to adjacent flocks, with the rate and speed depending on barriers and biosecurity routines between groups. The mortality rate may reach as high as 50%–60%. However, outbreaks with much lower mortality, sometimes even less than 5% do occur (6).

Pathology

Gross

Birds culled or found dead during the acute stage are often in good body condition and, in case of adult females, in full lay. Gross lesions are suggestive of generalized septicemia, with marked organ congestion, skeletal muscle dehydration and dark discoloration, petechiae in pericardial and abdominal fat, and occasional hemorrhages in heart muscle, skeletal muscle, and viscera in a serosal and/or mucosal position. The liver is friable and there is notable hepatosplenomegaly. White to yellow round nodules or irregular, well-demarcated pale multiple foci of

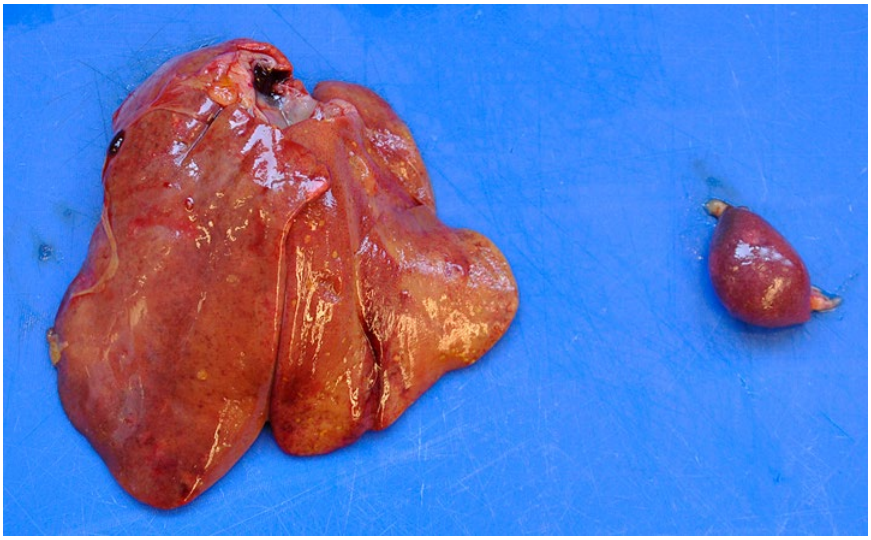


Figure 23.10 Liver and spleen from a laying hen with erysipelas. Marked hepato- and splenomegaly with multiple pale subcapsular foci of varying size (necroses) in both organs. Multiple small parenchymatous hemorrhages in liver. (D.S. Jansson, SVA)

suspected necrosis of varying size may be present in subcapsular position in liver and/or spleen (Figure 23.10). Fibrinopurulent exudate is usually absent unless the bird is coinfected with other bacteria such as *Escherichia coli*, but serosal egg yolk from ruptured follicles is sometimes observed in laying birds. The small intestinal content is often catarrhal or sanguinomucinous. Dark, thickened and crusty skin lesions are commonly observed in the turkey, especially on the head, and the snood and dewlap in toms often display severe cyanosis and turgidity. Some birds in affected flocks become chronically infected, gradually lose body condition and stop laying. Vegetative atrioventricular endocarditis and circulatory failure are common gross findings in such cases. Among the rarer findings associated with erysipelas in poultry, synovitis and arthritis has occasionally been reported in turkeys, geese, and ducks (5, 20, 43).

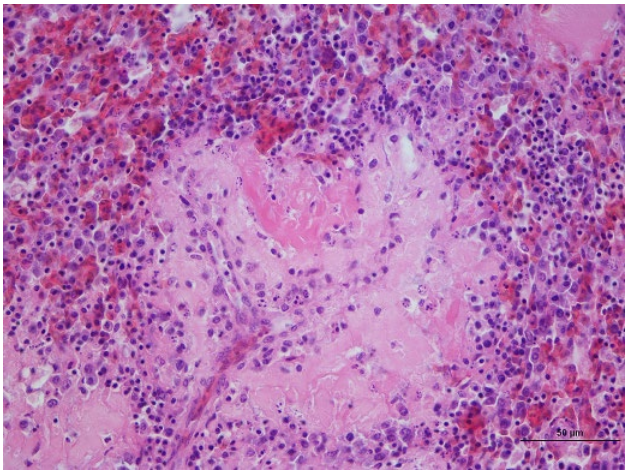


Figure 23.11 Fibrinoid necroses in spleen from a laying hen with erysipelas. H&E. Bar 50 µm. (D.S. Jansson, SVA)

Microscopic

The histopathology in acute erysipelas in poultry reflects the gross findings and are typically characterized by widespread vascular changes, fibrinoid necrosis, and a minimal inflammatory response. There is oedema and hemorrhage in the organs, especially in the lung and myocardium, generalized congestion, and fibrin thrombi are frequently observed in liver sinusoids, renal glomerular capillaries, and in the lung. In addition, rounding up of vascular endothelial cells, hyperplasia of sinusoidal lining cells and hyalinization of vascular walls have been described. Aggregations of bacteria may be found intravascularly, often trapped within fibrinous clots, and attached to or internalized in endothelial cells in vessels and on heart valves (42, 65). Engulfed bacterial cells are commonly observed in mononuclear phagocytes, including reticuloendothelial cells in the spleen and Kupffer cells in the liver.

In the liver, diffuse hepatocellular dissociation, cloudy swelling, and vacuolization are commonly observed, and there is often focal to widespread multiple foci of fibrinoid necrosis. Similar lesions are found in the spleen. Splenic necroses are primarily located in the white pulp around ellipsoidal capillaries (Figure 23.11). Degenerative changes may be seen in the proximal renal tubular epithelium, but necroses are rare. The cellular inflammatory component of the acute stage is usually very mild. Heterophil and mononuclear leukocytic infiltration may be present in association with parenchymatous necroses in liver and spleen, while reticuloendothelial cell proliferation is a more consistent feature. Lymphocyte depletion may be observed in the spleen, bursa of Fabricius, and thymus. Endocarditis involving the heart valves results in vegetative lesions of varying severity with aggregations of bacteria on the valves, in the connective

tissue of the valves, and in the inflammatory exudate. The latter consists of masses of fibrin and heterophils. Areas of necrosis may be seen.

Pathogenesis of the Infectious Process

Despite being a disease known for many years, the pathogenic mechanisms involved in *E. rhusiopathiae* infections have not been fully determined.

In vitro studies performed in nonavian species have indicated that adhesion to host cells may be an important feature in the initiation of the infection. This might be a result of various putative virulence factors with components that enable the bacteria to interact with the host extracellular matrix which subsequently may lead to adhesion and invasion. Once having infected its host, the bacterial capsule may assist in resistance to phagocytosis and intracellular survival in macrophages (48).

Shibatani et al. (47) showed that dysregulation of the coagulative system (disseminated intravascular coagulation) is induced in challenged chicks. Blood chemistry values indicating a hypofunction of the liver and kidney was also demonstrated.

Immunity

Active

In mammals, both innate (e.g., phagocytic cells) and specific (e.g., antibodies) immune responses are involved in controlling *E. rhusiopathiae* infections. However, immune responses during infections in poultry are hitherto poorly characterized. Upon experimental infection of young chickens, prompt blood heterophil and monocyte responses were noted, while blood lymphocyte numbers increased on days 5–11 postinfection (14). Hence a rapid activation of innate phagocytic cells may serve as a factor to control the infection. Seroconversion to *E. rhusiopathiae* after experimental infections of chickens has been observed from approximately 1 week postinfection (8, 17).

Vaccines (bacterins) have been used for a long time to prevent erysipelas outbreaks in poultry, but details on host vaccine responses and the development of protective immunity are still lacking. *Erysipelothrix rhusiopathiae*-specific IgY responses have, however, been recorded in chickens vaccinated with a commercial pig erysipelas vaccine (24). In nonavian species, it has been proposed that production of antibodies following vaccination aids in the elimination of *E. rhusiopathiae* through enhanced phagocytosis and prevention of intracellular replication (48).

Passive

The presence and potential role of maternally derived antibodies in protection of young poultry against erysipelas have not been studied.

Diagnosis

Isolation and Identification of Causative Agent

Gross lesions in birds that have died during an outbreak of erysipelas display signs of septicemia, and demonstration of *E. rhusiopathiae* in tissue samples is necessary for diagnosis. An indication may be provided by examination of smears from liver, spleen, heart blood, or bone marrow. Presence of Gram-positive, beaded, slender, and pleomorphic rods suggest erysipelas.

Samples from spleen and liver are usually sufficient for bacteriological investigation of birds in the septicemic phase. In the eventual case of decomposed specimens, *E. rhusiopathiae* may still be isolated from bone marrow.

For culture of organ samples from birds in the septicemic phase, direct culture on nonselective agar plates (e.g., horse blood agar) may be sufficient if samples are obtained with aseptic techniques. However, due to the risk of contaminated samples, initial incubation of the sample in a selective or inhibitory broth at +37 °C for 24–48 hours followed by culture on nonselective agar plates at +37 °C for 24–48 hours in ordinary atmosphere is recommended. For culture of samples containing a very diverse flora, for instance environmental samples, intestinal samples, or heavily contaminated organ samples, the broth should preferably be streaked on a selective or inhibitory agar plate to enable detection of *E. rhusiopathiae*.

Several media benefit from the ability of *Erysipelothrix* spp. to grow in the presence of sodium azide and crystal violet at concentrations that inhibit the growth of most other organisms. The combination of these substances was described by Packer (39). A liquid medium containing the combination of the antibiotics kanamycin, neomycin, and vancomycin was described by Wood as the '*Erysipelothrix* selective broth' (ESB) (66). Modified versions of ESB, sometimes excluding the vancomycin component, have been used with good results both as liquid media and selective plating medium. Other investigators have described other selective or inhibitory media, mainly based on the observations by Packer and Wood.

Conventional identification of *E. rhusiopathiae* is based on cultural morphology, Gram-staining, and biochemical tests. In recent years, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been introduced for bacterial identification. This method can identify *E. rhusiopathiae*, but the ability to differentiate *E. rhusiopathiae* from *E. tonsillarum* using commercially available databases need to be investigated (11).

Polymerase Chain Reaction

In addition to traditional culture, several polymerase chain reaction (PCR) methods have been established for

detection of *E. rhusiopathiae*. In 1994, Makino et al. (27) described a genus-specific conventional gel-based PCR system using a primer pair amplifying a 407-bp DNA segment targeting 16S rRNA. Using a primer pair for amplification of a 937-bp DNA fragment presumed to be associated with virulence of *E. rhusiopathiae* (capsule), a technique combining cultivation in an enrichment broth followed by conventional PCR was designed by Shimoji et al. (50). Also, a species-specific gel-based PCR method with 4 primer sets for *E. rhusiopathiae*, *E. tonsillarum* and strains representing serotypes 13 and 18 was developed and tested for diagnosis directly on tissue specimens, without prior cultivation (59). With the aim to distinguish *E. rhusiopathiae* from *E. tonsillarum*, Yamazaki (69) combined two primer pairs, one genus-specific and one species-specific for *E. rhusiopathiae* in a multiplex PCR. In 2009, Pal et al. (40) described a multiplex real-time PCR for direct detection and discrimination of *E. rhusiopathiae*, *E. tonsillarum* and strains representing serotype 18 from animal specimens. To et al. (60) developed a quantitative real-time PCR (qPCR) for detection of *E. rhusiopathiae* and discrimination from other *Erysipelothrix* species based on a gene involved in capsular formation. It was found that compared with conventional culture and direct qPCR, qPCR following enrichment significantly increased the diagnostic sensitivity.

Several methods such as immunohistochemistry, fluorescent antibodies, and PCR may be used to detect *E. rhusiopathiae* in formalin-fixed paraffin-embedded tissue samples (Figure 23.12) (16, 18, 37).

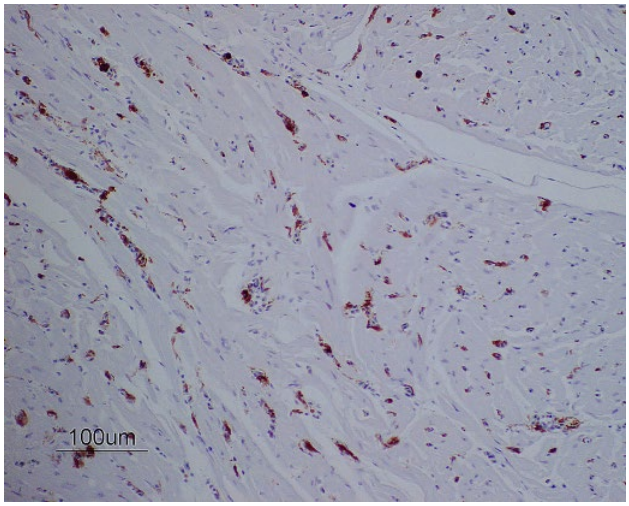


Figure 23.12 Abundant immunostained *Erysipelothrix rhusiopathiae* in capillaries and endothelial cells in myocardium of emu with erysipelas. Immunohistochemistry was performed using polyclonal antibodies to *E. rhusiopathiae* and diamino-benzidin-tetrahydro-chloride as chromogen. (D.S. Jansson, SVA)

Serology

Several serological methods have been used for erysipelas diagnostics, primarily in nonavian species. However, the development of immunity and the usefulness of serology in poultry have not been thoroughly studied. Hence, serological tests are mainly used for research.

Indirect enzyme-linked immunosorbent assays (ELISA) originally developed for use in pigs have been modified and applied to chickens (13, 24). Serological studies have indicated that chickens are commonly exposed to *E. rhusiopathiae* and an increase in seroprevalence with age was reported (13, 25, 57). The potential crossreactivity between serotypes and the fact that not all *E. rhusiopathiae* strains are pathogenic for poultry must be kept in mind.

Differential Diagnosis

The clinical picture in poultry flocks affected by erysipelas, often with sudden high mortality and lack of other symptoms, may resemble highly pathogenic avian influenza and Newcastle disease. The gross pathological findings are indicative of septicaemia hence colibacillosis, fowl cholera (pasteurellosis), and other bacterial septicemic infections are important differential diagnoses.

Intervention Strategies

Management Procedure

Normally, erysipelas outbreaks do not cease spontaneously. For animal welfare and economic reasons prompt action is recommended. Antibiotic treatment, vaccination, and euthanasia should be considered. Antibiotic treatment is usually effective to stop an ongoing outbreak, but the disease may reoccur. Likewise, vaccination may end an outbreak, but it requires at least 2 weeks before mortality is reduced to normal levels. Therefore, a combination of antibiotic treatment and vaccination may be the most efficient measure. When antibiotic treatment or vaccination is not feasible, euthanasia should be considered for animal welfare reasons.

During an outbreak, prompt removal of dead and diseased birds several times a day is fundamental to limit the spread of the infection. To avoid human infection, protective gloves should be worn. Following depopulation, thorough cleaning and disinfection of the house, equipment, and potentially contaminated surroundings is recommended as well as improved control of rodents, red mites, and other potential carriers and vectors. Vaccination of subsequent flocks is recommended since *E. rhusiopathiae* may persist in

the house or environment despite thorough cleaning and disinfection.

To minimize transmission to other animals, including wildlife, manure from infected flocks should be ploughed under and not spread on pasture.

For free-range flocks, there is a risk of increased bacterial load in outdoor pens during an outbreak and indoor confinement or transfer to noncontaminated pens should therefore be considered. However, moving an infected flock may contaminate the new pen.

No specific preventive measures for erysipelas can be given as bacterial sources and transmission routes are not fully understood. A high level of biosecurity, including effective hygiene barriers, may prevent outbreaks and further spread. Field observations in turkey and emu flocks have indicated that rainy, cold weather often preceded outbreaks in free-range flocks, and that the prevalence of erysipelas increased during the colder months of the year.

Vaccination

Types of Vaccine

For many years, inactivated erysipelas vaccines have been widely used and are registered for prevention of erysipelas in turkeys. Field experiences indicate that these vaccines may also be used in laying hens and other poultry species with good results. The inactivated vaccines are based on formalin-inactivated whole or lysed *E. rhusiopathiae* bacteria. Recent studies focusing on the immunogenic properties of the bacterium have identified proteins potentially involved in the immunity development which could be potential candidates for development of new vaccines. Autogenous vaccines are also used for prevention of erysipelas in poultry flocks.

Today, in some regions of the world, a live freeze-dried vaccine based on an avirulent culture of *E. rhusiopathiae* is also available for turkeys, and is administered orally via drinking water

Field Vaccination Protocols and Regimes

Vaccination is recommended for subsequent flocks on farms with previous outbreaks or in areas with history of erysipelas. The vaccine manufacturer's instructions for administration routes, age of the birds at administration, administration intervals, etc. should be considered when designing the vaccination programme for turkeys.

Laying hen flocks are usually vaccinated only once at placement, due to the labour-intensive vaccination protocol. This single vaccination is usually effective. However, recent field experience and reports indicate that flocks may suffer from an outbreak despite vaccination. A second dose for laying hen flocks on some farms may be necessary to prevent future outbreaks.

Treatment

Infected flocks have been treated with antibiotics with good results, although the disease may also reoccur after treatment. Therefore, to minimize the need for multiple rounds of treatment, antibiotic treatment may be combined with vaccination.

Any antibiotic should be used according to current treatment procedures and legislation. When antibiotic treatment is not feasible, for example due to too long withdrawal time for eggs, vaccination alone or euthanasia should be considered. Penicillin is the drug of choice for treating *E. rhusiopathiae* infections in poultry and other species (19), with no resistance reported. However, in some parts of the world penicillin is not registered for use in poultry. Field experience indicates that amoxicillin is effective and in some cases oxytetracycline. Antimicrobial susceptibility studies of poultry isolates are scarce but may aid in the choice of treatment.

Avian Intestinal Spirochetosis

David J. Hampson

Summary

Agents, Infections, and Diseases. Avian intestinal spirochetosis (AIS) is a condition of adult layer and breeder chickens, as well as other poultry. AIS is associated with wet feces and delayed and/or reduced egg production. The condition results from colonization of the cecum and/or rectum with anaerobic intestinal spirochetes of the genus *Brachyspira*. The commonest species involved are *Brachyspira intermedia* and *Brachyspira pilosicoli*, with *Brachyspira alvinipulli* and *Brachyspira hyodysenteriae* occurring less frequently. Infection of flocks is widespread, and the within flock prevalence increases with age.

Diagnosis. The relatively nonspecific clinical signs and specialized diagnostic requirements result in the condition often being unrecognized.

Intervention. Vaccines are not available and there are limited options for treatment. Strict biosecurity measures are needed to prevent transmission, particularly between flocks of different ages on the same site.

Introduction

Definitions and Synonyms

Avian intestinal spirochetosis (AIS) is a disease of adult chickens and other poultry species: it is most commonly associated with delayed and/or reduced egg production and wet feces. The condition is characterized by extensive colonization of the cecum and/or rectum with anaerobic intestinal spirochetes of the genus *Brachyspira*. The main species involved are *B. intermedia* and *B. pilosicoli*, with *B. alvinipulli* and *B. hyodysenteriae* occasionally being reported.

Economic Significance

Colonization with *Brachyspira* species is widespread amongst flocks of layer and breeder hens (80, 125). A study in the UK suggested that AIS resulted in a potential annual loss to the laying hen industry of around £14 million (US$17 million) (17, 18). Losses to the meat chicken industry associated with infection of breeder flocks also may be high (83, 124). In 1998 it was calculated that a commercial broiler flock hatched from eggs from a breeder flock with clinical AIS lost approximately £9,900 (US$ 15,500) per annum from reduced growth rates and poor feed digestion (115). Additional losses associated with reduced egg production and increased feed consumption were estimated at £10,600 (US$13,000) per flock per annum. Besides delayed and/or reduced egg production in laying hen flocks, AIS can reduce profitability due to mortalities, downgrading of fecally stained eggs, and increased labor costs associated with cleaning of cages and houses. There may be local adverse environmental effects through increased odor from wet feces, and attraction of flies. Due to its nonspecific signs AIS often goes undiagnosed, and its full economic significance is not appreciated.

Public Health Significance

Strains of *B. pilosicoli* from birds are closely related to strains from humans and other animals, and probably there is no barrier to cross-species transmission (42). Strains of *B. pilosicoli* isolated from humans have been used experimentally to colonize 1-day-old chicks (26, 90, 133, 142), and adult laying hens (48), and there seems to be no reason why avian strains could not colonize humans. *B. pilosicoli* has been isolated from lake and dam water frequented by ducks colonized by *B. pilosicoli*, emphasizing the potential for transmission by ingestion of contaminated water (102). *B. pilosicoli* has been found on the carcasses of spent laying hens in a supermarket, and this may be a potential source of transmission (147). The likelihood of healthy immunocompetent poultry industry workers developing severe infections with *B. pilosicoli* following contact with chickens is very low. The other *Brachyspira* species associated with AIS are not known to colonize or cause disease in humans.

History

Early accounts of intestinal spirochetes in birds are recorded in the AIS chapters in previous editions of this book and in a recent review (80). In the 1980s it was shown that intestinal spirochete infections were common in flocks of laying hens and broiler breeder hens in the Netherlands (23–29), and occurred in the UK (36). Colonization was associated with various syndromes including delayed and/or reduced egg production and wet feces. Subsequent work in the United States (92, 134, 139), Australia (83, 124), Europe, and Scandinavia (12, 17, 18, 30, 53) confirmed and extended these results, including the identification and naming of the main pathogenic *Brachyspira* species causing disease in poultry (84). In 1992, necrotizing typhlitis and high mortality associated with spirochetes was reported in common rheas (*Rhea americana*) (113), and since then forms of AIS have been reported in a number of other species of poultry including farmed geese and ducks (34, 50, 97).

Etiology

Classification

Spirochetes are bacteria classified in the order Spirochaetales. The intestinal spirochetes associated with AIS belong to the family Brachyspiraceae, genus *Brachyspira*.

Names and Synonyms

The genus *Brachyspira* currently contains 9 officially named species (*B. aalborgi, B. alvinipulli*, *B. hampsonii*, *B. hyodysenteriae*, *B. innocens*, *B. intermedia*, *B. murdochii*, *B. pilosicoli*, and *B. suanatina*), and several proposed species including "*B. canis*", "*B. pulli*", and "*B. corvi*" (80). All species colonize the large intestine, but only *B. pilosicoli* and *B. aalborgi* are known to attach by 1 cell end to cecal or colonic enterocytes (86). *Brachyspira* species have close similarities in their 16S rRNA gene sequences, indicating that they have evolved into different species relatively recently. A wide range of *Brachyspira* species are found in birds, and this suggests that they may have been the original hosts of an ancestral *Brachyspira*-like anaerobic spirochete when it first colonized the intestinal tract. Wild birds, and particularly aquatic species such as ducks and geese may act as reservoirs of *Brachyspira* species

that may be transmitted to and cause disease in other hosts (51, 55, 81, 102, 112).

Pathotypes

Based on experimental studies and naturally occurring colonization, *B. intermedia*, *B. pilosicoli*, *B. alvinipulli*, and *B. hyodysenteriae* are considered to be potential pathogens in birds. Of these *B. intermedia* is the most frequently encountered pathogenic species in poultry and *B. hyodysenteriae* is the least commonly reported. Their features are summarized in Table 23.3. Where the other species and proposed species occur in birds these are generally thought to be commensals (84); however, this assumption has not been thoroughly investigated.

Morphology and Staining

Brachyspira species are Gram-negative, helical-shaped bacteria with diameters ranging from 0.25 to 0.6 µm, lengths from 3 to 19 µm, amplitudes from 0.45 to 0.79 µm, and wavelengths from 2.7 to 3.7 µm. They can be identified in wet mounts by dark-field or phase contrast microscopy, or in histologic sections stained with silver. Each spirochete cell contains a central protoplasmic cylinder, multiple periplasmic flagella, and an outer envelope (Figure 23.13). The periplasmic flagella are endocellular and divided into 2 equal sets, with each set originating from opposite poles of the protoplasmic cylinder and overlapping with the other set in the middle of the cell. The number of periplasmic flagella has been used for spirochete classification. However, this has limited value as the numbers can vary between and within species. *B. pilosicoli* and *B. aalborgi* typically have 4 flagella at each cell end, whereas the other *Brachyspira* species have 8 or more at each end. The rotation of periplasmic flagella between the outer membrane and protoplasmic cylinder confers a corkscrew-like movement to spirochete cells. These morphologic features and motility permit spirochetes to traverse highly viscous liquids, such as mucus, which immobilize externally flagellated bacteria (93).

Table 23.3 Morphologic, biochemical, and other characteristics of the 4 main pathogenic *Brachyspira* species reported to cause disease in poultry.

	Species			
Characteristic	***B. intermedia***	***B. pilosicoli***	***B. alvinipulli***	***B. hyodysenteriae***
Pathogenicity	Moderate to mild	Moderate to mild	Moderate to mild (severe in geese)	Severe
Colonization of cecal epithelial surface	Random in lumen and crypts – not attached to epithelium	May be attached by one cell end to cecal enterocytes	Random in lumen and crypts – not attached to epithelium	Random in lumen and crypts – not attached to epithelium
ß-Hemolysis pattern	Weak	Weak	Weak	Strong (occasionally weak)
Indole production	+	– (occasionally positive)	–	+ (occasionally negative)
Hippurate hydrolysis	–	+ (occasionally negative)	+	–
Type strain	PWS/A^T – ATCC 51140	P43/6/78^T – ATCC 51139	C1^T – ATTC 51933	B78^T – ATCC 274164

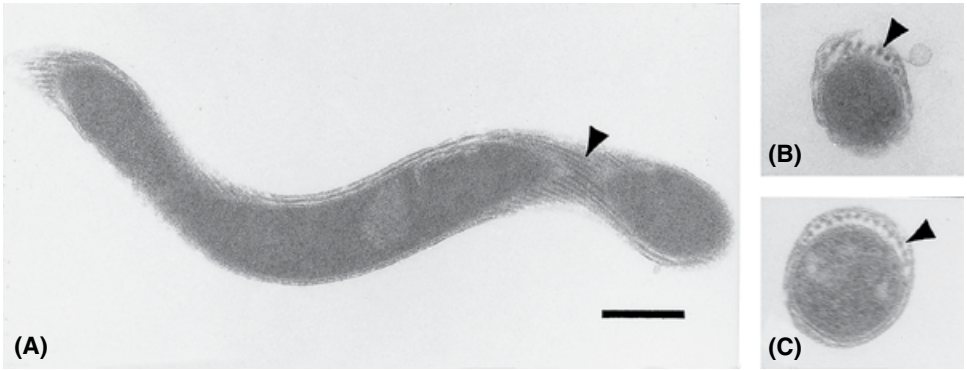


Figure 23.13 *Brachyspira alvinipulli*. Spirochete cell is helically shaped on longitudinal orientation (A). On transverse sections, an end (B) and the middle (C) of spirochete cells have 8 and 16 periplasmic flagella (arrows), respectively. (D.E. Swayne)

Growth Requirements

Brachyspira species are anaerobic but will tolerate brief exposure to air (118). Due to their slow growth rates and tendency to become overgrown by other more rapidly growing species, selective solid media systems are used for primary isolation, most of which were developed to isolate swine intestinal spirochetes (1, 57, 61). Typically, the media consist of trypticase soy agar with 5%–10% defibrinated ovine or bovine blood, and 1–5 selective antibiotics (including spectinomycin, rifampin, spiramycin, vancomycin, polymyxin, and/or colistin). As the different *Brachyspira* species vary in their tolerance to these antimicrobials, a recommended "general" brachyspira plate contains 400 μg/mL spectinomycin and 25 μg/mL each of colistin and vancomycin (57). Incubation is at 37 °C–42 °C for up to 10 days; however, for most avian isolates visible growth is usually present in 2–5 days. Typical gaseous environments are 94% H_2 and 6% CO_2, generated using anaerobic gas packs in an anaerobic jar, or 80% N_2, 10% H_2, and 10% CO_2 in an anaerobic chamber. The spirochetes can be further propagated in anaerobic brain heart infusion broth containing 10% fetal bovine serum, with 1% oxygen to enhance growth (118), or in a prereduced anaerobic trypticase soy broth medium ("Kunkle's medium") (62). Growth of 10^8 to 10^9 cells/mL can be obtained in these broths within 2–3 days.

Growth and Hemolysis on Blood Agar

Brachyspira species grow as a dull flat sheen on the surface of agar plates, forming confluent areas of growth with sharply defined edges, sometimes penetrating into the agar. They do not readily produce colonies. Most *Brachyspira* species are weakly hemolytic, although *B. hyodysenteriae*, *B. suanatina* and *B. hampsonii* are strongly hemolytic, and occasional avian strains of *B. intermedia* and other unidentified species may cause intermediate to strong hemolysis (50, 51). Growth can be confirmed by the characteristic morphology and motility of the bacteria on wet mounts using dark-field or phase contrast microscopy. As individual birds may be colonized with mixed *Brachyspira* strains and species, subculturing of isolates to purity is important.

Biochemical Properties

Brachyspira species typically contain alkaline and acid phosphatase, esterase, esterase lipase, ß-galactosidase, and phosphorylase activities. Differences in hemolysis patterns, production of indole, hippurate hydrolysis, and the presence or absence of ß-galactosidase and ß-glucosidase activities have been used to categorize isolates (31). A commercial API-ZYM system and numerical 5-digit coding system for enzyme activities has been used in differentiating avian and mammalian isolates (45). As these phenotypic properties can vary, more specific molecular-based techniques now have largely replaced biochemical testing for species identification.

Susceptibility to Chemical and Physical Agents

Strains of *B. pilosicoli* can survive for extended periods in water, particularly at colder temperatures (4 days at 25 °C; 66 days at 4 °C) (102). On the other hand, strains of *B. intermedia* and *B. pilosicoli* are relatively short lived in chicken feces (~3 days at 4 °C, at 10^9/g feces), and do not persist in the environment of chicken houses (103). The reduced viability in chicken feces is likely due to its typically dry and acidic nature. Most common disinfectants are efficacious against *Brachyspira* species (103), although it is best to remove organic matter first (21). Cleaning, disinfection, and resting of empty houses between batches of hens has the potential to break cycles of AIS on infected farms (103).

Virulence Factors

The mechanisms by which *Brachyspira* species cause disease in avian and mammalian species are incompletely understood. The genome sequences of 1 or more strains of all 9 officially named species are available publically, and pan-genomic comparisons have been undertaken (39). These data provide opportunities for new lines of investigation into disease mechanisms and identification of virulence factors, for example by comparing gene content and expression in pathogenic and nonpathogenic species. Nevertheless, research is still impeded by a lack of easy methods for genetic manipulation of these bacteria, and without this it is still difficult to assess the functional significance of individual genes or groups of genes. Moreover, in the case of AIS, generally it is not even clear whether and to what extent the underlying pathologic mechanisms are common to the different pathogenic species. Indeed, the development of AIS likely requires the activity of multiple virulence factors that may vary between the pathogenic species involved.

Virulence attributes of pathogenic *Brachyspira* species can be considered to consist of a set of "lifestyle" virulence factors involved in initial colonization and fitness for survival and proliferation in the microenvironment adjacent to the mucosa of the large intestine, and other "essential" virulence factors that are required for lesion production and/or disease. To some extent the lifestyle factors, including such things as ability to survive in an anaerobic environment, to use available substrates, to be motile, and to undergo chemotaxis are shared by commensal and pathogenic *Brachyspira* species, since all *Brachyspira* species are able to colonize the large intestine. Subtle differences in such lifestyle factors presumably cause differences in

behaviour – for example, some *Brachyspira* species show a limited host range (e.g., *B. aalborgi* is largely restricted to humans), whilst others have a much broader host range (e.g., *B. pilosicoli* colonizes many species of birds and animals). Despite *B. hyodysenteriae* being a major pathogen of pigs, even its host range and virulence trait determination remain poorly understood. For example, *B. hyodysenteriae* strains isolated from rheas caused severe necrotizing typhlitis and high mortality rates in rheas, but were apathogenic and failed to produce significant intestinal colonization in swine (122). Similarly, strains of *B. hyodysenteriae* recovered from swine vary in their virulence in experimentally inoculated swine (2).

As part of the colonization process *Brachyspira* spp. cells penetrate and move through the mucus overlying the epithelium of the large intestine. All *Brachyspira spp*. cells are motile, but they vary in their attraction to colonic mucin. Comparison of the genome sequences of *B. hyodysenteriae* and *B. pilosicoli* has shown that *B. pilosicoli* has fewer methyl-accepting chemotaxis genes than *B. hyodysenteriae*, with no *mcpC* genes, and hence these species are likely to have different chemotactic responses that may help to explain their different host range and colonization sites (150). Experimentally, strains of *B. intermedia* and *B. innocens* have been shown to be less attracted to mucin than virulent strains of *B. hyodysenteriae* (87). On the other hand, whilst cells of both *B. hyodysenteriae* and *B. pilosicoli* were shown to be attracted to and entered mucin solutions, this attraction was reduced at mucin concentrations above 6% for *B. hyodysenteriae* but not for *B. pilosicoli* (94). Even within a species it is evident that there are substantial strain differences; for example, *B. pilosicoli* strains varied in their motility and chemotactic responses to mucin (94), whereas 2 avirulent strains of *B. hyodysenteriae* were less attracted to mucin than were virulent strains tested under the same conditions (87).

Besides chemotaxis, the role of motility in colonization has been confirmed by experiments in which *B. hyodysenteriae* strains with disruptions introduced to their flagella genes (*flaA* and *flaB*) had reduced motility and a reduced ability to colonize (59, 111). Similar experiments have not been conducted with the other pathogenic *Brachyspira* species, but it can be assumed that motility is important for their colonization. For example, *B. pilosicoli* shows increased motility under viscous conditions, including increased mucus concentrations equivalent to those found in the colon (93, 94).

A characteristic feature of *B. pilosicoli* is the ablity of individual cells to attach by 1 of their pointed cell ends to the luminal surface of enterocytes in the large intestine, creating a "false brush border" on the surface (Figure 23.14). *B. pilosicoli* surface lipoproteins may be involved in facilitating this attachment by interactions with specific receptors on the cell surface (145, 150).

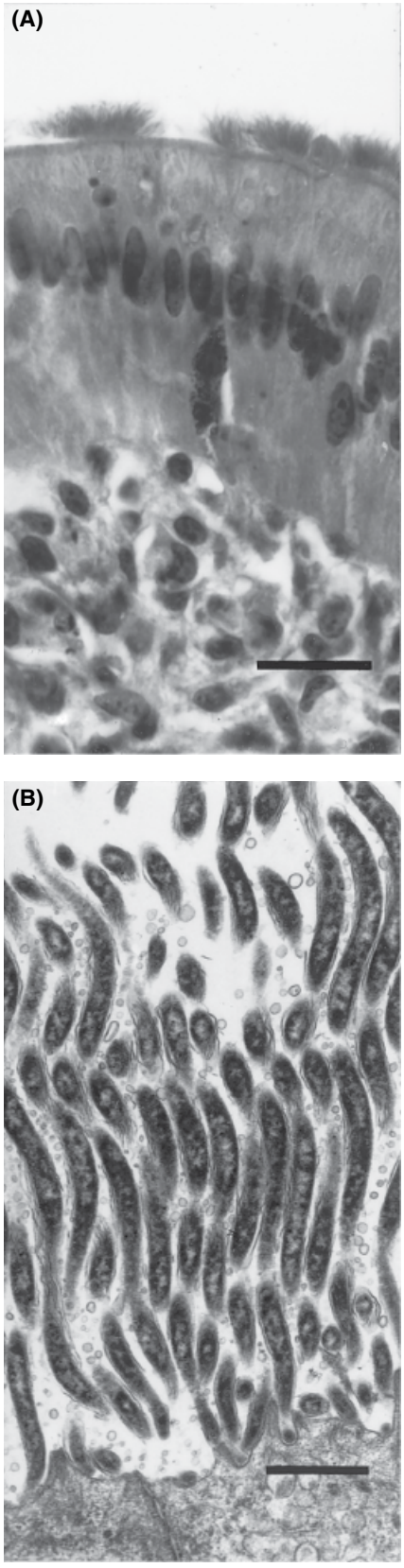


Figure 23.14 Tight association of *Brachyspira pilosicoli* with the luminal surface of cecal epithelium (A). The orientation is at right angles to the epithelial cells (B). (D.E. Swayne)

Where this layer of attached spirochetes is dense it may form a physical barrier that prevents the reabsorption of water and electrolytes, resulting in watery diarrhea.

An *in vitro* study using Caco-2 cell monolayers indicated that the cell junctions are the initial targets of attachment by *B. pilosicoli* (96). Colonized monolayers demonstrated a time-dependent series of changes, including accumulation of actin at the cell junctions, loss of tight junction integrity and condensation and fragmentation of nuclear material consistent with apoptosis. Using quantitative reverse transcription polymerase chain reaction (PCR), the colonized monolayers demonstrated a significant upregulation of interleukin-1ß (IL-1ß) and IL-8 expression. These cytokines/chemokines are likely to be responsible for attracting inflammatory cells to the colonization site. Candidates for inducing such cellular damage include the biological activity of lipo-oligosaccharides (LOS) and/or the action of membrane proteases, including a subtilisin-like serine protease similar to that of other Gram-negative bacteria (22, 150).

The LOS in the cell envelope of *Brachyspira* species have some of the same biological properties as lipopolysaccharides from other Gram-negative bacteria and is likely to contribute to lesion production. Studies with LOS extracted from *B. hyodysenteriae* has shown that it has a variety of effects, including being toxic for mouse peritoneal macrophages, increasing uptake of red blood cells by murine peritoneal cells via Fc and C3 receptors, acting as a mitogen for murine splenocytes, generating chemotactic factors in fresh swine serum (101), inducing interleukin-1 (IL-1) and tumor necrosis factor (TNF) from murine peritoneal cells, augmenting natural killer cell activity (35), and inducing production of proinflammatory cytokines such as interleukin-6 (IL-6) (100). The LOS of other pathogenic *Brachyspira* species has not been studied, other than to show that it is antigenically heterogenous amongst *B. pilosicoli* strains (73). *B. pilosicoli* sonicates (likely containing LOS) did cause significant upregulation of IL-1ß, TNF-α, and IL-6, whereas culture supernatants and sonicates of nonpathogenic *B. innocens* did not alter cytokine expression (96).

Most *B. hyodysenteriae* strains contain a ~36 Kb plasmid with 31 genes, including 6 *rfbA-D* genes that were predicted to be involved with rhamnose biosynthesis, and hence LOS structure (13, 14). Another block of 6 of these plasmid genes appear to be related to glycosylation and colonization efficacy, and are lacking in some strains with reduced virulence (67, 68). Interestingly, *B. hyodysenteriae* strain R301 isolated from a rhea lacks these 6 genes; moreover, it colonizes pigs poorly and does not cause disease, but colonizes and causes severe disease in rheas (122). This suggests that these genes may have a role in species-related specificity, perhaps associated with glycosylation patterns on surface receptors that are involved in spirochete attachment and colonization.

A final potential "essential" virulence determinant in *B. hyodysenteriae* is the spirochete's strong hemolytic activity. Currently, 8 genes encoding proteins with predicted hemolytic activity have been described in *B. hyodysenteriae* (13, 14), but their exact role and importance are unknown. The genetic basis of the strong hemolysis produced by *B. suanatina* and *B. hampsonii* also requires investigation (88, 91).

Pathobiology and Epizootiology

Natural and Experimental Hosts

Besides chickens, spirochetes have been recorded colonizing the ceca and recta of a variety of domesticated avian species, including common rheas (16, 113), pheasants (152, 153), partridges (50), turkeys (114), geese (97), ducks (34), and captive or free-living wild birds, especially acquatic birds of the orders Anseriformes and Ciconiiformes (50, 55, 102), but also in Corvid species (52) and other wild birds (56).

Incidence and Distribution

Cases of AIS in poultry have been reported in countries of Europe, Scandinavia, North and South America, and in Malaysia and Australia (5, 12, 23, 30, 36, 46, 53, 83, 85, 92, 124, 134, 139): the condition almost certainly occurs elsewhere but likely goes undiagnosed. Besides caged or housed flocks, outdoor free-range flocks and organic flocks with access to outside areas are commonly affected (18, 53, 149). Necrotizing typhlitis in rheas has been reported in the United States and Europe (16, 63, 113). Although this condition in rheas seemed to be common in the past, it now occurs less frequently.

There have been relatively few epidemiologic surveys of AIS, and among those reported, the incidence of colonization and disease has varied with the avian species examined and the methods used for demonstrating spirochetes. Surveys have shown the condition to be very common in flocks of laying hens and broiler breeder hens wherever these have been investigated. For example, in a survey conducted in the 1980s in chicken flocks from the Netherlands, using a direct fluorescent antibody test on feces, 27.6% of flocks with intestinal disorders were positive for intestinal spirochetes whereas only 4.4% of flocks without enteric signs were positive (24). A study in Western Australia used selective culture on feces collected from 37 randomly selected laying hen flocks and 30 broiler breeder flocks (83). Overall, 53% of the breeder hen flocks and 35% of the layer hen flocks yielded samples containing intestinal spirochetes. Moreover, 64% of the flocks with diarrhea or poor production were colonized, compared with only 24% of

flocks with normal feces. Within-flock prevalence varied from 10% to 95% of the samples tested. More recent surveys have supplemented or even replaced selective culture with PCR assays to identify the spirochete species present. Using these modifications, even higher overall prevalence rates were found in the Eastern States of Australia, where spirochetes were recovered from 43% of 28 randomly selected broiler breeder farms and from 68% of 22 laying hen farms (124). Within these farms, most of which contained multiaged flocks, infection was detected in 26% of 112 broiler breeder flocks (each in individual houses) and in 54% of 68 laying hen flocks. Within-flock prevalence varied from 10% to 100% of samples examined, with a mean of 47%. In this study there was a highly significant correlation between colonization and wet litter, with infected flocks on average having 14% greater fecal water content than flocks that were not colonized. Spirochetes were not detected in 45 broiler flocks on 19 farms that were surveyed. In a study in Northern Italy, 72.4% of 29 laying hen farms and 71.1% of 42 sheds contained chickens infected with intestinal spirochetes (12). There was a significant association between the presence of spirochetes and using deep pits rather than conveyor belts to remove feces. Sheds housing birds >40 weeks were significantly more likely to contain pathogenic spirochetes than younger flocks. Colonization was significantly associated with reduced egg production, but the increased fecal water content observed in colonized flocks just failed to reach significance. In a survey of laying hen flocks older than 40 weeks of age in Pennsylvania, 76% of 21 flocks contained chickens that were colonized by pathogenic *Brachyspira* species (92). In Canada, risks for infection again increased in older flocks (older than 60 weeks), with multiaged farms having the higest risk of infection (85). In Malaysia, most layer and breeder flocks sampled were colonized with *Brachyspira* species, with *B. intermedia* and *B. pilosicoli* being the most common pathogenic species found (5). There were significant positive associations between colonization and fecal staining of eggs, having open-sided sheds, and flocks being 40 weeks of age or older.

In surveys of various avian species held in zoological collections, colonization with intestinal spirochetes has been commonly detected in waterfowl of the order Anseriformes, but not in other species (102, 129).

Prevalence of Pathogenic Species

In the surveys described previously around 70% of laying flocks and 50% of breeding flocks contained birds colonized by intestinal spirochetes. Isolates from about 67% of these colonized flocks typically have belonged to pathogenic species, of which *B. intermedia* accounts for around two-thirds of the isolates and *B. pilosicoli* for most of the remainder (12, 92, 124, 128). *B. alvinipulli* and *B. hyodysenteriae* occasionally have been detected in chickens (30, 53), as well as in flocks of geese in Hungary (97). Individual flocks and hens may be colonized by more than 1 pathogenic species (53, 106, 124, 128).

Strains Present

Multilocus enzyme electrophoresis (MLEE) was used in 1 study to examine multiple *Brachyspira* isolates from 4 chicken farms (128). On 1 farm, 16 *B. murdochii* isolates were located in 14 different electrophoretic types (ETs), whereas 5 isolates of *B. pilosicoli* all belonged to the same ET. On the second farm, 5 of 6 *B. pilosicoli* isolates belonged to the same ET, and the sixth was distinct, whereas 2 *B. intermedia* isolates were different from each other. On the third farm, 3 isolates of *B. intermedia* all belonged to the same ET. On the fourth farm, the 4 *B. intermedia* isolates all belonged to different but related ETs. Hence some infected farms may have a dominant strain of a species present, but other strains of the same or other *Brachyspira* species also may be present. This heterogeneity was also found in a study on a Western Australian laying hen farm, where 20 *B. intermedia* isolates examined using pulsed field gel electrophoresis (PFGE) were divided into 4 different PFGE types (106). The existence of strain heterogeneity amongst isolates from a farm is important, because different strains may have different biologic properties that may affect the clinical outcome, including virulence traits and antimicrobial susceptibilities.

Where there are different strains on a farm, they may either have been independently introduced, or have arisen from "microevolution" of original strains that were present (8). Some of the pathogenic *Brachyspira* species appear to have a recombinant population structure, and the various species may undergo extensive genetic rearrangements and sequence drift that generates genetic diversity (77, 144, 156). Novel genetic information may be acquired from other species/strains through the activity of a prophage-like gene transfer agent observed in different *Brachyspira* species (82, 89, 123), and/or from horizontal gene transfer via bacteriophages with broad trophism, that themselves may have undergone extensive gene remodeling (37).

Anatomic Location

Intestinal spirochetes colonize the ceca and rectum, but not the small intestine. Spirochetes primarily are found in crypt lumina and to a lesser extent in the cecal contents adjacent to enterocytes. *B. pilosicoli* cells may be found in groups attached by 1 cell end to the surface epithelium (Figure 23.14), although they can colonize the ceca without attachment being seen.

Spirochetemia with *B. pilosicoli* has been reported in humans (10, 143). Although this has not been detected in birds, *B. pilosicoli* has been isolated from the liver of affected chickens, indicating that systemic spread occurs (78, 155).

Spirochete Persistence in the Ceca

Spirochetes can persistently colonize ceca (23, 25, 28). In 1 study, spirochetes were detected in cecal droppings from the time of experimental infection at 14 weeks of age until the termination of the experiment 23 weeks later (29); in another experiment the same spirochete strain 1380, later identified as *B. intermedia* (84), was still present in the feces of experimentally infected laying hens after 9 months (25).

Transmission, Carriers, and Vectors

Intestinal spirochetes are transmitted between birds by the fecal–oral route, either directly or indirectly. Aerosol transmission of feces between birds that are held in close proximity in confined conditions is likely. Transmission between flocks in different houses on a farm is most likely through the movement of personnel who have clothing or boots contaminated by chicken feces, and hence strict biosecurity measures are needed (85). Wild birds and animals such as rats and mice also potentially can introduce and/or spread infection. Insects such as flies, or species such as dogs or feral animals might serve as mechanical carriers. A major potential source of transmission is through the water supply. Wild ducks have been shown to shed strains of *B. pilosicoli*, *B. hyodysenteriae*, and *B. intermedia* in their feces (50, 51, 102), and these may survive in effluent ponds or dams supplying drinking water (102). Besides oral transmission, it has been suggested that transfer of *Brachyspira* species between waterfowl such as mallards in such environments may occur via the cloaca, with retrograde peristalsis carrying the spirochetes up to the caeca (54).

Incubation Period

The incubation period of AIS is variable, with the dose of the organism and environmental factors having profound influences. Disease signs can occur in chickens as early as 5 days after experimental inoculation (135), although it may take several weeks for significant levels of colonization to occur, and clinical signs to develop (40, 41).

Influence of Age on Spirochete Colonization

Experimentally, the pathogenicity of avian intestinal spirochetes is greatest when they are inoculated into 1-day-old birds via crop gavage (135), although natural infection of such young birds does not appear to occur. In commercial laying hens it is unusual to detect colonization before 15 weeks of age, and more colonized hens are found as the flocks become older (12, 85, 124). This age-related distribution likely reflects increasing levels of exposure rather than differences in age susceptibility.

Influence of Diet and Microbiota on Spirochete Colonization

For *Brachyspira* species to colonize they must reach the large intestine, then establish and interact successfully within the local microenvironment. It is assumed that the spirochetes survive passage through the upper intestinal tract inside boluses of food or feces. Studies in swine have shown that once in the large intestine the spirochetes interact with various anaerobic bacterial species forming part of the normal microbiota of the cecum and colon, including species such as *Clostridium perfringens*. These species act synergistically with *B. hyodysenteriae* to facilitate spirochete colonization and augment inflammation and lesion production (154).

Dietary influences on colonization with *B. intermedia* have been shown in experimentally infected laying hens. In particular, diets based on wheat seem to promote colonization with *B. intermedia* compared with diets based on barley or barley and sorghum (104). Furthermore, different wheat varieties have been shown to vary in their influence on promoting colonization by *B. intermedia* (105). In a study of laying hens fed wheat-based diets the addition of exogenous enzymes designed to hydrolyse the nonstarch polysaccharides in the wheat reduced *B. intermedia* colonization following experimental infection (40). In the same study, and in a subsequent study, addition of zinc bacitracin (ZnB) to the diet reduced colonization with *B. intermedia* (40, 41), but dietary ZnB enhanced colonization with *B. pilosicoli* (47, 126). As ZnB typically acts on Gram-positive bacteria rather than on the spirochetes themselves, these conflicting results indicate that there are likely to be complex positive and negative interactions between different components of the cecal microbiota and different *Brachyspira* species in chickens.

Taken together, these studies suggest that different clinical outcomes may occur in infected hens depending on their diet, intestinal microenvironment, and microbiota, as well as the particular *Brachyspira* species involved in the colonization. These findings may help explain some of the heterogeneity in clinical signs and pathology seen in different commercial flocks with AIS.

Clinical Signs and Pathology

Information about clinical signs and pathology of AIS is limited, and available data have come from 3 main sources. The first is from experimental infection of

1-day-old chicks. These data provide a guide to the pathogenic potential of certain isolates, but the results must be viewed with caution as the associated colonization and disease may not be representative of natural infections in adult birds. The second comes from experimental infection of adult chickens using defined isolates. This system is more representative of natural disease, but also has limitations. The experimental hens are usually individually caged, appropriately fed and relatively free of stress, and these conditions do not reflect the situation in many commercial flocks where AIS occurs. Disease is often quite mild or absent under these experimental conditions; for example, only slight reductions in egg production and/or increased fecal water content have been observed in the absence of obvious histologic changes in the ceca of some experimentally colonized birds (38, 126). Furthermore, as these experiments are time consuming, testing tends to be done with only 1 or a few spirochete strains under a restricted set of standard dietary and other conditions. The birds are not co-infected with other species or strains of spirochetes, or with other enteric pathogens, as may occur in commercial flocks. The third source of information comes from observations of natural cases of AIS. These are important data of direct industry relevance in terms of observing changes in production, but are limited by the fact that often there may be co-infections that are unrecognized, or which make the attribution of production losses or pathology associated with AIS difficult to allocate. Another problem with some of the earlier descriptions of AIS in the field is that it was not known what species of spirochete were involved (23, 36).

Besides the *Brachyspira* species and strain involved in the colonization, the initiation and severity of clinical disease is likely influenced by the host species, husbandry, nutrition, environment, and genetics. Some specific predisposing factors for AIS that have been observed in the field include molting, recent onset of egg production, poor or inappropriate feed quality, floor housing, and light-laying breeds of hens (17, 36, 134, 139). Crowding induces stress and increases the opportunity for spirochete transmission between individuals held in close proximity. Exposure to the stress hormone norepinephrine increases the potential virulence of *B. pilosicoli* by enhancing its growth, attraction to mucin and attachment to Caco-2 cells (95). As norepinephrine may be present in the intestinal tract, it is likely that this effect also occurs *in vivo*, and possibly with other pathogenic *Brachyspira* species under stressful conditions.

Naturally occurring or experimental colonization of birds with intestinal spirochetes broadly may result in: (1) subclinical colonization, (2) mild to moderate clinical disease, or (3) severe clinical disease.

Subclinical Colonization

Colonization by spirochetes without disease has been reported in chickens (83, 92, 124), and is often associated with apparently commensal *Brachyspira* species such as *B. innocens, B. murdochii*, and/or "*B. pulli*" (84). In wild birds, especially waterfowl, most spirochetes are not associated with enteric disease, and are considered to be commensals forming part of the normal microbiota. From an epidemiologic perspective it is also important to note that wild birds may carry pathogenic species without showing obvious clinical signs (50, 51, 102).

Mild to Moderate Clinical Disease

The "mild to moderate" disease spectrum is seen particularly in association with strains of *B. intermedia, B. pilosicoli*, and *B. alvinipulli*, often in layer hens and broiler breeder hens. These infections tend to be associated with diarrhea and/or reduced egg production, but cecal changes are mild or inapparent.

***Infections with* B. intermedia.** In early studies *B. intermedia* strain 1380 was used to experimentally infect broiler chicks (27), laying hens (28), and 14-week-old broiler hens and cocks, where the eggs were collected and hatched (29). Infected chicks showed variable reductions in growth rate, wet droppings with increased fat content, and increased serum content of protein, lipid, carotenoids, and bilirubin (27). Laying hens showed increased fecal fat content (28), developed slimy, wet, frothy feces (25), or had wet droppings and produced significantly fewer eggs (29). Spirochetes were found penetrating the cecal mucosal lining between undamaged columnar cells, in "gap-like" lesions running through the epithelium and accumulated just under the epithelium. There was some erosion of the superficial mucosa but no clear signs of inflammation in the connective or lymphatic tissues. The eggs from infected hens were significantly lighter, had paler yolks and had a lower carotenoid content than those from uninfected birds. Broiler chicks hatched from eggs from the infected hens had pale, mucoid and wet feces, and the chicks were significantly lighter than control chicks at 2 and 3 weeks of age. They tended to develop rickets and had low blood plasma concentrations of carotenoids and alkaline phosphatase activity. They were not themselves colonized by spirochetes suggesting a lack of vertical transmission.

Experimental infection of laying hens with *B. intermedia* strain HB60 caused reduced growth rates, increased fecal water content, and decreased egg production and egg weight, but did not induce any characteristic pathological changes in the ceca (38, 41, 105). Due to the diversity of the species (108), other strains/genetic groups need to be tested.

***Infections with* B. pilosicoli.** A review of *B. pilosicoli*-induced AIS recently has been published (70). *B. pilosicoli* is an extremely diverse recombinant species with strains varying in genome sizes and arrangements (39, 77, 98, 99, 144). Newly hatched broiler chicks have been infected with human, porcine and/or canine isolates of *B. pilosicoli* (26, 90, 141, 142). Clinical signs either were not observed (26), or the chicks developed watery diarrhea (141, 142), sometimes with a depressed growth rate (142). Gross cecal lesions were not seen, but there were variable histologic changes that included the characteristic presence of a dense mat of spirochetes attached by 1 cell end to cecal enterocytes (90, 141, 142), sometimes with a diffuse thickening of the cecal epithelial brush border (90). There was variable crypt elongation, crypt lumina were dilated and there was mild focal infiltration of the lamina propria with heterophils. Sometimes spirochetes were found between enterocytes or producing gap-like lesions; subepithelial accumulation of spirochetes and focal erosion without an inflammatory reaction also were recorded (26). Vacuolation and protein deposition were observed in the apical cytoplasm of some luminal enterocytes. Sometimes microvilli were obscured, damaged, or obliterated by large numbers of attached spirochetes, and there was disruption to the terminal web microfilaments. Individual spirochetes invaginated into the cellular membrane and indented into the terminal web cytoplasm but did not penetrate it.

Experimental infection of broiler breeder hens with avian *B. pilosicoli* strain CPSp1 resulted in a transient increase in fecal water content, fecal staining of eggshells and/or a significant reduction in egg production (126, 127). The ceca of infected birds were gassy and the contents were frothy, fluid and pale, but no gross or histologic lesions were observed. Spirochetes were isolated but they were not found attached to the cecal epithelium. Infection of laying hens with strain CPSp1 resulted in no disease signs (47), whilst infection with a human isolate of *B. pilosicoli* resulted in a persistent and significant increase in fecal water content (48). Again, neither attachment of spirochetes nor gross pathologic changes were observed.

Natural infection of 2 layer flocks with *B. pilosicoli* was associated with a 5% reduction in egg production, diarrhea in up to 25% of chickens, wet droppings, feces smeared on feathers around the vent ("pasty vents"), lethargy, and depression (139). The apical surfaces of cecal enterocytes were covered by a dense layer of spirochetes aligned parallel to each other and perpendicular to the mucosal surface (Figure 23.14). Four turkey flocks that had increased mortalities were infected with *B. pilosicoli* (114). Large numbers of spirochetes were attached along the surface epithelium of the ceca, extending into the middle of the crypts. Focal mucosal erosions occurred in some ceca, with spirochetes attaching directly to the exposed basement membrane or invading the lamina propria. This was accompanied by an increase in the number of subepithelial mononuclear inflammatory cells.

Experimental infection of day-old ducklings with *B. pilosicoli* or *B. alvinipulli* strains failed to induce gross or histologic changes in the intestinal tract (137). This suggests that although ducks may carry pathogenic *Brachyspira* species they may be relatively refractory to disease, or at least disease caused by these 2 species.

***Infections with* B. alvinipulli.** Experimental infection of 1-day-old chicks and 14-month-old hens with *B. alvinipulli* strain 91-1207/C1 resulted in yellow, golden, or orange cecal droppings (135). The ceca were dilated and contained pale-green to yellow fluid to frothy contents. Infected birds had moderately severe lymphoplastic typhlitis and proctitis with lymphocyte and/or heterophil exocytosis, mild cecal villus epithelial cell hyperplasia, edema in the lamina propria of villus tips, and submucosal lymphocytic follicles (Figure 23.15). Some chicks had mildly dilated cecal crypts. Mats of spirochetes were present over the villus surface and in the crypts, with spirochete cells oriented randomly on the cecal epithelial luminal surface or in the crypt lumina. Spirochetes rarely invaded between and below the cecal epithelial cells (Figure 23.16).

Brachyspira alvinipulli strain 91-1207/C1 was originally identified in 2 flocks of laying hens where 5% of the chickens had wet feces, clinical diarrhea, pasty vents and produced dirty, fecal-stained eggshells (134). Spirochetes were present within the crypts and/or in the lumina of the ceca, and chickens with pasty vents had mild lymphocytic typhlitis.

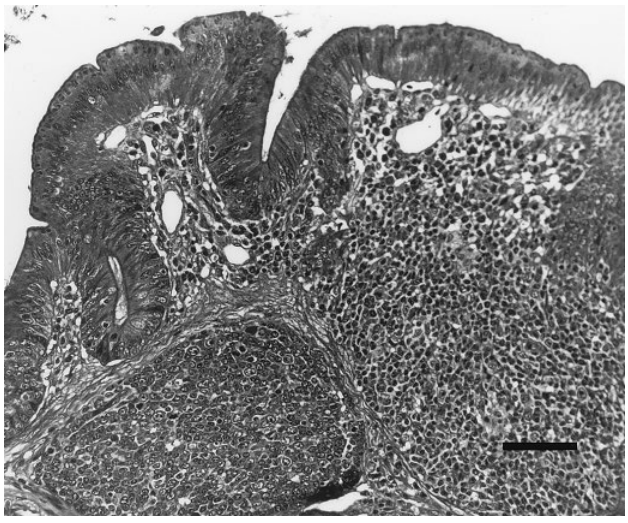


Figure 23.15 Mild lymphocytic typhlitis and mild epithelial hyperplasia in a chicken colonized with *Brachyspira alvinipulli*. Bar = 50 µm. (D.E. Swayne)

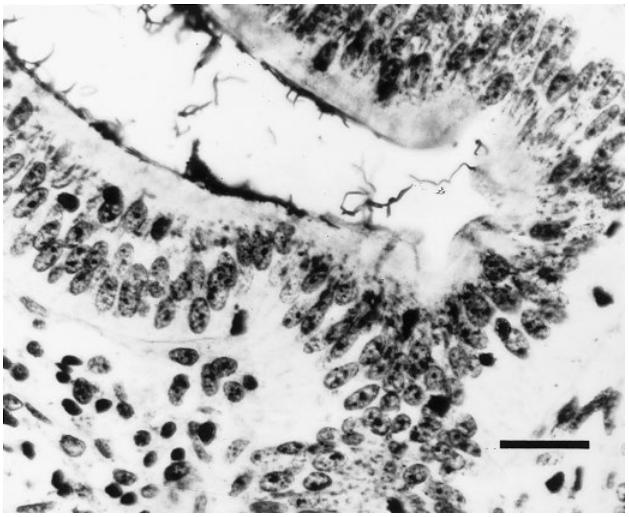


Figure 23.16 Randomly oriented *Brachyspira alvinipulli* spirochetes on the villous surface epithelium in the cecum. Warthin–Starry silver stain. Bar = 20 µm. (D.E. Swayne)

Cases of AIS Where the Spirochetes Were Not Identified. In an early study in the Netherlands, a weakly hemolytic spirochete designated "strain K1" was isolated from a hen on a laying hen farm where there was prolonged intermittent diarrhea and an early decrease in egg production (23). Naturally infected birds had a mild typhlitis. There was a slight increase in numbers of goblet cells, focal "gap-like" lesions in the cecal epithelium that were filled with spirochetes, and mild degeneration and mononuclear cell infiltration beneath the gaps. Ten-week-old hens experimentally infected with mucosal homogenates or strain K1 showed a transient increase in fecal water content, and this recurred after 8–9 weeks.

A study in the UK reported retarded growth rate and delayed onset of egg production in 22-week-old pullets (36). The mucosal crypts of the ceca were distended with sloughed epithelial cells and inflammatory debris, and there was marked mononuclear leukocytic infiltration of the lamina. Spirochete cells were randomly oriented on the cecal epithelial luminal surface or in crypt lumina.

In the Netherlands, observations were made on 8 broiler breeder flocks with a history of AIS caused by uncharacterized spirochetes (115). Flocks with clinical signs had decreased egg production and increased feed consumption. Three percent of eggs produced were too light for successful hatching. Commercial broiler flocks hatched from eggs layed in periods when clinical signs of AIS were present in the breeder flocks showed increased feed conversion and consumption. Weak chicks, retarded growth, and poor feed digestion occurred in the broiler flocks. Antibiotic treatment of the breeder hens before the onset of lay resulted in offspring that performed normally.

Severe Disease

Descriptions of severe disease associated with AIS include the typhlitis seen in rheas naturally infected with *B. hyodysenteriae* (16, 113), where mortality rates can range from 25% to 80%; a similar syndrome in geese infected with *B. alvinipulli*, where 18%–28% mortality was recorded over a period of 2–3 months (97); and in duck flocks infected with *B. hyodysenteriae* or *B. pilosicoli* where there was hemorrhagic to fibrinonecrotic typhlocolitis and 17%–18% mortality (34). Ducks also had renal degeneration with fibrosis and mineralization, hepatic and splenic amyloidosis, and swelling of some metatarsal and phalangeal joints.

Most clinically affected rheas colonized with *B. hyodysenteriae* are older than 6 months (16). Adult birds can be affected, but these cases usually involve concurrent stress such as recent shipping. Clinically, 1–2 days prior to death a few birds may show depression, have reduced body weights, and pass watery feces with caseous cores; however, rheas often die suddenly without clinical signs (113). Ceca are dilated and have thickened walls with ulcerations and lumina containing thick pseudomembranes (113, 133). Cecal walls have severe mucosal necrosis, crypt elongation, hyperplasia of glandular epithelial and goblet cells, and the cecal lumina contain mucus, colonies of spirochetes, bacilli, and fibrinonecrotic debris. Experimental inoculation of 1-day-old chickens and turkeys with intestinal spirochetes from rheas produced similar although less severe lesions (58).

Following the original reports of AIS in rheas, a strongly ß-hemolytic spirochete identified as *B. hyodysenteriae* was isolated (58). Inoculation of 1-day-old common rhea chicks reproduced the gross and histologic lesions within 5–9 days (133). In other cases in rheas, unclassified weakly ß-hemolytic spirochetes have been isolated (113).

Experimental infections of 1-day-old chicks with porcine strains of *B. hyodysenteriae* have resulted in severe changes. These include reduced weight gain, atrophic and thickened ceca with mucus in the cecal lumina, necrosis of the epithelium at the tips of plicae, abundant spirochetes in crypts, an edematous lamina propria with accompanying heterophilic inflammation, epithelial and goblet cell hyperplasia, and crypt elongation (3, 130, 131, 141).

Severe disease associated with *B. alvinipulli* infection has been recorded in 2 flocks of geese in Hungary (97). Following molting at the end of the first egg-laying season, 28% of the 1,500 laying birds in flock A died during an 8-week period and 18% of the 4,500 laying birds in flock B died during a 12-week period. Affected geese had hemorrhagic to necrotic inflammation of the colon/rectum and fibrinonecrotic typhlitis accompanied by severe degeneration. Spirochetes were present in the mucus membrane of the large intestine. The kidneys

were swollen, and some geese had visceral gout. The large intestine had a necrotic epithelial layer and the lamina propria contained hemorrhage as well as infiltration with lymphocytes, histiocytes, and heterophilic granulocytes. Sometimes necrosis extended into the upper third of the lamina. Kidneys had degeneration of the tubular epithelial cells, focal or diffuse intertubular fibroblast cell proliferation, with atrophy of the glomeruli and tubules, and mineral deposition. Lymphohistiocytic inflammation of the liver was observed. Nine isolates were identified as *B. alvinipulli* whereas another (from flock A) was strongly ß-hemolytic but indole negative, and was tentatively identified as *B. hyodysenteriae*. In retrospect, because of its phenotype, it is possible that this may have been *B. hampsonii* (88).

Immunity

Little is known about immunity to intestinal spirochetes in birds, and prolonged colonizations of individual experimentally infected birds have been observed (25). Humoral antibodies to *Brachyspira* spp. preparations may or may not be produced following naturally occurring colonization. Antibodies can be detected in birds from which spirochetes cannot be isolated, and other birds may yield spirochetes on culture but be serologically negative (129, 133).

Diagnosis

Introduction

Gross pathologic and histologic examinations are rarely sufficient to allow an unequivocal diagnosis of AIS. Hence the diagnosis of AIS is usually confirmed using microbiological techniques to identify the associated spirochetes in birds with clinical, pathologic, and/or production data consistent with AIS. This helps to explain why AIS is not frequently diagnosed, despite evidence from surveys that it occurs widely.

Demonstration of Spirochetes

Visualization of helical-shaped bacteria in feces or cecal droppings by dark-field or phase contrast microscopy can be followed up by electron microscopy to look for periplasmic flagella, which are characteristic of spirochetes. Demonstration of spirochete antigens by direct or indirect fluorescent antibody tests (IFAT) or immunohistochemical methods using polyclonal antisera also is possible; for example, IFAT using antiserum raised against *B. hyodysenteriae* was used in the early epidemiologic surveys for AIS (23, 24). However, neither ultrastructural morphology nor identification of antigens by IFAT or immunohistochemical methods using polyclonal antisera will reliably distinguish between spirochete groups or species. Monoclonal antibodies to cell envelope proteins of *B. pilosicoli* have been described (72, 136), and these could increase the specificity of IFAT for identifying this species in chickens. Similar species-specific reagents are needed for the other avian pathogenic spirochetes.

Isolation of Causative Spirochetes

Culturing and further characterization of isolates is important to help identify the spirochete species, and to allow strain typing and determination of antimicrobial sensitivity. The level of detection of culture is dependent on the number of organisms and type and condition of the sample, with fresh cecal droppings or cecal mucosa being optimal material to culture.

Identification of Causative Spirochetes

Phenotypic Properties

Isolated bacteria can be confirmed as spirochetes by their characteristic morphology and motility under dark field or phase contrast microscopy, by the presence of periplasmic flagella observed under transmission electron microscopy, and/or by their reactivity in immunofluorescent microscopy using specific antisera (see previously). As discussed under "etiology", the observation of hemolytic patterns on blood agar and the patterns of biochemical reactivity can allow a presumptive identification of some pathogenic *Brachyspira* species (Table 23.3).

Protein Profiles

In recent years matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry has been adapted for rapid and inexpensive identification of *Brachyspira* species based on their protein profiles, including ribosomal proteins and others with housekeeping functions (20). To date this method has mainly been used with porcine *Brachyspira* species (151), but as the databases expand they will be increasingly useful for identifying all officially named and proposed *Brachyspira* species.

Genotypic Properties

The use of molecular methodology has greatly improved identification of spirochetes, with PCR assays being routinely used on isolated spirochetes or on the growth on primary isolation plates to identify and differentiate *Brachyspira* species (7, 9, 132). PCRs also have been used on spirochetes recovered by laser capture from fixed cecal mucosa of turkeys (114). To date the most reliable PCR assays for amplification of DNA from *B. pilosicoli*

have been based on the 16S rRNA gene, which contains a signature sequence for this species. PCR assays for *B. intermedia* have been less reliable, but, in retrospect, this may be due to the diversity amongst strains currently identified as *B. intermedia* (108). Problems of sensitivity and specificity have arisen using PCR systems for *B. intermedia* based on the 23S rRNA gene sequence and or the NADH-oxidase (*nox*) gene (33, 132), although an improved PCR based on modified *nox* primers appears to work well for this species (30, 92, 106, 107). Whether or not it will prove necessary to develop other PCR systems to identify the different subgroups identified amongst strains currently designated as *B. intermedia* will depend on what is discovered about their biological properties and pathogenic significance (108). Both *nox* and *tly* gene PCRs are regularly used for *B. hyodysenteriae* (32, 64), but to date no reliable direct PCR assays have been described for *B. alvinipulli*.

A number of other schemes have been developed to identify *Brachyspira* species. These involve PCR amplification of specific gene sequences, followed by restriction enzyme digestion of the products to give species-specific banding patterns after gel electrophoresis (restriction fragment polymorphism analysis: RFLP). Genes that have been used in RFLP analysis for identifying different (mainly swine) *Brachyspira* species include the 16S rRNA gene (120), the 23S rRNA gene (75), and the *nox* gene (110, 138). In addition, several studies have used PCR to amplify *Brachyspira*-specific portions of the 16S rRNA or *nox* genes, and then sequenced these products to help identify the species from which they originated (9, 12, 30, 92).

Another adjunct to diagnosis is the use of a fluorescent *in situ* hybridization technique. This uses fluorescent oligonucleotide probes specific for sequences present in the 16S or 23S rRNA of different *Brachyspira* species to visualize spirochetes associated with the mucosa in formalin-fixed tissues (15). This technique has been further modified so that visualized spirochetes are isolated by laser capture microdissection, subjected to direct 16S rRNA gene PCR with subsequent DNA sequencing and analysis (60). The advantage of these techniques is that they provide simultaneous identification and localization of the spirochetes associated with the intestinal mucosa. They should prove particularly useful in investigating aspects of the pathogenesis of AIS.

Strain Typing

Typing of strains of individual *Brachyspira* species can provide important epidemiologic information to help devise control measures. Early studies used MLEE to differentiate intestinal spirochete isolates into species and strains (71, 74, 84, 119, 128), but this technique is too slow and cumbersome for routine diagnostic use and has been replaced by multilocus sequence typing (MLST) (66, 99, 109). Most recently, the advent of next generation sequencing has allowed inexpensive whole genomic sequencing so that MLST can be performed *in silico* without the need to amplify and sequence individual genes: at the same time the genome potentially can be screening for other genes involved in virulence and/or antimicrobial susceptibility (69). Other methods that have been used for strain typing of *Brachyspira* species include PFGE (6, 8, 106, 132, 144), random amplified polymorphic DNA (RAPD) analysis (11, 33), and multiple-locus variable-number tandem repeats (MLVA) analysis (44, 98).

PCR to Detect Spirochetes in Feces

A 2-step nested duplex PCR has been described for direct detection of *B. intermedia* and *B. pilosicoli* DNA extracted from washed chicken feces (107). The first round of PCR amplified genus-specific portions of the 16S rRNA and *nox* genes, whereas the second round used a nested *B. pilosicoli*-specific 16S rRNA gene PCR and a *B. intermedia* specific *nox* PCR. Washing removed potential PCR inhibitors, and a 2-step amplification procedure compensated for any loss of sensitivity associated with the washing step. This assay was rapid and should enhance diagnostic capacity for AIS, especially if it could incorporate a *B. alvinupulli*-specific PCR in the second round of amplifications.

A further advance was the development of a multiplex-quantitative polymerase chain reaction assay for detecting and quantifying *B. hyodysenteriae*, *B. pilosicoli* and *B. intermedia* in chicken and pig feces (116). This was based on amplification of a 198 base pair portion of the *nox* gene, followed by the use of TaqMan probes. The assay could detect 10^2 to 10^3 cells per 0.2 g of feces, giving an improved detection threshold compared to standard PCRs. Multiplex-quantitative polymerase chain reaction assays of this type should become valuable tools for detecting and quantifying low numbers of pathogenic intestinal spirochetes in chicken feces; however, this specific assay has been criticized for failing to detect all strains of *B. pilosicoli* from pig feces due to allelic variation in the *nox* region where the probe bound (19).

Serology

Several serologic tests have been developed to determine exposure of swine to *B. hyodysenteriae*, including an ELISA using recombinant surface proteins (65, 117). Similar assays have not yet been developed for the other *Brachyspira* species that infect birds.

Differential Diagnosis

Spirochetes identified in fecal specimens from poultry should be distinguished from other helical bacteria including *Campylobacter*, *Arcobacter*, *Helicobacter*, and

Spirillum. Wet droppings have a number of causes, and these may interact in a multifactorial way to result in severe problems. In cases of chronic diarrhea or pasty vents, nutritional problems such as excess dietary salt, fats, or raw soybean meal should be investigated. Increased urinary output due to excessive drinking, kidney damage, and incorrect amounts of calcium and electrolytes in the diet can result in wet litter, as can water spillage. Other infectious causes of chronic diarrhea include enteric salmonellosis, colibacillosis, coccidiosis and histomoniasis.

In the case of necrotizing typhlitis other potential causes include *Salmonella*, especially group B serotypes, *Clostridium difficile*, *C. perfringens*, *C. sordelli*, and *Histomonas meleagridis*.

Intervention Strategies

Biosecurity

In farms that do not have AIS, strict biosecurity measures should be put in place to prevent entry. There should be good physical containment (security fencing) around the farm, and bird-proof netting around openings in the houses. Entry of personnel should be restricted, preferably with shower-in and shower-out facilties. Replacement hens should only be obtained from sources known to be free of AIS. The food and water supply should be free of contamination, and particularly protected from fecal contamination from feral waterfowl and other birds. Maintaining biosecurity becomes particularly difficult where birds are not housed exclusively indoors.

Management Procedures in Flocks with AIS

Farms with AIS should practice the same precautions as outlined above, but they also should decrease contact with potentially infectious feces by raising hens off floors, frequently changing litter and removing manure, implementing good rodent and insect control programs, minimizing dietary and molting stress, and providing high-quality feed ingredients whilst avoiding ingredients that enhance spirochete colonization (e.g., wheat). To prevent transmission between flocks on a site, and particularly from older to younger flocks, clean coveralls and boots, and disinfectant boot-dips should be provided at the entrance to each house.

In the case of rheas, it is best to avoid raising them on swine farms, and visiting other rhea farms should be discouraged. Proper cleaning and disinfection of clothing, shoes, and equipment should be done before returning to the home flock following visits to rhea shows, rhea farms, or swine operations. New rhea stocks should only be introduced following a minimum 60-day quarantine with 2–3 negative cloacal cultures for *B. hyodysenteriae*. Birds should be segregated into age groups, and strict biosecurity measures implemented to minimize potential transmission of *B. hyodysenteriae* to susceptible rhea chicks from asymptomatic adolescent or adult birds.

Vaccination

No commercial vaccines are available to prevent AIS. Vaccination with an autogenous *B. intermedia* bacterin failed to prevent colonization of experimental laying hens following challenge with this strain (4).

Treatment

Probiotics

Cell-free extracts of 2 *Lactobacillus* strains have been shown to suppress the growth and motility of *B. pilosicoli in vitro*, and to significantly reduce adherence and invasion of the spirochete in a 3-dimensional avian cecal organ culture model (76). Subsequently, when *L. reuteri* LM1 was added to drinking water of chickens approaching lay prior to experimental infection with *B. pilosicoli*, colonization and signs of AIS were reduced (79).

Essential Oils

Various components of essential oils were evaluated *in vitro* with avian *B. intermedia* isolates, and of these cinnamaldehyde had the lowest minimum inhibitory concentration (MIC). When coated trans-cinnamaldehyde was added to the feed of rearing pullets that were experimentally infected with *B. intermedia*, this material was shown to have both preventative and curative effects (148). Consequently, essential oil components may prove useful to assist in controlling AIS, particularly where the spirochetes show resistance to antimicrobials.

Antimicrobial Treatment

Introduction. No chemotherapeutic compounds appear to have been approved and registered for the treatment or prevention of AIS. Nevertheless, compounds used to treat or prevent swine dysentery and/or porcine colonic spirochetosis should have similar efficacy for treatment of AIS, although limited data are available.

In Vitro *Testing of Antimicrobials.* Antimicrobial sensitivity testing should be conducted using either an approved agar dilution or broth dilution method to establish an MIC. There are only a few publications on *in vitro* antimicrobial susceptibility testing of intestinal spirochetes from birds. A US study examined 2 isolates of *B. pilosicoli* and 2 of *B. alvinipulli* from chickens, and 3 isolates of *B. hyodysenteriae* and 1 of uncertain identity from rheas (140). All 8 isolates were susceptible to tiamulin, lincomycin, and carbadox,

were resistant to streptomycin, and gave strain-dependent results for chlortetracycline, oxytetracycline, tylosin, bacitracin, erythromycin, neomycin, and penicillin.

A larger study investigated susceptibilities of predominantly Australian isolates of *B. intermedia* ($n = 25$) and *B. pilosicoli* ($n = 17$) from chickens (43). Isolates of both species generally were susceptible to tiamulin, lincomycin, metronidazole, and tetracycline. The *B. intermedia* isolates tended to be less susceptible to tiamulin and more susceptible to lincomycin, tylosin, and ampicillin than the *B. pilosicoli* isolates. Although not classed as resistant, 4 isolates of *B. intermedia* had an elevated MIC range for tiamulin (1–4 mg/L), 11 isolates of *B. intermedia* and 5 of *B. pilosicoli* had an elevated MIC range for lincomycin (10–50 mg/L), 1 isolate of *B. pilosicoli* had an elevated MIC range for tetracycline (10–20 mg/L), and 1 isolate of *B. intermedia* and 5 of *B. pilosicoli* had an elevated MIC range for ampicillin (10–50 mg/L). A clear lack of susceptibility to tylosin (MIC >4 mg/L) was seen in 11 isolates each of *B. intermedia* and *B. pilosicoli*, and to ampicillin (MIC >32 mg/L) in 2 isolates of *B. pilosicoli*.

Twenty *B. intermedia* isolates that were recovered from layer flocks in Belgium and the Netherlands between 2008 and 2010 were tested using a broth microdilution method (146). The MIC distribution patterns for tylosin, tilmicosin, lincomycin, and doxycycline were found to be bimodal, with acquired resistance against doxycycline in 3 strains, against the macrolides in 2 strains, and against lincomycin in 1 strain. The MICs of tiamulin and valnemulin showed a monomodal distribution, but with tailing toward the higher MIC values, suggesting low-level acquired resistance in 6 isolates.

A Swedish study investigated antimicrobial susceptibility of 30 *Brachyspira* spp. isolates from commercial laying hens and 18 from free-living wild mallards (*Anas platyrhynchos*) (49). Presumed pathogens (*B. alvinipulli*, *B. intermedia*, *B. pilosicoli*), commensals (*B. murdochii*, *B. innocens*, "*B. pulli*"), and isolates of undetermined species affiliation were included. The laying hens had not been exposed to therapeutic levels of antimicrobials for at least 50 weeks before sampling, and low levels of environmental antimicrobial exposure were presumed in mallards. No isolates with decreased susceptibility to tylosin, valnemulin, tiamulin or doxycycline were found. Decreased susceptibility to lincomycin (MIC 16 µg/mL) was detected in 2 isolates (*Brachyspira* sp.) from laying hens. Five isolates showed decreased susceptibility to ampicillin (MIC 16 to >32 µg/mL), including 2 "*B. pulli*" and 1 *B. alvinipulli* from laying hens, and isolates of *B. pilosicoli* and "*B. pulli*" from mallards. Decreased susceptibility to ampicillin was associated with ß-lactamase activity in 4 isolates. Isolates with decreased susceptibility to ampicillin were present in flocks where fully susceptible isolates of the same species or other genotypes occurred.

Overall these *in vitro* data from different regions suggest that drugs such as tiamulin, lincomycin, and metronidazole should prove useful in the treatment of AIS, regardless of the *Brachyspira* species involved. Nevertheless, *in vitro* susceptibility testing should be undertaken on several representative isolates before antimicrobial therapy is started. To avoid possible toxicity, tiamulin should not be used in combination with ionophores (e.g. monensin, salinomycin, and narasin).

In Vivo *Treatment with Antimicrobials.* Treatment of laying hens with some antimicrobials is problematic because of the withdrawal times needed to avoid residues being present in the eggs. In addition, drugs such as the nitroimidazoles are not available for use in food-producing animals in many legislative areas. There have been several reports on the outcome of antimicrobial treatment of flocks with AIS, as well as of treatment of individual experimentally infected hens.

In a study on a UK laying hen unit with AIS, treatment of immature hens with 125 ppm dimetridazole in-feed for 10 days resulted in improved condition and egg production, and spirochetes were not isolated at postmortem (36). Similarly, in the Netherlands, in-water treatment of infected broiler breeder flocks with 120 ppm Ridzol S (a 5-nitroimidazole) for 6 days resulted in a temporary increase in egg production (115). Lasting effects required early treatment, whereas late treatment did not improve production. Re-infection of birds in some flocks may have resulted from contact with infected litter or from ineffective treatment of parts of a flock. Longer intervals between medications were suggested to increase the numbers of spirochetes shed in the feces.

In an Australian study, 2 houses each of 8,000 40-week-old broiler breeder hens with AIS were treated with antimicrobials in the water (124). The first house received lincospectin at 50 mg per bird per day for 7 days, whereas the second received tiamulin at 25 mg/kg body weight for 5 days. Treatment with lincospectin resulted in slimy feces persisting for several weeks. Hens in the lincospectin-treated house remained negative for spirochetes for 3 months, after which time 30% of fecal samples were spirochete positive. Three weeks after tiamulin treatment ceased, approximately 30% of fecal samples from the house again became spirochete positive, increasing to 80% after another 3 months. Both houses were then water medicated with oxytetracycline at 60 mg/kg for 4 days. This removed the low level of infection from the first house, but only reduced the prevalence from 80% to 60% in the second house. Subsequently, the prevalence in the second house built up to 70% after 4 weeks. It was assumed that reinfection occurred either from the

environment of the houses or from birds that had not received adequate medication to remove the infection.

In a UK study, 3 flocks of approximately 12,000 laying hens on a multiage site were found infected with *B. pilosicoli* (18). In-water treatment with tiamulin at 12.5 mg/kg body weight for 3 days resulted in increased egg production and reduced mortality. More recently, a UK study reported that addition of either 59, 113 or 225 ppm of Denagard (tiamulin hydrogen fumerate) to the drinking water of laying hens that had been experimentally infected with *B. pilosicoli* resulted in significantly reduced spirochete colonization and clinical signs compared with unmedicated birds (155).

In laying hens experimentally infected with *B. intermedia*, both ZnB at 50 ppm in the food and 256 ppm of a dietary enzyme designed to hydrolyse the nonstarch polysaccharides in wheat (Avizyme 1302) resulted in less colonization (41). In a subsequent experiment, 100 ppm ZnB inhibited colonization with *B. intermedia*, whilst hens treated with tiamulin at 25 mg/kg body weight for 5 days became spirochete negative and maintained egg production, although they later became re-infected (40). The use of ZnB is not necessarily recommended for the control of AIS, as 50 ppm in the food resulted in an increased susceptibility of laying hens to infection with *B. pilosicoli* (47, 126). Treatment of broiler breeder hens with either tiamulin at 25 mg/kg body weight for 5 days or with lincomycin at 20 mg/kg for 5 days removed experimental infection with *B. pilosicoli* (127).

Taken together, these studies suggest that treatment with tiamulin, lincomycin/lincospectin, dimetronidazole or even chlortetracycline should assist with control of AIS in adult hens. Regular treatments with courses of antimicrobials, for example given at 1–2 month intervals, together with thorough house cleaning and implementation of strict biosecurity measures to prevent spread of infection between houses may be required for effective long-term control.

For rheas with severe AIS, treatment with dimetridazole (25–50 mg/kg body weight once or twice daily), lincomycin (25 mg/kg twice daily), or erythromycin (15–25 mg/kg once daily) for 5–7 days has been successful in reducing illness and deaths (133).

Tuberculosis

Susan Sanchez and Richard M. Fulton

Summary

Agents, Infections, and Diseases. *Mycobacterium avium* is the main etiological agent of avian tuberculosis. Other mycobacterium species such *M. tuberculosis*, *M. bovis*, *M. genavense*, *M. fortuitum*, and *M. gordonae* have also been reported in pet birds. *M. genavense* is the most common agent of tuberculosis in zoological collections. Growth of these organisms and subsequent speciation is difficult, leading occasionally to incorrect identifications.

Diagnosis. The gold standard for a diagnosis of avian tuberculosis is culture and molecular identification through sequencing. Most commonly it is made based on gross lesions and demonstration of acid-fast bacilli in smears or histological sections. Serological tests are available, but false positives are common. The tuberculin test is useful to determine the presence of tuberculosis in a flock.

Interventions. Removal of the sources of contamination are paramount. In production flocks this includes the removal of all affected birds and equipment from premises. Control of tuberculosis in exotic birds includes avoiding contact with other infected birds and implementation of quarantine before the addition of new birds.

Introduction

Tuberculosis of poultry, often termed avian mycobacteriosis, avian tuberculosis, avian TB, or TB, is a contagious disease caused by *Mycobacterium avium*. Avian tuberculosis is a chronic infection. Persistence in a flock, once established, induces unthriftiness, decreased egg production, and finally causes death. Although tuberculosis in commercial poultry in the United States is rarely diagnosed, tuberculosis still occurs sporadically in backyard poultry and game birds, and it remains an important problem in captive exotic birds.

Public Health Significance

The literature contains a number of instances in which it was claimed that *M. avium* was responsible for a tuberculous infection in humans. In the United States, the first case of avian tuberculosis in humans (with adequate proof) was published in 1938 (18).

With a decline in the incidence of tuberculosis due to *M. tuberculosis* in humans, increasing interest has been directed toward other mycobacteria, that is *M. avium* (14, 72) Moreover, *M. avium* infections have been common in patients with acquired immune deficiency syndrome (AIDS) (38). In the United States, different *M. avium* serovars are isolated from AIDS patients than

from non-AIDS patients (16). One serovar of *M. avium* in particular was commonly isolated from wild birds as well as AIDS patients (27). Based on pulsed-field gel electrophoresis (PFGE), *M. avium* isolates recovered from humans and animals have some relatedness, but the human isolates are more closely related to pig isolates rather than those from birds (17). Classic avian strains are clearly molecularly distinct from human, other mammal, and environmental isolates (35, 48, 70). Thus, it would appear that most human *M. avium* infections in people would more likely be due to human-to-human or human-to-environment contact rather than bird-to-human contact.

History

Tuberculosis in chickens was first described in 1884. Initially, Koch maintained that tubercle bacilli were a single species irrespective of host. However, Rivolta and later Maffucci (32) showed that the microorganism of tuberculosis in chickens was dissimilar to that of bovine tuberculosis. Eventually, Koch (29) declared that tuberculosis of poultry was unlike tuberculosis of humans and that the disease in humans was dissimilar to that of cattle.

Cases of avian tuberculosis in domestic poultry have declined with the development of integrated commercial poultry farming. Cases in chickens and turkeys are predominantly in hobby flocks. The occurrence of avian tuberculosis in birds in zoo aviaries has become an important disease with increasing economic consequences. Certain species of exotic birds have increased in value as they near extinction, thereby increasing the significance of mortalities from avian tuberculosis. Management and control of the disease is difficult because exotic species are often maintained for years. The ability of the *M. avium* to survive in the soil and the lack of adequate procedures for cleaning and disinfecting contaminated premises have become a major obstacle to the elimination of avian tuberculosis from zoologic collections. This is made more complex by the lack of efficacious vaccines or suitable drug-treatment regimens.

Etiology

The cause of avian tuberculosis in chickens and many other bird species across the world is *M. avium* subsp. *avium*. The subspecies (subsp.) classification of bacteria belonging to *M. avium* in recent years has been clarified with the advent of molecular techniques. This classification has further elucidated correlation of certain characteristics such as host predilection and pathogenicity to particular subspecies. Thus, newer methods of classification have allowed *M. avium* subsp. *avium* to be further subdivided into *M. avium* subsp. *avium* and *M. avium* subsp. *hominissuis* depending on their molecular classification. *M. avium* subsp. *avium* is only found to cause disease in birds (11, 34, 40, 53). Furthermore, the presence of IS901 in 97.8% of strains of *M. avium* subsp. *avium* from sick birds is clearly related to their pathogenicity (11, 57). Other subspecies of *M. avium* and other *Mycobacterium* species are also known to cause disease in other birds such as pigeons, wood pigeons, and other free-ranging birds. These cases are sporadic and not associated with poultry production.

Growth Requirements and Colony Morphology

In contrast to *M. tuberculosis* and *M. bovis*, all subspecies of *M. avium* grow at temperatures ranging from 25 °C to 45 °C, although the most favorable temperature range is 39 °C–45 °C. *M. avium* is aerobic. However, for primary isolation, growth is enhanced by an atmosphere of 5%–10% carbon dioxide (49).

Special media designed for culturing tubercle bacilli is desirable for isolation from field materials. Colonies are larger if the medium contains glycerin. Some subspecies of *M. avium* such as *paratuberculosis* and *sylvaticum* require mycobactin as a growth factor for initial and subsequent growth (49). On media containing whole egg or egg yolk and incubated at 37.5 °C–40 °C, small, slightly raised, discrete, grayish white colonies form in 10 days to 3 weeks. If the inoculum has abundant bacteria, colonies will be numerous and coalesce. Colonies are hemispheric and do not penetrate the medium. They gradually change from grayish white to light ocher and become darker as the age of the culture increases.

Subcultures on solid media result in growth within 6–8 days and reach maximal development in 3–4 weeks. Such cultures usually appear moist and unctuous; the surface eventually becomes roughened. The colonies are creamy or sticky and are readily removable from the underlying medium. In liquid media, growth occurs at the bottom of the tube as well as at the liquid surface. Recently, 3 culture media were evaluated to determine the best media to use when culturing tissue and fecal samples from *M. avium*-infected Japanese quail. Modified Herrold egg yolk with mycobactin, Lowenstein–Jensen, and Lowenstein–Jensen with cycloheximide, nalidixic acid, and lincomycin were evaluated. Lowenstein–Jensen media (without additives) provided more positive cultures, had greater numbers of colonies on positive tubes, and had shorter incubation times than the other media (60). Liquid mycobacteria growth indicator tube (MGIT) media was shown to detect more positive samples faster than conventional media; however, a combination of traditional

media and MGIT was necessary to isolate *M. avium* from all positive samples (56).

Traditionally a relationship appears to exist between the type of colony and virulence; *M. avium* with smooth transparent colonies were virulent for chickens; in contrast, variants with smooth-domed, smooth-opaque, or rough colonies were avirulent regardless of source (3). Colony morphology is transient and variable and difficult to standardize. Modern molecular typing methods are more reliable and easy to standardize. In addition, changes in the classification of the group into subspecies may have implications in virulence and colony morphology.

The most characteristic morphologic feature of *M. avium* is its acid-fastness. The organisms are bacillary in morphology, but club-like, curved, and crooked forms are also seen in some preparations. Cords are not formed. Branching infrequently occurs. Most of the bacteria have rounded ends and vary in length from 1 to 3 mm. Spores are not produced and the organisms are nonmotile. Spherical or conical granules occur anywhere within the cytoplasm.

Biochemical Properties

Mycobacterium avium and its subspecies together with *M. intracellulare* form the *Mycobacterium avium* complex (MAC). There are no significant biochemical differences between *M. avium* and *M. intracellulare*. However, they do have features that separate them from other species or groups of mycobacteria. MAC does not produce niacin, does not hydrolyze Tween-80, is peroxidase-negative, produces catalase, does not have urease or arylsulfatase, and does not reduce nitrate; there are variations in these features, particularly in the results of tests for arylsulfatase. MAC lack most amidases except for pyrazinamidase and nicotinamidase. Detailed listings of the biochemical features of MAC and related microorganisms are available (49).

Further identification of mycobacterial cultures may be performed using high-performance liquid chromatography (HPLC) for the identification of mycolic acids or through the use of molecular tests such as 16S rRNA, *hsp65* and *groEL2* sequencing, as well as polymerase chain reaction (PCR) detection of insertion elements (31, 70).

Disease in poultry is primarily attributed to 1 subspecies, namely *M. avium* subsp. *avium*. Culture and clinical presentation may be sufficient for assignation. However, in pet and wild birds, speciation of mycobacterium isolates is warranted because infection has been reported with several subspecies of *M. avium* and other species of *Mycobacterium* including *M. tuberculosis*, *M. bovis*, *M. fortuitum*, and *M. genavense* (25, 26, 28, 46).

Sensitivity to Antituberculosis Drugs

Generally, *M. avium* is more resistant to the commonly used antituberculosis drugs as compared with *M. tuberculosis* and *M. bovis*. This generalized increase in resistance is attributed to its lipid-rich cell wall (42).

In approximately 50 strains of *M. avium* from chickens and swine and 11 from humans, in egg yolk agar, most strains will grow in the presence of 10 mg, but not in 50 mg, of streptomycin/mL, in more than 10 mg of p-aminosalicylic acid/mL, and in more than 40 mg isoniazid/mL medium. On the same kind of medium, *M. avium* is relatively resistant to ethambutol, thionamide, viomycin, and pyrazinamide. The inhibitory concentration is variable, depending on the medium and procedure (49).

Strain Classification

Mycobacterium avium belongs to the slow growing nontuberculous bacteria group and together with *M. intracellulare* form the MAC. The reservoir for MAC organisms is the environment. All members of the MAC have been isolated from animals. Strains of *M. avium* have traditionally been identified by serologic procedures (50). A numbering scheme was developed on the basis of the production of similar polar glycolipid surface antigens for reporting MAC serotypes or serovars (74). Serological typing allowed for the recognition of at least 28 serovars of *M. avium* (serotypes 1–6) and *M. intracellulare* (serotypes 7, 12–20, and 22–28) (71). Using molecular techniques *M. avium* was further subdivided into *M. avium* subsp. *paratuberculosis* isolated from ruminants and free-ranging birds, *M. avium* subsp. *silvaticum* isolated from wood pigeons and other exotic birds, and *M. avium* subsp. *avium* isolated from birds and other domestic animals (13, 33). While *M. avium* subsp. *paratuberculosis* has been recovered by culture from wild birds, it has only been shown to cause lesions in 1 crow despite testing more than 250 birds (2, 8). In 2002, Mijs and collaborators (35) suggested the *M. avium* subsp. *avium* can be further subdivided based on phenotypic and genotypic grounds into *M. avium* subsp. *avium* for isolates originating from birds (serovars 1, 2, and 3) and *M. avium* subsp. *hominissuis* (serovars 4, 6, 8–11, and 21) for isolates recovered from humans and animals. Serovars 1 and 2 occur mainly in animals, whereas 4–20 are commonly found in humans. Some serovars of *M. avium* found in swine (serovars 4 and 8) also have been isolated from humans (73). Serovars 1 and 2 are most commonly isolated from chickens, and serovar 3 is recovered sporadically from wild birds (36).

Distinguishing serovars provides a means for studying origin and distribution of specific strains. This typing method is simple and can be conducted in microtiter plates. However, not all isolates can be reliably serotyped

using this method because some are untypeable; furthermore, interlaboratory reproducibility is low and antigen failure is common. In the past few years it has been demonstrated that a combination of molecular techniques is by far a more accurate method of classifying *M. avium* isolates. Strains have been identified based on the presence or absence of insertion elements (IS) and are further subdivided by restriction fragment length polymorphisms (RFLP). All *M. avium* subsp. *avium* are of the genotype IS1245^{+}, IS901^{+}, IS1311^{+}, and have the IS1245 RFLP unique 3-band pattern called "bird type" for those isolates from birds which correspond to serovars 1, 2, and 3. *M. avium* subsp. *sylvaticum* has the same genotype except that the IS1245 RFLP differs in band size (6, 10, 11, 41). The presence of IS901 in isolates has been associated with virulence for birds (41). Nevertheless, these insertion elements are by nature mobile, and there is the possibility that they can be found in unrelated bacteria. Therefore, they should only be used for further typing isolates already known to be part of the MAC and not for diagnostics. Molecular techniques with more accurate strain classification allow better epidemiologic study of these organisms. Chief among them are sequence-based classifications of the ribosomal operon and housekeeping genes (54). Sequencing of the *hsp65* housekeeping gene is preferred for mycobacteria species identification but will not achieve subspecies separation within *M. avium* unless the 3′ end sequence of *hsp65* is used and then it has only been shown to distinguish among *M. avium* subsps. *avium*, *M. avium* subsps. *paratuberculosis*, and *M. avium* subsps. *hominissuis*. With future progress of molecular tools, newer and more discriminatory typing methods for this dynamic group of organisms will emerge (45, 70).

Pathobiology and Epidemiology

Incidence and Distribution

Avian tuberculosis in chickens is caused by *M. avium* subsp. *avium* (serovars 1, 2, and 3) and is worldwide in distribution, but occurs most frequently in the North Temperate Zone. As stated previously, avian tuberculosis is diagnosed rarely in commercial poultry. It has been diagnosed in 21 hens in the Czech Republic, in small flocks of 30–400 chickens in Canada, in relatively small free-range commercial flocks of 2,000 chickens in Australia, and in a commercial egg laying flock in Spain (20). More recently, an outbreak of *M. avium* was described in a commercial flock of Pekin ducks in China (75). Historical evidence has shown that the highest incidence of infection in the United States was found in flocks of the north central states: North Dakota, South Dakota, Kansas, Nebraska, Minnesota, Iowa, Missouri, Wisconsin, Illinois, Michigan, Indiana, and Ohio. The incidence of the disease in western and southern states is low. The explanation for this is not entirely obvious, although there are several possible contributing factors such as climate, flock management, and duration of infection. The necessity of keeping birds closely confined during winter provides favorable conditions for the spread of the disease.

The difficulty of tuberculin testing all chickens in the United States, or even a majority of the flocks, makes it impossible to obtain exact data on the incidence of *M. avium* infection of chickens. Slaughter data maintained in the United States by the National Agricultural Statistics Service for the years 1995 through 2005 revealed that avian tuberculosis was the cause for the condemnation of young chickens only in 1997 and 1998, at a rate of 7.5 and 6.2 birds/10 million birds slaughtered, respectively. In mature chickens during the same period, avian tuberculosis was diagnosed during 1996, 1999, 2000, 2001, 2002, 2003, 2004, and 2005 at a rate of 2.1, 1,870, 1,630, 14.6, 0.59, 2.4, 18.4, and no birds/10 million slaughtered, respectively. The same agency reported that there were no condemnations at slaughter due to tuberculosis during from 2006 through February 2017. Avian tuberculosis was diagnosed in mature turkeys only in 2003 at a rate of 0.04/10 million turkeys (30, 44). Because visual inspection alone is used to derive these numbers, this figure may represent an under- or overestimation of the true incidence. The diagnosis at inspection was most likely based only on emaciation and the presence of granulomas (5).

From 1985 to 2001, a midwestern animal diagnostic laboratory (Fulton, unpublished data) received 6,059 avian submissions involving 15,097 birds. Only 27 cases (0.45%) involving 36 animals (0.24%) were diagnosed with tuberculosis. Of these cases, only 3 cases (0.05%) (4 animals) were chickens from hobby flocks; 2 cases were in peafowl; and 1 case each in pigeons, doves, quail, and partridges. By far, the largest group represented was exotic captive birds (parrots, toucans, budgerigars, and finches). Three different zoos had diagnoses of avian tuberculosis in such species as penguins, a crane, a duck, an ostrich, and toucans. No cases were seen in commercial chickens or turkeys. From 2008 through 2016, the same laboratory had 1044 avian submissions. Only 1 case of M. *avium* subsp. *avium*, based on 16S RNA sequencing, was observed in a zoo-kept King Penguin during that time period (Fulton, unpublished data).

Avian tuberculosis also occurs in some Latin American countries such as Brazil, Uruguay, Venezuela, and Argentina.

Overall, there has been significant reduction in the prevalence of avian tuberculosis due in part to the changes in poultry husbandry. Increasing emphasis has been placed on the desirability of maintaining all-pullet flocks, rather than older hens.

Historical information from the 1960s reports infection of animals by *M. avium* in certain European countries. It is said to be rare in Finland, but not uncommon in Norway and Denmark; *M. avium* infection occurs in Germany and the United Kingdom. In Australia, avian tuberculosis is unknown in Queensland and West Australia but occurs in other states. In South Africa, the incidence in poultry is low. Infections probably occur in domestic and wild fowl in other countries, but the incidence and distribution cannot be determined because bacteriologic studies are not universally performed. In Kenya, avian tuberculosis has been reported in lesser flamingoes (20). Historical prevalence of tuberculosis in animals may be found in articles by Thoen (63) and Thorel et al. (68).

Natural and Experimental Hosts

Birds

All species of birds can be infected with *M. avium*. Generally speaking, domesticated fowl or captive wild birds are affected more frequently than those living in a wild state. Avian tuberculosis has been reported in domesticated or captive-raised ducks, geese, swans, peafowl, pheasants, quail, partridge, pigeons, doves, turkeys, birds of prey, and other captive and/or wild birds. Pet birds including parrots, cockatoos, budgerigars, finches, flycatchers, and canaries have been infected (18, 58).

Although uncommon, infections and disease may be expected to develop in wild birds in contact with farm premises where avian tuberculosis is prevalent in chickens. Pheasants seem to be unusually susceptible to infection by *M. avium* (58). The disease has also been observed in sparrows, crows, barn owls, cowbirds, blackbirds, eastern sparrow hawks, starlings, wood pigeons, Canada geese, wild turkeys, American bald eagles, painted quail, red-legged partridge, and sandhill and whooping cranes (37).

Avian tuberculosis has been reported in ostriches, emus, and rheas housed in zoologic parks and in a 3-year-old female emu in a commercial flock (55)

Avian tuberculosis, although reported, is not common in turkeys and usually is contracted from infected chickens. Avian tuberculosis has been reported in wild birds. The susceptibility of studied species suggests that domestic chickens, sparrows, ring-necked pheasants, grey partridges, and laughing gulls are highly susceptible; guinea fowl and domestic turkeys are less susceptible; domestic geese and domestic ducks are moderately resistant; while domestic pigeons, collared turtle doves, and the rook are highly resistant to infection.

Avian tuberculosis is more common among birds in many zoologic gardens than in domestic fowl (36). Infections usually result from *M. avium* serovar 1 or serovar 2 as was reported in a zoologic collection of waterbirds (12). Tuberculosis in psittacine birds also may be due to *M. tuberculosis* or *M. bovis* (34). During a 9-year period of identification of *Mycobacterium* spp.-infected pet birds in Switzerland it was found that *M. genavense* (71.8%) was the predominant species followed by *M. avium* complex (16.7%), *M. fortuitum* (4.2%), *M. tuberculosis* (4.2%), *M. gordonae* (2.2%), and *M. nonchromogenicum* (2.2%) (26). In other studies (43), *M. genavense* is a common isolate of zoological collections.

Mammals

Mycobacterium avium can infect and cause disease in some domesticated mammals, but lesions are usually localized (20). Microorganisms may multiply in tissue for a considerable period and induce sensitivity to tuberculin. Disseminated tuberculosis caused by *M. avium* has been reported in rabbits and swine (66).

Although spontaneous infection of mammals may not be of comparable severity to that developed in fowl, it is possible to produce extensive changes in many species of mammals by introducing the infective agent artificially. The relative pathogenicity of *M. avium* for many of the domesticated mammals is summarized in Table 23.4.

In the United States and Europe, *M. avium* serovar 2 is the most common cause of tuberculous lesions in swine (64). Tuberculosis will remain an unnecessary economic burden on the swine industry until it is eliminated from chickens and other barnyard fowl. There has been a gradual but definite decrease of tuberculosis in swine in

Table 23.4 Comparative pathogenicity of *Mycobacterium avium* for certain mammals.

Animal	Susceptibility
Cat	Highly resistant
Cattle	Infection occurs; usually localized
Deer	Infection reported
Dog	Highly resistant
Goat	Assumed to be relatively resistant
Guinea pig	Relatively resistant
Hamster	Susceptible (intratesticular)
Horse	Infection reported
Llamas	Susceptible
Marsupial	Infection reported
Mink	Readily infected
Monkey	Susceptible
Mouse	Relatively resistant
Rabbit	Readily infected
Rat	Relatively resistant
Sheep	Moderately susceptible
Swine	Readily infected

the United States (64). Reasons for the decrease may be the lower incidence of avian tuberculosis in poultry as a result of the increasing practice of maintaining one-age flocks and a change to confinement rearing of swine. In the past when pork prices were high, pork production would expand by using vacant farm buildings (outdated chicken houses). With the advent of contract pork production, a market no longer exists for swine raised in this fashion.

Age of Host Commonly Affected

Avian tuberculosis appears to be less prevalent in young fowl not because the younger birds are more resistant to infection, but because in older birds the disease has had a greater opportunity to become established through a longer period of exposure. Although tuberculosis lesions are usually less severe in young chickens than adult birds, extensive or generalized avian tuberculosis in young chickens has been observed. Such birds are an important source of dissemination of virulent tubercle bacilli and must be considered a source to other fowl and susceptible mammals.

Tuberculosis causes important death losses in captive wild birds of zoo aviaries (36). The significance of these findings is emphasized by reports of disease in valuable endangered species. Numerous reports also are available on tuberculosis in pet birds (20).

Transmission

The tremendous number of tubercle bacilli exuded from ulcerated tuberculous lesions of the intestine in poultry creates a constant source of virulent bacteria. Although other sources of infection exist, none equals infective fecal material in the importance for dissemination of avian tuberculosis. Fecal discharges also may contain tubercle bacilli from liver lesions and mucosa of the gallbladder expelled through the common bile duct. The respiratory tract is also a potential source of infection, especially if lesions occur in tracheal and bronchial mucosa.

The contaminated environment, especially soil and litter, is the most important source for the transmission of the bacilli to uninfected animals. The longer the premises have been occupied by infected birds and the more concentrated the poultry population, the more prevalent the infection is likely to be. In addition, researchers Nishiuchi et al. looking at likely sources of nontuberculous mycobacterial infections in humans found that *M. avium* was found in sediment samples taken from water dams, potting soil, garden soil and house dust (39).

Mycobacterium avium may persist in soil for up to 4 years (51). *M. avium* bacilli remained viable in carcasses buried 3 feet deep for 27 months. Virulent strains of *M. avium* have been found to survive in sawdust for 168 days at 20 °C and 244 days at 37 °C (52).

Mycoacterium avium has been isolated from eggs of naturally infected chickens, but hatched chicks failed to develop avian tuberculosis. *M. avium* does not survive in eggs after 6 minutes of boiling, and in preparation of scrambled eggs, 2 minutes of frying was sufficient to kill the bacteria (19).

Mycobacterium avium can be disseminated in carcasses of tuberculous fowl and offal from chickens dressed for food. Cannibalism might play a part in transmission.

Dissemination of *M. avium* on shoes, equipment, and materials (crates and feed sacks) used in the care and maintenance of infected poultry can be involved in transfer from diseased to healthy flocks.

Wild birds such as sparrows, starlings, and pigeons may be infected with *M. avium* and may spread *M. avium* to poultry flocks. Although not very likely, swine may have ulcerative intestinal lesions from *M. avium* and, thus, constitute a source of infection for other animals and birds (21, 24, 59).

Clinical Signs

Clinical signs are not pathognomonic. In advanced infections, the bird is less lively than its pen mates, fatigues easily, and may have a depressed appearance. Although appetite usually remains good, progressive and striking loss of weight commonly occurs, evident as atrophy of breast muscles with a prominent keel. In extreme cases, the body fat eventually disappears, and the face of the affected bird appears smaller than normal.

Feathers assume a dull and ruffled appearance. Comb, wattle, and earlobes often appear pale and thinner than normal and have a dry epidermis. Occasionally, the comb and wattles have a bluish discoloration. Icterus, indicative of advanced hepatic damage, may be noted.

Even when the disease is severe, the temperature of the affected bird remains within the normal range. In many instances, the bird reveals a unilateral lameness and walks with a peculiar jerky, hopping gait and even paralysis. In those cases, investigation of bone and joints for tuberculous lesions is warranted. Lesions may rupture and discharge fluid with caseous material.

With advanced emaciation, nodular masses can be palpated along the intestine. However, the hepatomegaly that many tuberculous birds possess may make this procedure difficult or impossible. Intestinal nodules may be ulcerative, resulting in severe diarrhea.

Affected birds may die within a few months or live for many, depending on severity or extent of the disease. A bird may die suddenly as a consequence of hemorrhage from the rupture of the affected liver or spleen.

In a flock situation, clinical signs may vary between infected birds. Two clinical syndromes were described in an infected commercial flock of laying hens. One group had good body condition and continued to lay eggs but had nodular masses in the infraorbital sinuses, liver, and

intestines. Another group within the same flock was emaciated, did not lay eggs, lacked sinus lesions, and had numerous nodular masses in internal organs (22).

Pathology

Gross

Lesions are seen most frequently in liver, spleen, intestines, and bone marrow. Some organs, such as heart, ovaries, testes, and skin, are affected infrequently and cannot be considered organs of predilection. For turkeys, ducks, and pigeons, lesions predominate in the liver and spleen but also occur in many other organs.

Lesions of avian tuberculosis in chickens are characterized by pinpoint to several centimeter, irregular grayish yellow or grayish white nodules in spleen, liver, and intestine (Figure 23.17A, B, D). Involvement of liver and spleen results in enlargement, which can result in fatal hemorrhage from rupture. Large nodules have an irregular knobby contour, with smaller nodules present over the capsular surface of affected organs. Lesions near the surface in such organs as liver and spleen are easily enucleated from adjacent tissues. Nodules are firm but can be incised easily. Mineralization is rare. On cross-section, a nodule may contain a variable number of small yellowish foci or a single soft yellowish caseous center surrounded by a fibrous capsule. The capsule continuity may be interrupted by small circumscribed necrotic foci. The fibrous capsule is of variable thickness and consistency, depending on the size and duration of the lesion. It is barely discernible or apparently absent in small lesions and measures 1–2 mm in thickness in larger nodules. Intestines may have white, firm nodules that bulge from the serosal surface. Involvement of lungs is usually less severe than that of liver or spleen.

Granuloma formation is frequent within bone marrow (Figures 23.17C and 23.18). Infection of bone marrow probably occurs very early in the course of the disease and results from the bacteremia.

Microscopic

The basic lesion of *M. avium* infection consists of multiple granulomas with central caseous necrosis. Granulomas consist of the accumulation of large numbers of macrophages with abundant cytoplasm (epithelioid macrophages). Epithelioid macrophage populations expand within the granuloma and fuse near the periphery to form multinucleate giant cells. In larger nodules, the central area of the granuloma may have coagulative or caseous necrosis. In large nodules, only the multinucleate giant cells may persist as a mantle around the necrotic core. Immediately peripheral to the multinucleate giant cells is a collection of both epithelioid and histiocytic macrophages (Figure 23.19). A fibrous capsule consisting of fibrocytes and minute blood vessels also occurs near the outer portion of the peripheral area. Acid-fast bacilli are numerous in the central or necrotic zone of the tubercle but can be found in large numbers in the epithelioid zone adjacent and distal to multinucleate giant cells (Figure 23.20).

The outermost region of the granuloma is encapsulated by fibrous connective tissue, macrophages, some lymphocytes, and an occasional granulocyte. Calcification of the tubercle rarely occurs in fowl. Amyloid deposition in the surrounding parenchymal elements has been reported in liver, spleen, and kidney.

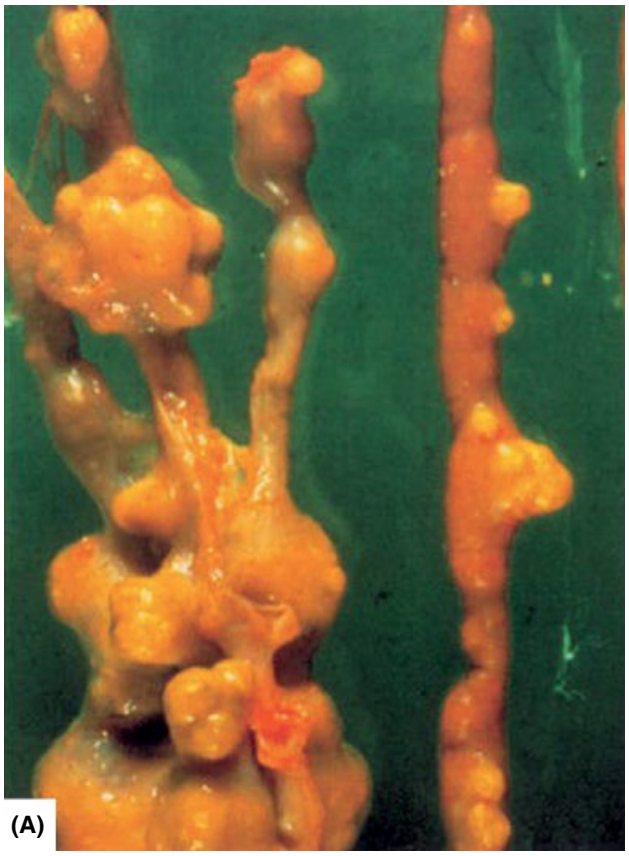
Microscopically, lesions of avian tuberculosis in turkeys vary considerably but are similar to avian tuberculosis of chickens. In other instances, lesions are diffuse, with extensive destruction of surrounding parenchyma. Cytoplasmic masses or large giant cells may be numerous, and large numbers of granulocytes are commonly present. Some lesions become circumscribed by a broad, dense zone of fibrous connective tissue.

Pathogenesis of the Infectious Process

Ingestion of the bacillus results in intestinal infection and eventual bacillemia. Bacillemia allows for the transfer of bacilli from the intestine directly to the liver. The bacillemia, which probably occurs intermittently and perhaps early in most instances, also provides for a generalized distribution of lesions. No tissue, with the possible exception of the central nervous system, appears to be exempt from infection.

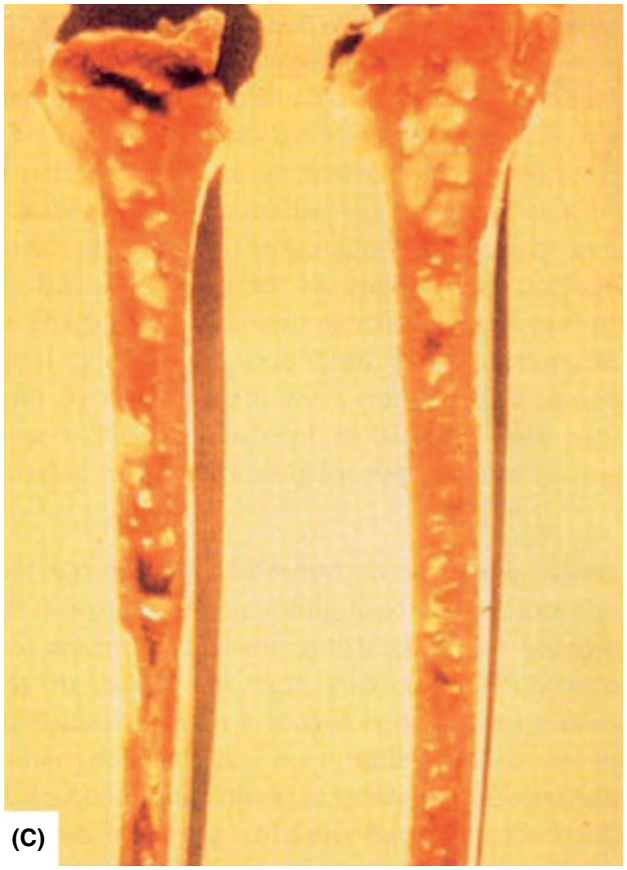
Cheville and Richards (7) studied experimental *M. avium* infection in chickens. The disease course in young chicks lasted for 30 days after intravenous challenge. Single acid-fast bacilli were first found 5 days postinfection in cells of periarterial lymphoid sheaths of the spleen without other histologic evidence of infection. Many bacilli were found within aggregated histiocytic macrophages of the sheath 10 days postchallenge. By day 14, miliary tubercles were found within lymphoid sheaths. Delayed type hypersensitivity (DTH), as judged by wattle thickness, first occurred 2 days postinfection and increased in intensity as the disease progressed (see Figure 23.17E). This response decreased as the disease became more severe. The disease process was divided into 3 periods: a latency period, a lesion development period, and a cachexia period.

The latency period occurred for the first 7 days of the infection. During this period, there were no microscopic lesions, but DTH reactions appeared and increased in intensity with time. The lesion development period occurred from days 8 to 17 postinfection. Bacilli multiplied in lymphoid sheaths during this time. Serum antibody titers developed, the thymus atrophied, and small tubercles with few bacilli developed. Cachexia lasted from day 18 until death. During this period, massive









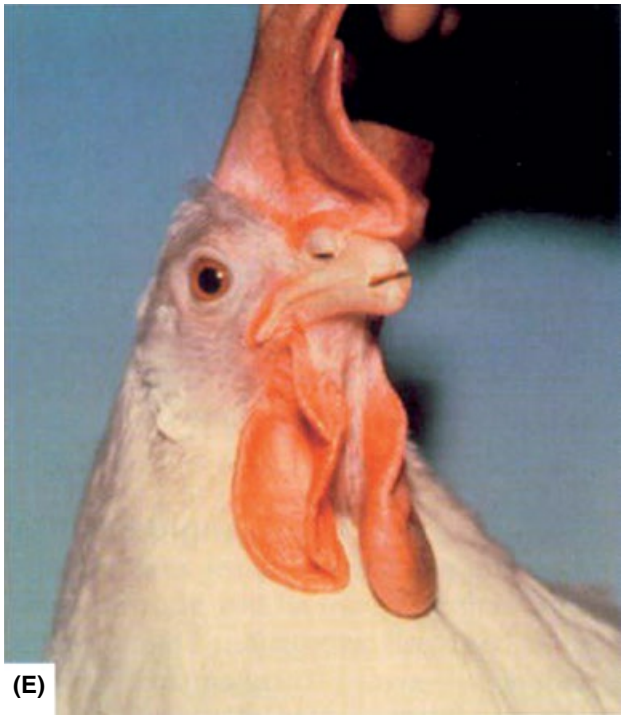


Figure 23.17 Tuberculous lesions in intestine (A), liver (B), bone marrow (C), and spleen (D) of naturally infected chickens. Note the variation in size of granulomas in the liver and spleen. (E) Positive reaction in the left wattle of tuberculous chicken 48 hours after intradermal infection of avian tuberculin. (M.C. Peckham) (*For color detail, please see the color section.*)

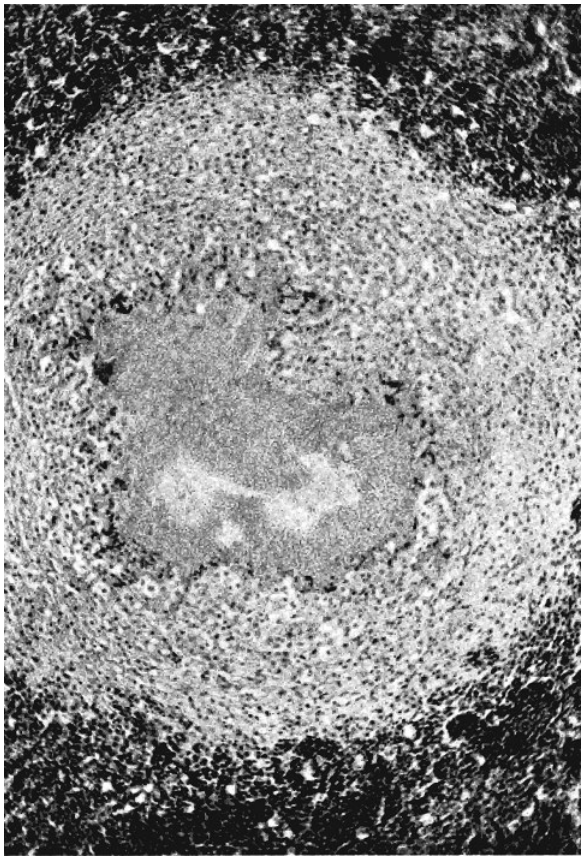


Figure 23.18 Small tuberculous granuloma in bone marrow of a naturally infected chicken. The central necrotic region is surrounded by a zone of dense connective tissue. ×100.



Figure 23.19 Developing tubercle in lung of chicken. ×100.

tubercles with large numbers of bacilli developed, there was lymphoid atrophy, DTH disappeared, and amyloid was deposited at the periphery of tubercles. In addition to lymphocyte-intact chickens, these studies also used both bursectomized and thymectomized chicks. There was very little difference in the pathogenesis between lymphocyte-intact and -depleted chicks.

The capacity of *M. avium* to produce progressive disease may be related to cell wall constituents and certain complex lipids present in the cell wall, such as cord factor, sulfur-containing glycolipids (sulfatides), or strongly acidic lipids (47, 67). Although not yet proven in the avian species, lipoarabinomannan, an outer cell wall component of all mycobacteria including *M. avium*, also may play a role in the pathogenesis through its oxygen radical scavenging, inhibition of protein kinase C, and blocking activation of γ interferon. *M. tuberculosis* and *M. avium* prevent fusion of phagosomes (the vacuole where they reside intracellularly) with lysosome and maturation of the resulting phagolysosome. It appears, however, that the effect of the aforementioned components alone or together on phagosome-lysosome fusion cannot account for virulence.

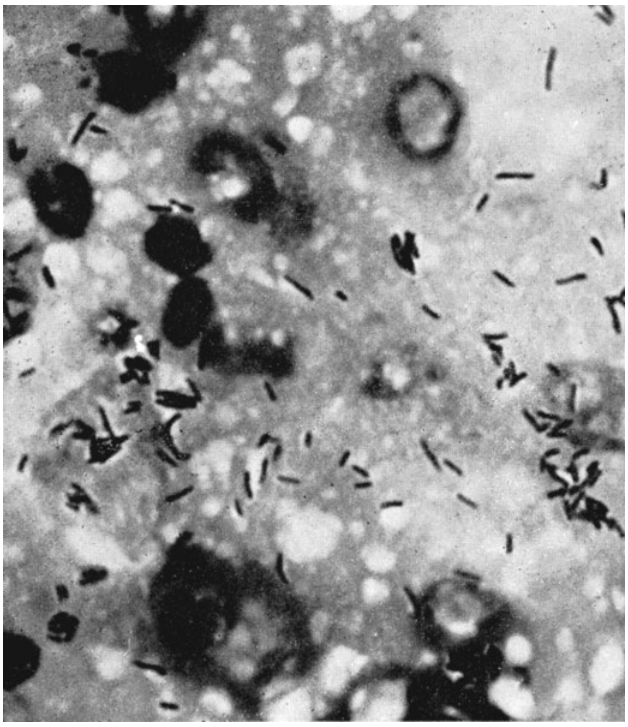


Figure 23.20 Numerous tubercle bacilli in smear preparation from a small lesion of lung of a naturally infected chicken. Ziehl–Neelsen stain, ×1,600.

Delayed type hypersensitivity develops following exposure to mycobacteria; once activated, macrophages demonstrate an increased capacity to kill intracellular *M. avium*. The DTH responses are mediated by lymphocytes, which release lymphokines that act to attract, immobilize, and activate blood-borne mononuclear cells at the site where virulent bacilli or their products exist. Tumor necrosis factor, alone or in combination with interleukin-2, but not γ interferon, has been associated with macrophage killing of *M. avium* serovar 1 (4).

The DTH contributes to accelerated tubercle formation and is, in part, responsible for cell-mediated immunity in tuberculosis. Activated macrophages that lack sufficient subcellular microbiocidal components to kill virulent tubercle bacilli are destroyed by the intracellular growth of the organism, and a lesion develops. A combination of toxic lipids and factors released by virulent *M. avium* may: (1) cause disruption of the phagosome, (2) inhibit phagolysosome formation, (3) interfere with the release of hydrolytic enzymes from the attached lysosomes, and/or (4) inactivate lysosomal enzymes released into the cytoplasmic vacuole. Toxic oxygen metabolites are not responsible for killing activated macrophages. *M. avium* has been shown to induce caspase-1 activity in macrophages and may serve as a mechanism for its pathogenicity.

Diagnosis

A presumptive diagnosis of avian tuberculosis in fowl usually can be made based on gross lesions. Demonstration of acid-fast bacilli in smears or histologic sections of liver, spleen, or other organs strengthens the diagnosis and is sufficient for most diagnostic cases. Inoculation of suitable media to isolate and identify the causative agent confirms the diagnosis of avian tuberculosis and allows speciation of the causative agent (28). In live, suspected infected birds, fecal smears for culture, staining, and/or PCR may be attempted but these tests are not reliable due to intermittent or no fecal shedding of bacilli (69). Fecal positivity increases as the disease course progresses (61). PCR has been used to detect mycobacteria including *M. avium* and *M. genavense* in formalin-fixed tissue, which may further aid diagnostic considerations (23). PCR also may be used to detect mycobacteria in organ samples as well as further differentiate isolates (55).

Tuberculin Test

When administered properly, the tuberculin test provides a satisfactory procedure for determining the presence of avian tuberculosis in a flock. The technique involves intradermal injection of the wattle with 0.03–0.05 mL of a United States Department of Agriculture (USDA) supplied purified protein derivative tuberculin prepared from *M. avium* in a manner previously described (1). The injection site then is monitored for a reaction (see Figure 23.17E). Tuberculin testing in poultry may reveal a false-negative result twice during the course of infection: once during early infection and again during late infection, when there is immune system exhaustion or anergy. This test is also unreliable in some bird species (62).

Serology

Enzyme-linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) has detected mycobacterial antibodies in sera of chickens experimentally inoculated with *M. avium* serovar 2, but false-positives may be common (65). ELISA is less specific than the tuberculin test.

Rapid Agglutination Test

A whole-blood agglutination test has been described for diagnosis of avian tuberculosis in fowl and is a better test than tuberculin for waterfowl. The agglutination test has been more useful for detecting infected birds in a diseased flock; however, occurrence of false-positive agglutination reactions in healthy birds is a drawback.

Differential Diagnosis

The most expedient way to diagnose the disease is by necropsy. Granulomas are rather characteristic, but other conditions must be differentiated. These include coligranulomas (Hjarre's disease), pullorum disease, other *Salmonella* infections, *Staphylococcus* infection, fowl cholera, aspergillosis, and neoplasia. Presence of numerous acid-fast bacilli in lesions is significant. When available, culture and identification of *M. avium* is helpful but not essential for a diagnosis.

Intervention Strategies

Management Procedures

In backyard poultry and captive birds, the tuberculin test should be used to detect avian tuberculosis. Removal of chickens that react reduces environmental contamination and subsequent infections. The whole-blood agglutination test also may serve to detect infected birds and may be more reliable. However, if the residual flock is permitted to occupy the same contaminated premises, contaminated soil may be a continuing source for infection. Furthermore, neither

the tuberculin nor the agglutination tests can be depended upon for detection of every tuberculous fowl. As long as 1 infected bird remains in a flock, dissemination of the disease to healthy fowl is possible. Consequently, termination of the entire flock and repopulation on noninfected soil may be the best approach to control avian tuberculosis.

Procedures for establishing and maintaining avian tuberculosis-free backyard flocks should include the following: (1) Abandon old equipment and establish other facilities on new soil. Ordinarily, it has been impractical to render an infected environment satisfactorily safe by disinfection. (2) Provide proper fencing or other measures to prevent unrestricted movement of chickens, thus preventing exposure from previously infected premises. (3) Eliminate the old flock, burning carcasses of birds that show lesions of tuberculosis. (4) Establish a new flock in the new environment from avian tuberculosis-free stock. If chickens in a clean flock are prevented access to an infected environment and are protected against accidental exposure to an infected environment and accidental exposure to *M. avium*, it is reasonable to believe that they will remain free from avian tuberculosis.

Recommendations for control of avian tuberculosis in exotic birds include the following: (1) Prevent contact with tuberculous birds; premises and housing previously used by them are to be avoided. (2) Quarantine additions to the aviary for 60 days and retest with avian tuberculin.

Vaccination

Use of experimental vaccines containing inactivated and/or live mycobacteria for protecting chickens against tuberculosis has been evaluated. The best results were obtained in chickens vaccinated with live *M. intracellulare* serovar 6 (*M. avium* serovar 6) given orally. These fowl showed 70% protection after intramuscular challenge with *M. avium*. Encouraging results were also reported in chickens after combined intramuscular vaccination with inactivated plus live *M. intracellulare* serovar 7 and serovar Darden (*M. avium* serovars 7 and 19). Recently, vaccination of chickens using various fractions of a homologous strain of *M. avium* for vaccine production and challenge revealed that the number of lesions and bacilli/gram of liver were decreased; it did not, however, prevent infection (15).

Treatment

Treatment with antibiotics is impractical and is rarely performed to treat domestic backyard poultry. However, combinations of antituberculosis drugs have been used to treat certain exotic birds maintained in captivity. Clinical remission was observed in 3 birds that received a combination of isoniazid (30 mg/kg), ethambutol (30 mg/kg), and rifampicin (45 mg/kg). The recommended duration of therapy was 18 months, provided that there were no adverse side effects. More information concerning treatment may be found in Dhama et al. (9).

Miscellaneous and Sporadic Bacterial Infections

Tahseen Abdul-Aziz

Summary

Agent, Infection, and Disease. A variety of bacteria have been isolated frequently or infrequently from different lesions in birds. Infections with some bacteria included in this subchapter are uncommon and of unknown prevalence, being reported only from certain parts of the world. Other bacteria, such as *Pseudomonas aeruginosa*, commonly cause infection and high mortality in young chicks.

Diagnosis. Diseases caused by miscellaneous bacteria usually have lesions that arouse suspicion of a bacterial etiology. However, swabs and/or fresh tissues need to be collected for bacterial isolation and identification and/or for other ancillary test such as histopathology and molecular methods.

Prevention. Knowing the route and source of infection is an important factor in developing a strategy to prevent infection with different bacteria. Sanitation and reasonable hygienic measures should be applied to minimize the possibility of exposure to harmful bacteria, most of which are typically found in the environment.

Introduction

Surveys of bacteria isolated from poultry often include a variety of unusual bacteria of low incidence. Similarly, microbial surveys of eggs, dead embryos, chicks with omphalitis and yolksacculitis, and chick mortality frequently reveal a diversity of bacteria, generally in low incidence that typically are found in the environment and are not normally associated with disease. Because the importance of isolates recovered infrequently in surveys is unknown, or not considered significant, they are not covered in detail in this review. Additionally, interest in achieving and maintaining

intestinal homeostasis in meat-producing birds has led to methods to quantify microbial populations by molecular methods. Organisms identified in these studies also are not discussed, nor are organisms considered to be normal flora.

Acinetobacter

The genus *Acinetobacter* belongs to the family Moraxellaceae. *Acinetobacter* spp. are nonfermentative, nonmotile, oxidase-negative, strictly aerobic, small, Gram-negative coccobacilli (224). The organism is occasionally recovered from dead-in-shell embryos and weak chicks (108, 130). *A. lwoffi* and *A. calcoaceticus* were isolated from outbreaks of septicemia in hens. Mortality was approximately 15%, and there was multifocal necrosis and green discoloration of the liver (69, 110). Turkeys also are affected as *Acinetobacter* has occasionally been isolated from dead-in-shell embryos and weak poults, respiratory disease, septicemia, and inflamed joints (70). Other clinical presentations include pigeons with arthritis (66) and ducks with arthritis, septicemia, or airsacculitis (18, 233).

Aegyptianella

Aegyptianella pullorum is an obligate intracellular organism in the family *Anaplasmataceae*, order Rickettsiales, which is most closely related to *Anaplasma* spp. (180). It causes the tick-borne disease aegyptianellosis (84). The disease occurs in tropical and subtropical areas and has been identified in a variety of birds including chickens, turkeys, and guinea fowl. With the exception of wild turkeys in the Rio Grande area of Texas (35) and an Amazon parrot imported into England from South America (170), the organism has only been identified in Europe, Asia, and Africa.

Affected birds experience increased mortality and develop severe anemia, which can predispose them to ascites and right ventricular heart failure (100). Aegyptianellosis occurs primarily in free-ranging poultry and wild birds that are infested with fowl ticks of the genus *Argas*. Diagnosis depends on identifying the typical organism in erythrocytes of infected birds (84). In stained blood smears the organism appears as purple, 0.3–4 µm diameter intracytoplasmic inclusions (180). Treatment with tetracyclines and supportive care are generally effective (84). Prevention is the same as that for spirochetosis (see *Borrelia*, later).

Aeromonas

Aeromonas is a small Gram-negative rod that is commonly found in aquatic environments. It frequently colonizes the intestines of animals and can contaminate poultry carcasses during processing. *Aeromonas* has public health significance because it can cause intestinal and extraintestinal diseases in people including gastroenteritis, septicemia, necrotizing fasciitis, and myonecrosis (103).

Aeromonas hydrophila, either alone or in combination with other organisms, causes localized and systemic infections in avian species including poultry (78, 194). *Aeromonas* was recovered from turkeys experiencing severe diarrhea. Inflammation and hemorrhage of the intestinal mucosa were characteristic findings in affected poults. Experimental inoculation of chicks with the turkey isolate caused significant mortality (76). *Aeromonas* was among the organisms identified from cellulitis lesions in turkey carcasses at processing (165). *A. hydrophila* has been isolated from ducks with salpingitis (19), septicemia (129), airsacculitis (233), and granulomatous inflammation of salt glands (115). A bacterin prepared from 3 strains that caused high mortality in experimentally inoculated ducklings successfully controlled losses in commercial duck flocks (129). *A. formicans* has been isolated infrequently from arthritic lesions in ducks at processing (21). *Aeromonas* and *E. coli* were the most frequently isolated bacteria from geese with necrotic inflammation of the phallus (see Goose Venereal Disease, later) (138). *Aeromonas* caused severe necrotizing enteritis and septicemia in a 10-year-old ostrich. Pure cultures were isolated from the intestine, liver, lung, and trachea of the bird (72).

Aeromonas is among environmental bacteria that can be recovered from dead-in-shell embryos and weak chicks (130). Microbial contamination of ostrich eggs by *Aeromonas* is associated with reduced hatchability (61).

Arcanobacterium (Actinomyces)

The genus *Arcanobacterium* belongs to the family Actinomycetaceae. Organisms are pleomorphic, Gram-positive, facultative anaerobes that do not form spores. Colonies are small, hemolytic, and grow slowly under microaerophilic conditions. They colonize the skin and mucous membranes (227). *A. pyogenes* was originally classified as *Actinomyces pyogenes*, then as *Corynebacterium pyogenes*, and now as *Trueperella pyogenes*.

A chronic, disseminated granulomatous disease of turkeys suspected to be actinomycosis has been observed sporadically (191). Serious outbreaks of osteomyelitis involving the proximal tibiotarsi and/or thoracic vertebra caused by *A. pyogenes* in commercial male turkey

flocks resulted in considerable economic loss (27). Lame birds in 20 affected flocks averaged 20% (range 5%–50%), age averaged 16 weeks (range 12–20 weeks), and weekly mortality averaged 2.8% (range 0.5%–10.5%). Hen flocks were not affected (10). Club-shaped, pleomorphic, Gram-positive bacilli in smears of lesions provided a rapid diagnosis (27). Osteomyelitis was reproduced in 15-week-old male turkeys inoculated intravenously with a representative isolate.

Septicemia, visceral lesions, cutaneous abscesses, mortality of nearly 14%, and a decrease in egg production of more than 27% occurred in caged layers infected with *A. pyogenes*. Portal of entry was through skin lesions caused by poor caging (53).

Bacillus

Bacillus spp. occasionally have been associated with embryo mortality and yolk sac infections in chickens (40, 60, 61, 225), turkeys (28), ducks (12), and ostriches (60, 61). *Bacillus* spp. and *E. coli* were the most commonly cultured bacteria from reproductive disorders of hens (83). *Bacillus cereus*, an organism that can cause foodborne illness in people, infected turkey hens following artificial insemination and was found in 25% of their unhatched eggs. The prevalence fell to 4% after the infection was controlled (28).

Borrelia

Borrelia are highly motile, helical spirochetes that stain well with aniline dyes, hematologic stains, and silver impregnation (Figure 23.21). Spirochetes can be readily identified in wet smears of blood or tissues by dark-field or phase microscopy (44).

Borrelia anserina causes nonrelapsing, tick-borne spirochetosis in avian species including chickens, turkeys, pheasants, geese, and ducks in tropical and subtropical areas. Occasional outbreaks have been identified in the southwestern United States in chickens, turkeys, and pheasants (52). Extensively reared free-range flocks are more likely to be affected than confined flocks, and indigenous breeds of chickens are generally more resistant than exotic breeds (176). The disease is usually an acute septicemia characterized by high morbidity and mortality, but may be mild if birds are infected with low-virulent strains (11).

Birds can also develop asymptomatic infections with *B. burgdorferi*, the cause of Lyme disease in people, and serve as hosts for ticks capable of spreading the spirochete to mammals (46, 111, 114, 136, 188). Wild turkeys also are hosts for *B. lonestari* and *B. miyamotoi* (105, 189).

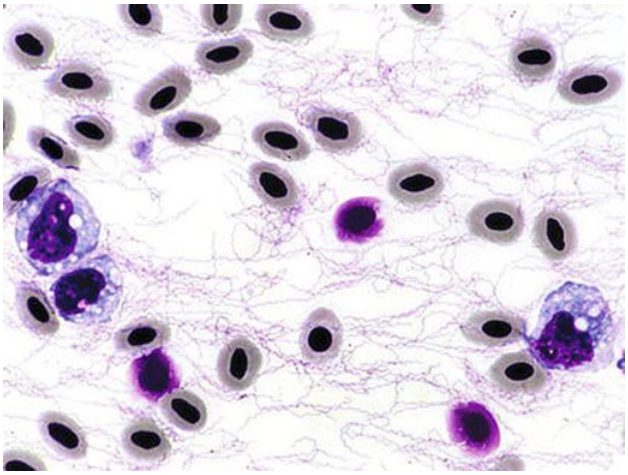


Figure 23.21 *Borrelia anserina* in blood film during the acute stage of infection. Giemsa, ×1,200.

No clinical disease has been recognized in birds infected with *Borrelia* species other than *B. anserina*.

Occurrence of spirochetosis corresponds with the distribution of fowl ticks of the genus *Argas*, which serve as both the reservoir and primary vector. Attempts to transmit *B. anserina* with the tick *Amblyomma cajennense* were unsuccessful (121). In addition to ticks and other biting arthropods (mosquitoes, mites), infection can result from: cannibalism; scavenging on carcasses; multiple use of syringes and needles; or ingestion of infective blood, droppings, or infected ticks. Virulent strains are capable of penetrating unbroken skin. *B. anserina* is not resistant outside of the host. Recovered birds are not carriers; organisms disappear from tissues at or shortly after they disappear from the circulation (11).

Birds infected with virulent strains of *B. anserina* are visibly sick, with cyanosis evident in the comb and wattles, ruffled feathers, dehydration, inactivity, and anorexia. A marked elevation in body temperature begins shortly after infection accompanied by rapid weight loss. Affected birds pass fluid green droppings containing excess bile and urates, and have increased water consumption. Late in the disease, birds develop paresis or paralysis, become anemic, and are somnolent to comatose. Body temperatures are subnormal just prior to death. Birds recovering from the disease are often emaciated and have temporary residual weakness or paralysis (11). Infection with low-virulent strains may be mild or inapparent (52).

Marked enlargement and mottling of the spleen is typical of spirochetosis (Figure 23.22) but may not be evident when birds are infected with low-virulent strains (53) or early in the disease. Livers often are enlarged and contain small hemorrhages, pale foci, or marginal infarcts. Kidneys are swollen and pale with excess urates distending the ureters. Green, mucoid intestinal



Figure 23.22 An enlarged, mottled spleen is characteristic of spirochetosis caused by highly virulent strains of *Borrelia anserina* in chickens. Low-virulence strains may not produce splenic lesions. Spleens may appear differently in other avian species depending on the amount of necrosis and hemorrhage. A few serosal hemorrhages on the proventriculus also can be seen in this bird.

contents are usually present, and often there are variable amounts of hemorrhage, especially at the proventriculus-ventriculus junction. Fibrinous pericarditis occurs infrequently. Extensive hemorrhage and muscle necrosis occur in naturally infected pheasants (11).

Splenic lesions result from macrophage and lymphoid hyperplasia, erythrophagocytosis, and hemosiderin deposition. Multifocal necrosis and hyalinization of white pulp and/or extensive hemorrhage may be present in some birds. The liver is congested with increased periportal infiltrates of mixed lymphocytes, hemocytoblasts, and phagocytic cells with vacuolated cytoplasm. Erythrophagocytosis and hemosiderin are seen in Kupffer cells. Extramedullary hematopoiesis may be present. Lymphoplasmacytic infiltrates occur in kidneys and intestinal lamina propria of some birds. Occasionally, there is mild to moderate lymphocytic meningoencephalitis (8, 11).

Spirochetosis can be tentatively diagnosed by finding characteristic lesions in birds with signs consistent with the disease. Larval ticks on the birds, evidence of tick bites, or presence of ticks in the bird's environment increases the likelihood of spirochetosis. Diagnosis is confirmed by demonstrating *B. anserina* in blood or tissue sections. In chickens exposed to ticks (*Argus miniatus*) infected with *B. anserina*, spirochetes were found in blood smears prepared from the exposed birds between day 5 and day 12 postexposure, with the peak number of spirochetemic birds occurring between days 7 and 9. Spirochetes were not found in blood smears from any of the exposed birds after day 13 (132).

Borrelia cannot be cultured on routine bacteriologic media but will grow in chick embryos following yolk sac inoculation or in susceptible young chicks or poults (43). It can be grown in liquid medium but loses virulence (127). Bursectomy or dexamethasone treatment of chicks may be necessary to detect low-virulent strains (58). Isolates are usually maintained in ticks, day-old chicks, chicken or turkey embryos, or by cryopreservation (-70 °C or in liquid nitrogen) in 5% glycerol or dimethylsulfoxide added to infective blood (43, 120). Several serologic methods have been used to detect antibodies in immune birds. Spirochetal antibodies occur in the yolk of eggs from immune hens (43).

Arsenicals and most antibiotics, including penicillin, chloramphenicol, kanamycin, streptomycin, tylosin, and tetracyclines, are effective in treating infected birds. Intramuscular injections of penicillin at 20,000 IU/bird given 3 times in 24 hours or 20 mg oxytetracycline given daily for 2 days represent current treatment regimens (11).

Active immunity follows recovery or immunization. Immunity is serotype-specific; infection with other *B. anserina* serotypes can occur in recovered or vaccinated birds. An autogenous or polyvalent vaccine containing multiple serotypes may be necessary to provide full protection (226). Controlled infection followed by antibiotic treatment 3 days later also has been used to induce active immunity. Passive maternal immunity provides protection for 5–6 weeks (11). Preventing fowl tick infestation is the best method to control spirochetosis in endemic areas. Young chickens in dense poultry areas during the summer are more likely to be infested with fowl ticks (193). Adult ticks can remain alive without feeding and carry the spirochete for as long as 3 years (11).

Citrobacter

Citrobacter is a genus in the *Enterobacteriaceae* family. The organism commonly colonizes mucous membranes of the respiratory and digestive tracts of normal birds, but can be an opportunistic pathogen. *Citrobacter* is 1 of many environmental bacteria that are occasionally isolated from unhatched eggs, weak chicks, and yolk sac infections (130, 225). It has been isolated from the liver of 2-week-old turkey poults with respiratory disease (70) and was isolated from 10% of airsacculitis lesions in processed broilers (197). *C. freundii* infrequently has been isolated from young ducks with salpingitis (19). *C. murliniae* was isolated from visceral organs of clinically sick and dead ducks, quail, and chickens (90).

Coenonia

Coenonia is a genus in the family Flavobacteriaceae that contains a single species, *C. anatina*, which was previously identified as *Riemerella anatipestifer*-like taxon 1502. It causes an exudative septicemia in ducks and geese (222).

Coryneform Bacteria

A Gram-positive, pleomorphic, coryneform bacterium with certain characteristics of *Erysipelothrix*, *Lactobacillus*, and *Listeria* was isolated from an outbreak of polyarthritis in chickens (148). *Corynebacterium* spp. accounted for 18% of 132 isolates from blood samples, livers, and hock joints of clinically ill commercial broilers within 2 weeks of processing (6).

Coxiella

Coxiella burnetii is an obligate intracellular, Gram-negative bacterium that causes Q fever in humans. It can also infect different animal species including mammals, reptiles, fish, and ticks. Phylogenetically, it is related to the genus *Legionella*. The bacterium resides within cytoplasmic parasitophorous vacuoles in the infected cell (56, 224, 239).

Infection with *Coxiella*-like organisms has been described in psittacine birds, a toucan, and lorikeets. Affected birds were either asymptomatic or weak and lethargic. Emaciation, hepatomegaly, and splenomegaly were seen in affected birds. Histologic lesions included hepatitis, hepatic necrosis, epicarditis, myocarditis, cardiomyopathy, enteritis, splenic granulomas, nephritis, pneumonitis, and lymphohistiocytic encephalitis. A consistent lesion in parakeets was histiocytic vasculitis and perivasculitis with formation of small microgranulomas adjacent to affected vessels. Infiltrating macrophages had cytoplasmic, lightly basophilic, granular inclusions that represented the organisms. There are variations in the reported results of the staining properties of the inclusions in tissue sections with different histochemical stains (196, 224, 239). Infection of poultry has not been reported, but there is serologic evidence that it occurs (154).

Enterobacter

Enterobacter is a normal inhabitant of the avian digestive tract (17). Similar to other Gram-negative bacteria in the *Enterobacteriaceae* family, it can infect eggs and young birds causing embryo loss, omphalitis, yolk sac infections, and mortality in young birds (70, 130, 185, 225, 234). *Enterobacter* has been isolated infrequently from turkeys with cellulitis (163).

Flavobacterium

Flavobacterium is a dominant proteolytic bacterium in the upper respiratory tract of chickens and turkeys (32) that is rarely associated with clinical disease. It has been recovered from ducks with arthritis (18), an adult goose with salpingitis (19), chickens, a pigeon, a finch with septicemia and/or arthritis (221), and unhatched eggs and weak chicks (130). Heavy, pure cultures of *F. meningosepticum* were obtained from a 5-week-old ostrich chick that failed to grow and thrive and had airsacculitis, pneumonia, and thymic atrophy/hypoplasia (125).

Gallibacterium

The genus *Gallibacterium* is a member of the family *Pasteurellaceae*. Bacterial isolates of avian origin formerly identified as taxons 2 and 3 were classified within the genus *Gallibacterium*. These isolates were from salpingitis in ducks, salpingitis and septicemia in geese, septicemia and pneumonia in pigeons, septicemia in turkeys, pneumonia in pheasants, and septicemia in parakeets and budgerigars. Four species (*G. anatis, G. salpingitidis, G. melopsittaci*, and *G. trehalosifermentans*), 3 new genomespecies (*Gallibacterium* genomespecies 1, 2, 3), and an unnamed taxon (Group V) were proposed. *G. salpingitidis* includes isolates from salpingitis in ducks and geese (23, 41).

Gallibacterium anatis was formerly classified as *Pasteurella hemolytica*-like, *Actinobacillus salpingitidis*, or *Pasteurella anatis* (41). The organism is known to be a normal inhabitant of the upper respiratory tract and lower reproductive tract of chickens, but is also considered a cause of salpingitis and peritonitis in laying hens (104). Chickens, ducks, geese, and ostriches have been affected. In a study involving 31 flocks of table egg layers, *G. anatis* was isolated from the heart, liver, spleen, intestine, and reproductive tract of hens in several flocks (158). Field isolates from septic laying hens and a well-characterized strain of *G. anatis* were pathogenic for pullets and layers following inoculation (25, 207, 208). Mortality was higher in birds inoculated intravenously compared with those inoculated intraperitoneally, and lesions were more severe in experimentally immunosuppressed birds (25). Twelve-week old specific-pathogen-free leghorn chickens inoculated intranasally with 1 of 3 strains of *G. anatis* developed histologic lesions of varying severity in the trachea, lung, air sacs, and liver, with no clinical signs or mortality (244).

Bacteria originally reported as atypical *Actinobacillus lignieresii*, which were later classified as taxon 2 and taxon 3 (currently classified as *Gallibacterium*) (23), were isolated from lesions of salpingitis in egg-laying ducks and geese. Isolation from the cloaca and penis of normal geese suggests that salpingitis probably results from an ascending infection (19). A similar conclusion was reached about the role of *Actinobacillus* in goose venereal disease (see previously) (137).

Hafnia

Hafnia is a Gram-negative rod-shaped bacterium in the family *Enterobacteriaceae. H. alvei* infrequently has been identified as a cause of septicemia in pullets and laying hens (33, 177). Infections were characterized by loss of appetite, diarrhea, opisthotonus, decreased egg production, and increased mortality. Scattered pale foci in the liver, hepatomegaly, splenomegaly, and catarrhal to hemorrhagic enteritis were seen on necropsy. Microscopically (1) degeneration, multifocal necrosis, and inflammation of the liver, (2) lymphocytic depletion and necrosis of the spleen, and (3) intestinal hyperemia, hemorrhage, and catarrhal enteritis were identified. Gram-negative bacteria were numerous within lesions, occurring frequently as intravascular emboli.

Helicobacter

A distinct group of bacteria, previously identified as *Campylobacter*-like organisms, has been placed into the genus *Helicobacter* based on their phenotypic characteristics and 16S rRNA sequences (166). *Helicobacter* species are Gram-negative, curved or spiral-shaped bacteria. Species in this genus are separated into 2 groups: gastric helicobacters and enterohepatic helicobacters (135).

Helicobacter pullorum, a species in the enterohepatic group, has been identified in intestinal contents of broiler chickens, laying hens, guinea fowl, and turkeys (135, 243), and livers and intestines of layers with lesions characteristic of "vibrionic hepatitis" (31). The organism has been found infecting chickens in Europe and Australia (37, 142). *H. pullorum* may have public health significance, because there are reports of its association with gastroenteritis, bacteremia, and liver and gall bladder diseases in humans (31, 109, 199, 201, 213).

Helicobacters can be cultured using procedures for isolating campylobacters; however, they are inhibited by polymyxin B, which was used in some older media formulations. A PCR to detect the organism has been developed (31, 77, 199). A multiplex PCR is useful for identifying and differentiating *Arcobacter*, *Campylobacter*, and *Helicobacter* (81, 157). Specific identification requires a combination of phenotypic and genotypic analyses (77, 141, 166, 200).

Helicobacter canadensis, a species closely related to *H. pullorum*, has been found in geese, guinea fowl, and pheasants. It also has been identified from people with diarrhea (156, 181, 229).

Two additional *Helicobacter* species, *H. anseris* and *H. brantae*, infect Canada geese. Although these 2 *Helicobacter* species are suspected to be possible human pathogens, they have not been implicated in human disease. Environmental contamination of parks by feces from geese infected with these organisms is considered a potential public health concern. Another avian species (*H. pametensis*) has been described from a tern (64) and other unnamed, distinct strains have been isolated from avian species (192).

Klebsiella

Klebsiella is an environmental contaminant that occasionally causes embryo mortality, yolk sac infections, and mortality in young chickens, turkeys, and ostriches (108, 130, 167, 172, 185, 186, 225, 234).

The organism has been associated with cutaneous, respiratory, ocular, systemic, and reproductive diseases of poultry. *Klebsiella* was among aerobic bacteria isolated from turkeys with cellulitis (80). *Klebsiella* was isolated from turkey flocks with adenoviral inclusion body tracheitis that experienced respiratory disease and increased mortality. An outbreak of ocular disease caused by *Klebsiella* affected a flock of 4-week-old chickens (133). *Klebsiella* and *Staphylococcus aureus* were isolated from a septicemic disease in 20-week-old layers experiencing increased mortality. Mortality and clinical disease followed oral inoculation of young chicks with 3 *Klebsiella* biotypes. Chicks inoculated with *K. pneumoniae* had the highest mortality (62). *Klebsiella* has been infrequently isolated from reproductive diseases including salpingitis and oophoritis in hens (14, 195). Localized and systemic infections with *Klebsiella* occur in young ostriches causing "ostrich fading chick syndrome," an often-fatal disease of birds younger than 3 weeks of age (214). Hydroponically grown alfalfa sprouts being fed to the birds were heavily contaminated with the organism and believed to be the source of infection (234).

Lawsonia

Lawsonia intracellularis is an obligate, intracellular, *Campylobacter*-like organism that causes proliferative enteropathy in a variety of animals, especially pigs, horses, and hamsters (124). Among avian species the disease has been reported in young ostriches and emus (47, 126) and broiler chickens (163). Infection of ratites was associated with increased mortality, poor growth, diarrheal disease, tenesmus, and rectal prolapse. Affected intestinal mucosa was thickened and rugose. Enterocyte proliferation, crypt changes, and infiltration of the mucosa with mixed inflammatory cells were seen microscopically. Intraepithelial, comma-shaped bacteria were visible with Warthin–Starry silver staining in the apices of enterocytes, which were identified as *L. intracellularis* by specific immunofluoresence. Affected birds responded to treatment with chlortetracycline (126). Proliferative

enteritis and typhlitis caused by *L. intracellularis* was also reported in 2 broiler chickens. Silver staining and electron microscopy revealed curved bacteria morphologically compatible with *Lawsonia* in the villous epithelium which was identified as *L. intracellularis* by immunohistochemical staining (163). Chickens were not susceptible to experimental infection with porcine strains of *L. intracellularis* (44). The organism was not found in normal chickens or those with malabsorption syndrome in the southeastern United States (140). Genomic analysis of organisms from several animal species including ostriches showed they are closely related (48).

Listeria

Outbreaks of listeriosis caused by *Listeria monocytogenes* occur sporadically in chickens, turkeys, waterfowl, pigeons, pheasants, partridges, and other avian species (85, 86, 101, 117, 128, 242). Young birds are most susceptible (13). The organism is important because of its ability to cause human infections following contact with infected birds (85) or consumption of contaminated poultry or poultry products, especially those that are precooked and "ready to eat" (54). Intestinal colonization of poultry and presence of *L. monocytogenes* in feces represent potential sources of the organism for listeriosis in ruminants (65).

Septicemic and encephalitic forms of listeriosis are recognized in birds. Emaciation and diarrhea, and lethargy followed by death characterize birds with septicemia. Neurological signs including depression, incoordination, ataxia, torticollis, and opisthotonos are seen in the encephalitic form (50, 51). Torticollis is especially common in affected birds. In the septicemic form, there is splenomegaly, multifocal hepatic necrosis, myocardial necrosis, and pericarditis. Myocardial degeneration, necrosis, and inflammation are often extensive (55, 128, 173). Ascites and petechial hemorrhages in liver, heart, spleen, kidneys, and brain were seen in affected broilers (228). Salpingitis developed in hens following the acute systemic phase of the infection (113).

Birds with the encephalitic form have necrotic and inflammatory foci in the brain but usually lack gross lesions (50, 51, 85, 101, 117, 228, 242). Microscopically, gliosis and satellitosis in the cerebellum and hemorrhages, fibrin thrombi, and abscesses containing Gram-positive bacteria are present in the midbrain, cerebellum, and medulla oblongata of birds with encephalitic listeriosis. Lesions tend to be most severe in the medulla oblongata (50, 117).

The organism is commonly found in feces and soil in temperate areas of the world. Infection can follow inhalation, ingestion, or wound contamination. An outbreak of listeriosis occurred in broilers shortly after beak trimming (228). Cold, wet conditions causing excessively moist litter were associated with an outbreak of encephalitic listeriosis and the organism was isolated from litter, water, and soil samples (50). In another outbreak, the poultry house had been flooded 10 days before onset of the disease, and conditions were hot and humid (117).

Listeria can be readily isolated and does not require special procedures (49) except it may be difficult to recover from birds with the encephalitic form of the disease. However, direct culture of brain stem was positive in 4 of 5 samples collected in an outbreak of encephalitic listeriosis (51). Chicken embryos are readily infected and can be used for isolation. *L. monocytogenes* is the only species that has been implicated in poultry disease and it needs to be differentiated from other species of *Listeria* (49). There are 13 serotypes; the majority of human and animal infections are caused by serotypes 4B, 1/2a, and 1/2b (49). Demonstrating antigen in fixed tissues that have lesions of a septicemic disease is useful for confirming a diagnosis of listeriosis when culture is not possible (173). A comprehensive review of diagnostic methods for identifying *Listeria* has been published (73).

Prevention of listeriosis depends on identifying and eliminating sources of infection. Based on the history of published outbreaks, avoiding wet conditions would seem prudent even though the risk for developing listeriosis has not been proven. The organism is often resistant to most commonly used antibiotics.

Moraxella

Moraxella has been isolated occasionally from turkeys with respiratory disease (70). *M. osloensis* caused a fowl cholera-like disease in commercial turkeys. Affected birds had at least 1 consolidated, pneumonic lung, multiple hemorrhages and inflammation of serous membranes, and abnormal spleens and livers. The organism could be distinguished from *Pasteurella multocida* by its growth on eosin-methylene blue and MacConkey's agar. The disease was reproduced in experimentally inoculated turkeys (68). *Moraxella* spp. has also been recovered from salpingitis in layers (21) and from dead-in-shell embryos or weak chicks (130). An ostrich developed granulomatous conjunctivitis from which *M. phenylpyruvica* was isolated (87).

Mycobacterium avium subsp. *paratuberculosis*

Natural infections of poultry with *Mycobacterium avium* subsp. *paratuberculosis* have not been reported, but chickens are susceptible to experimental infection (218, 220) and develop an immune response following

exposure to the organism (42). A related mycobacterial strain that causes chronic intestinal disease of wood pigeons (*Columba palumbus*) in Europe (215, 219) produces lesions consistent with paratuberculosis in experimentally inoculated calves (45, 216). Both the wood pigeon strain and *M. avium* subsp. *paratuberculosis* may have significant public health importance, because they have been associated with Crohn's disease and sarcoidosis in people (139). Granulomatous liver lesions with intralesional acid-fast bacteria identified by PCR as *M. avium* subsp. *paratuberculosis* have been reported in a sparrow (144).

Neisseria

Neisseria are nonfermentative, rod-shaped diplococci that do not grow on MacConkey's agar and are nonhemolytic, oxidase-positive, and catalase-positive. *N. weaveri* has been isolated from tracheas and lungs of chickens and turkeys in flocks with respiratory disease. Lesions in affected birds included tracheitis and pneumonia. Turkeys were more frequently affected than chickens. Ages of infected birds ranged from 5 weeks to 3 years. Usually other bacteria or viruses were identified in affected flocks. Its role in respiratory disease is unknown (39). *Neisseria* are also commonly identified in goose venereal disease (see below) and it can cause pneumonia in young ostriches (98).

Nocardia

Nocardia is an aerobic actinomycete. It is Gram-positive, very long, thin, obviously branched, finely beaded, and weakly acid-fast (227). The organisms are widely distributed in the soil as saprophytes. *Nocardia* typically causes granulomatous lesions, especially in the respiratory system. The organism has rarely been isolated from poultry although chickens are susceptible to experimental infection following oral or intraperitoneal inoculation (164). A 26.8% mortality rate due to systemic nocardiosis has been reported in pigeons (59).

Oerskovia

Oerskovia is a Gram-positive bacterium that morphologically resembles *Nocardia*. It is widely distributed in the environment. On agar, colonies are yellow and the organism forms branching vegetative hyphae that penetrate into agar, without aerial hyphae (227). Infection of animals and humans with *Oerskovia* is rare. The organism was isolated, along with α-hemolytic *Staphylococcus*, from a clinically ill pigeon that had a large granulomatous mass at the base of the heart adjacent to the esophagus and trachea. Colonies of Gram-positive bacilli were present within granulomas (235).

Pelistega

Bacterial isolates associated with respiratory disease in pigeons have been placed into a novel genus, *Pelistega*, as a single species, *P. europaea*. It is a Gram-negative, nonmotile, rod-shaped bacterium taxonomically related to *Taylorella equigenitalis*, the cause of contagious equine metritis (223).

Plesiomonas

Plesiomonas shigelloides, the one species in the genus, is closely related to species in the genus *Aeromonas*. It is commonly found in freshwater environments and has been infrequently isolated from avian species, mainly aquatic birds (102). Concurrent infections with *P. shigelloides* and *Edwardsiella tarda* caused fatal septicemia in young penguins (159).

Proteus

Proteus is a genus in the family *Enterobacteriaceae* that inhabits the lower intestinal tract. The organism is capable of penetrating the eggshell following fecal contamination. Experimental inoculation of fertile eggs resulted in 100% embryonic mortality (29). Temperature influences egg penetration and survival time within the egg (1).

Proteus occasionally causes embryonic death, yolk sac infections, and mortality in young chickens, turkeys, and ducks (12, 108, 130, 167, 172, 183, 185, 186, 225). However, experimental inoculation with an isolate from ducklings failed to cause disease (183).

Septicemia due to *Proteus* has occurred in quail (146, 184), pheasants infected with low pathogenic avian influenza virus (212), and broilers suspected of having immune deficiency (174). *Proteus* has been recovered occasionally from a low percentage of salpingitis and oophoritis lesions in layers (14, 21, 195), and has been associated with respiratory disease in chickens (131, 210, 241). An isolate from chickens with respiratory disease caused 50% mortality in experimentally inoculated 4-week-old chickens (131). *P. mirabilis* was isolated from the lung, trachea, and kidney of chickens experiencing respiratory signs, diarrhea, paralysis, and high mortality. The disease was reproduced with isolates of the organism (241). *Proteus* was isolated infrequently from turkeys

with cellulitis (80,165) and white leghorn pullets with necrotic dermatitis that seroconverted to reticuloendotheliosis virus (97). In waterfowl, *Proteus* can occasionally produce arthritis, salpingitis, airsacculitis, septicemia (18, 19, 233), and granulomatous inflammation of salt glands (115).

Pseudomonas

Pseudomonas causes localized or systemic disease in young and growing poultry, invades fertile eggs causing death of embryos and newly hatched birds, and reduces shelf life of contaminated meat. Pseudomonads are capable of digesting eggshell cuticle if the humidity is high (24).

They are ubiquitous, often associated with soil, water, and humid environments. *Pseudomonas* is generally considered to be an opportunist that produces respiratory infections, including airsacculitis (197), sinusitis (70), keratitis and keratoconjunctivitis (116), or septicemia and its sequelae when introduced into tissues of susceptible birds. Above normal mortality in young birds due to omphalitis and yolk sac infections acquired in the hatchery have been described (231). Chickens (9, 63, 118, 145, 178, 231), turkeys (7, 80, 88), ducks (18, 115, 183, 233), pheasants (96), ostriches (99, 149, 168, 234), geese (203), and a variety of pet and captive birds have been affected. Although birds of any age may be infected, young birds are most susceptible, as are severely stressed or immunodeficient birds. Concurrent infections with viruses and other bacteria, especially mycoplasmas, are common and may enhance susceptibility to *Pseudomonas* (171, 174, 203). Morbidity and mortality are usually 2%–10% but can be much higher, approaching 100%.

Pseudomonas aeruginosa is the most common species causing infections, especially yolk sac infections and septicemia in young chicks. Virulence varies among isolates. Mortality following yolk sac inoculation of chicks ranged from 0% to 90% (231). Isolates examined in another study were highly virulent, causing 50%–100% mortality in experimentally inoculated 4-week-old chickens (131).

Pseudomonas fluorescens caused death of turkey embryos following dipping of eggs in contaminated antibiotic solution, and it has been associated with multicausal respiratory disease of chickens (131) and turkeys (93). *P. stutzeri* was isolated from chickens with respiratory disease but produced only low mortality in experimentally inoculated chickens (131). A *P. putida*-like organism has been isolated from osteomyelitis lesions in processed tom turkeys (H.J. Barnes, unpublished data).

Mortality characterizes most *Pseudomonas* infections. Death usually occurs rapidly, often within 24–72 hours after infection. Clinical signs vary depending on whether infections are localized or systemic, but may include: anorexia; stunting; lassitude; lameness; neurologic signs; swelling of head, wattles, and sinuses; swelling of hock joints or foot pads; respiratory distress; diarrhea; or conjunctivitis (63, 88, 116, 118, 153). Infections of the nasal glands in ducks resulted in granulomatous adenitis (115).

Lesions are consistent with clinical findings and include: subcutaneous edema and fibrin, occasionally with hemorrhage; exudate in affected joints; inflammation of serous membranes mimicking lesions of colisepticemia (airsacculitis, pericarditis, hepatic serositis); pneumonia; swelling and necrotic foci in liver, spleen, kidney, and brain; conjunctivitis; sinusitis; and occasionally keratitis (63, 88, 116, 131, 153). Chicks that survived yolk sac inoculation had retained, inflamed yolk sacs when examined 14 days postinoculation (231). Unilateral panophthalmitis characterized by corneal perforation and phacolysis occurred in young turkeys with ocular *Pseudomonas* infection (Figure 23.23). The rapidly progressive destruction of the eye may be related to proteases produced by the organism (7). Large numbers of bacteria, often in and around affected blood vessels within most tissues, including brain (Figure 23.24), are typically seen microscopically in acute lesions. Heterophilic exudate in the pharynx and pulmonary foci were present in respiratory infections of pheasants (96). A similar case characterized by pseudodiphtheritic membranes and granulomatous lesions in the respiratory and upper digestive tract occurred in a group of young ostriches experiencing high mortality. Intralesional bacteria were identified as *P. aeruginosa* by immunohistochemistry and confirmed by culture (149). *Pseudomonas* was isolated infrequently from adult hens with salpingitis and oophoritis (14, 195), from turkeys with cellulitis (80, 165), and from geese with venereal



Figure 23.23 Panophthalmitis with corneal perforation in an 18-day-old turkey poult. A heavy pure growth of *Pseudomonas aeruginosa* was obtained on culture and intralesional bacteria were present in tissues on histopathology.

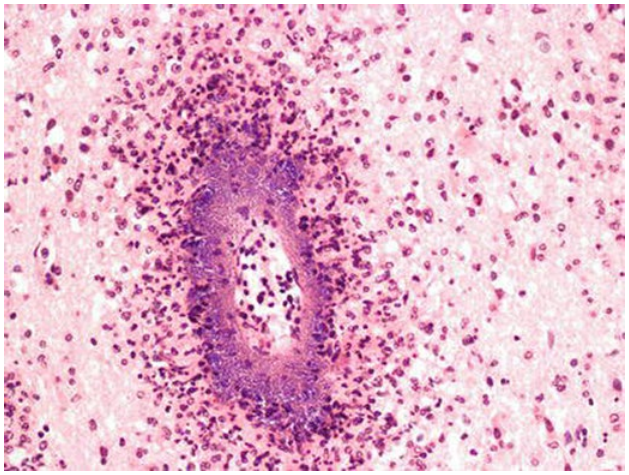


Figure 23.24 A blood vessel in the brain of a 3-day-old chick with marked neurologic signs is disrupted by numerous bacteria. A perivascular zone of necrotic debris is present. A heavy pure growth of *Pseudomonas aeruginosa* was isolated from the brain.

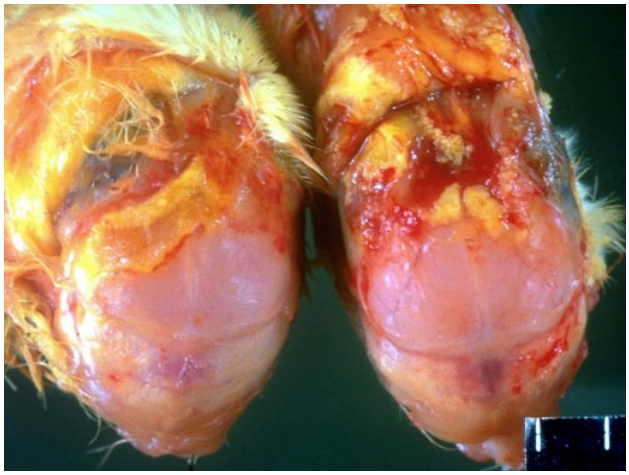


Figure 23.25 Subcutaneous lesions in the upper neck area of chicks following the use of a Marek's disease vaccine contaminated by Pseudomonas. (L. Munger)

disease (see later), and was the most common bacterium recovered from abnormal joints of broiler chickens in a study of leg weakness (26).

Pseudomonas is among a variety of bacteria often recovered from dead embryos and sick newly hatched chickens, turkeys, pheasants, ducks, and ostriches (29, 61, 108, 130, 167, 183, 225). With the exception of a respiratory outbreak in pheasants attributed to exploding contaminated eggs in the incubator, presence of *P. aeruginosa* in embryos is not considered a source of infection for other birds. Severe outbreaks have followed injection of large numbers of birds with contaminated vaccines (Figure 23.25) (145, 217) and antibiotic solutions (36, 236). In these cases, contamination resulted from poor hygiene during mixing and handling, not from the products themselves. Contact with infected birds (153) and intense, continuous broiler production with different ages being raised at the same facility (63) can result in spread of *Pseudomonas* infection. In some outbreaks, the source of the organism and how it spread could not be determined.

Diagnosis requires isolation and identification of the organism. Various methods including serologic, phage, and aeruginocine typing methods, and molecular testing (182) may be useful in epidemiologic studies.

Prevention and control are based on identifying and eliminating the source of the organism. Measures to prevent yolksacculitis and omphalitis by different bacteria are applied to the prevention of infection of newly hatched chicks with *Pseudomonas*. Good hygiene, especially in hatcheries and when birds are injected, is fundamental to preventing the infection. Cleaning and disinfection of equipment and use of sterile techniques in preparing vaccines and injectables will prevent *Pseudomonas* infections resulting from inoculation (36).

Sensitivity of isolates to hatchery disinfectants needs to be determined (238). *P. aeruginosa* is known for its resistance to disinfectants; it has been isolated from the stock solution of most commonly used biocides (134). A commercial quaternary ammonium disinfectant was ineffective at completely inactivating high concentrations (10^9) of *P. aeruginosa* that had been isolated from chicks with omphalitis. It was generally effective when numbers of organisms were low (10^3) and had a variable effect on intermediate concentrations (10^6). Potentiation of the disinfectant with ethylenediaminetetraacetic acid (EDTA) substantially improved its efficacy when tested *in vitro* (230). *Pseudomonas* spp. were found to adapt to growth in increasing concentrations of disinfectants following serial passage (134).

Antibiotics can be useful in reducing losses if initiated early in the disease, but because the organism has high antibiotic resistance, antimicrobial susceptibility testing is essential. Resistance to antibiotics is due to low permeability of the outer membrane to chemicals, as well as to efflux mechanisms that remove many antimicrobial agents (134).

Rothia

The genus *Rothia* belongs to the family *Micrococcaceae*. *Rothia* spp. are Gram-positive, aerobic, rod-shaped or coccus-shaped bacteria that are included within a group of bacteria called "coryeneforms" (227). *Rothia* was the only bacterium isolated from osteomyelitis and joint lesions in lame or recumbent tom turkeys in an affected flock. Intravenous inoculation of unaffected turkeys reproduced the clinical signs and lesions and the organism was reisolated from the birds (H.J. Barnes, unpublished data).

Segmented Filamentous Bacteria

Segmented filamentous bacteria (SFB) are Gram-positive, anaerobic, endospore-forming bacteria that appear microscopically as filamentous, septate organisms with peculiar morphological characteristics. Because they have not been cultured *in vitro*, this group of organisms does not have an official taxonomic name, although they have been given the provisional name *Arthromatus candidatus* (198).

They are commensals in the intestinal tracts of mammals (including humans), birds, fish, and insects (34). Histologically, they can be seen embedded in the apical cytoplasm of enterocytes, displacing microvilli (240). In turkeys, SFB are 0.6–1.1 µm wide and up to 13.5 µm long (3). Challenge and gene sequencing studies suggest there are different host-specific types or species. Chickens are refractory to infection with SFB from mice even following corticosteroid treatment (2), and the intestinal tract of mice are not colonized by SFB from rats, and vice versa (211). Additionally, SFB from rats, mice, and chickens show differences in 16S rRNA sequences that are sufficient to suggest the existence of different, but closely related, species (198).

Often SFB are markedly increased in young chickens, turkeys, and quail with gastrointestinal diseases, especially during cold periods (82). Although most frequently found in ill birds, SFB may not be pathogens, but rather they overgrow when conditions are altered because of enteric disease. High numbers of SFB were present in the jejunum of poults with experimental stunting syndrome (3), but subsequent studies using filtered inocula showed they were not the cause of the disease (190). However, depressed growth (11%–14%) occurred when poults were inoculated with 2 isolates of similar filamentous bacteria (152).

Among intestinal microflora, SFB are the only ones known to play a role in postnatal maturation and development of intestinal mucosal immunity, specifically inducing and regulating T-cell responses (34).

Streptobacillus

Streptobacillus moniliformis, a Gram-negative, often beaded, nonbranching, filamentous bacterium, can infect turkeys, usually following rat bites or exposure to infected rats. Polyarthritis and synovitis occur in infected birds; other tissues are usually normal. The disease can be reproduced in turkeys following experimental inoculation of the organism by intravenous, subcutaneous, or footpad routes, but not by oral administration. Chickens are not susceptible. Diagnosis requires isolation and identification of the organism. Infection can be prevented through rodent control (79, 147).

Suttonella

A unique Gram-negative, rod-shaped bacterium isolated from the lungs of passerine birds (tits) experiencing episodes of mortality in the United Kingdom was described as a new species, *Suttonella ornithocola* (71, 112). The association between *S. ornithocola* and mortality was uncertain until recently when the organism was isolated from pneumonic lungs of passerines (123). The organism has not been associated with disease in poultry.

Vibrio

Vibrio spp. are Gram-negative, comma-shaped bacteria that are widespread in coastal and estuarine environments. *Vibrio cholerae*, the cause of cholera in humans, results from infection with serogroup O1 or O39 strains that produce cholera toxin (toxigenic strains). Other non-O1 isolates of *V. cholerae* may also pose a potential risk of gastroenteritis in humans (5).

Non-O1 *V. cholerae* has been isolated from geese that died following weight loss and lassitude of 2–3 days' duration (187), from nasal cavities of apparently healthy ducks (22), and from tissues of ducks with airsacculitis or septicemia (233). Individuals working with ill birds who have contact with coastal waters and shellfish need to be aware that birds can be a source of human infection (187). Conjunctivitis caused by *V. cholerae* NAG, a potential human pathogen, occurred in ducklings (20). The organism also was isolated from the intestines and water where the ducks were being kept. Exposure of domesticated ducks was believed to have come from free-living birds. Both O1 and non-O1 *V. cholerae* have been frequently isolated from the feces of aquatic birds and their environments (162).

Vibrionic hepatitis is probably a misnomer, because the disease is suspected to be caused by *Campylobacter* spp., although the etiology has not been definitively identified (245). *V. metschnikovii* (*metschnicovii*) is occasionally isolated from waterfowl (92) and there are a few reports of *V. metschnikovii* isolated from humans with bacteremia, neonatal sepsis, postoperative wound infection, pneumonia, diarrhea, or foot and leg ulcers (169).

Vibrio alginolyticus, a dominant proteolytic bacterium in the upper respiratory tract of chickens and turkeys, does not cause disease but may enhance pathogenicity of avian influenza viruses by providing a mechanism for cleavage of the virus hemagglutinin (32).

Yersinia pseudotubeculosis

The genus *Yersinia* belongs to the family Enterobacteriaceae. *Y. pseudotuberculosis* is a Gram-negative, nonspore-forming, facultative anaerobic coccobacillus. Infection

with *Y. pseudotuberculosis* was diagnosed in pigeons, turkeys, ducks, as well as nonpoultry species. It causes systemic (septicemic) infection, with gross lesions consisting primarily of 1–2 mm diameter necrotic foci in the liver and spleen. On histopathology, there is multifocal hepatic and splenic necrosis, with intralesional large, dense clusters of bacteria, which are also present in the lumens of blood vessels of different organs and tissues (205, 232). Turkeys infected with *Y. pseudotuberculosis* also had an osteomyelitis lesion from which the bacterium was isolated (232).

Diseases Caused by or Associated with Bacteria

Beak Necrosis

A Gram-positive bacterium with affinity for keratin was associated with beak necrosis that affected nearly half of the birds in a flock of 1-year-old broiler breeder hens and caused approximately 10% mortality (38). Feeding fine feed (mash) predisposes birds to oral and beak lesions although the exact mechanism of how lesions develop is unknown. Oral lesions resolve rapidly after birds are put on pelleted feed (74). Injury to the epidermis occurs initially and is followed by necrosis, ulceration, and bacterial growth. Affected birds have decreased leukocytes and anemia (75). Beak deformity, loss of the distal end of the mandible, and osteomyelitis occur in severely affected birds. Weight loss and mortality result from impaired feeding (67). Males are more often affected than females (67). Use of 40-mm grids over feeders to restrict male access to feed resulted in a higher occurrence of oral and beak lesions (94). Visibly affected birds need to be culled for welfare reasons. In one study, beak lesions were the major cause for culling male broiler breeders (95).

Goose Venereal Disease

An infectious venereal disease of uncertain etiology characterized by inflammation of the phallus and cloaca of ganders in breeding flocks was first described in Hungary (209). Subsequently, flocks have been affected in other European countries, Russia, and the Middle East. Initially, the base of the phallus becomes swollen and inflamed, with the process extending to the cloaca. Later, there is necrosis, ulceration, and eventually considerable scarring of the mucosa, often making reproduction impossible. Similar lesions may develop in the cloaca of hens following breeding. Morbidity ranges from 20%–100%, and newly introduced birds readily contract the disease. Decreased fertility and gander mortality of approximately 5% are flock problems resulting from the disease (204).

A variety of bacteria, especially *Neisseria*, *Mycoplasma* spp., and *Candida albicans* affecting the phallus of ganders and cloaca of hens, has been associated with the disease (16, 137, 202, 204). Normal phallus microflora of unaffected ganders has been established (160) and is similar, with the exception of mycoplasmas and *C. albicans*, to that of affected ganders (16). Use of antimicrobials effective against mycoplasmas substantially reduces disease severity (57). A similar disease attributed to *C. albicans* spread through goose flocks in Israel (15). Dramatic improvement followed treatment with mycostatin and antibiotics. Vaccination with ethanol-inactivated fungus provided good control of the disease (119). A zygomycete, *Mucor janssenii*, found in 1 flock of affected ganders reproduced a similar disease in experimentally inoculated birds (138). Exposure of specific pathogen-free (SPF) Muscovy ducks and geese to isolates alone and in various combinations produced only mild clinical signs and lesions except in a female contaminated by an affected gander and inoculated with *C. albicans*. Trauma is considered to be a likely initiating factor followed by infection with opportunistic bacteria and fungi (137).

It is recommended that ganders be examined at each breeding season and affected birds removed from the flock. Artificial insemination can be used in affected flocks to improve fertility (16).

Intracellular Infection in Ducks

Mortality in Muscovy ducks (*Cairina moschata*) caused by an intracellular organism primarily affecting endothelial cells in the lungs was initially attributed to *Haemoproteus* infection (106). However, subsequent examination of additional cases revealed that the organism was not a protozoan, but probably a bacterium capable of forming spores or an unidentified microorganism. Recently the organism has been identified as a yeast in the family Saccharomycetales (143). Muscovy ducks are most susceptible and can contract the infection from asymptomatic infected Pekin ducks. Experimental transmission is possible using blood from infected ducks.

At necropsy, lungs are dark red-purple, slightly edematous, and firm. Microscopically, air capillaries are obliterated because of marked swelling of endothelial cells, which are often packed with intracellular organisms, and interlobular septa are widened and contain inflammatory cells and edema. Organisms stain poorly with hematoxylin and eosin but are readily demonstrated with periodic-acid-Schiff or silver stains (107, 175).

Liver Granulomas and Related Granulomatous Disorders

Granulomas are occasionally seen in livers of turkeys and, less frequently, chickens at processing. Affected livers and carcasses are condemned. The incidence in individual flocks may reach 50%.

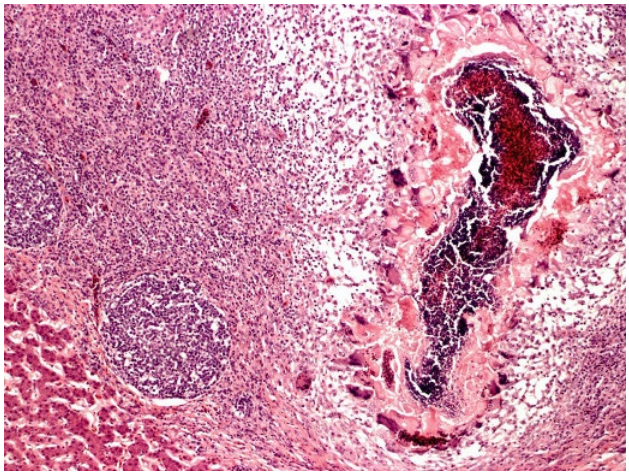


Figure 23.26 Caseous granuloma caused by *Eubacterium tortuosum* in the liver of a 20-week-old tom turkey. The granuloma consists of central caseous debris covered by multinucleated giant cells and surrounded by mononuclear inflammatory cells and fibroblasts. Lymphoid nodules are seen at the periphery. Warthin–Starry stain revealed many intralesional organisms morphologically compatible with *Eubacterium tortuosum*.

Granulomas are focal or multifocal, single to coalescing lesions that are grossly visible as firm, lobulated, roughly spherical, pale yellow to white masses ranging in size from a few millimeters to several centimeters. Advanced lesions have a rough appearance and may be "gritty" when cut. Bile stasis of adjacent normal hepatic tissue is often marked. Liver granulomas are caused by a variety of infectious and parasitic agents. *E. coli*, *Eubacterium*, and other bacteria are among the more common etiologic agents (206). Similar granulomas are occasionally seen in the spleen and rarely in other tissues.

Microscopically, lesions are typical heterophilic granulomas (150) that contain a central caseous mass covered by a layer of multinucleated giant cells, which is confluent except in areas where the process is still active (Figure 23.26). A more diffuse zone of heterophils, macrophages, fibroblasts, and lymphocytes surrounds the caseated center. Heterophils can be seen migrating through the layer of giant cells and are especially numerous in areas where giant cells are absent or discontinuous. Diffuse and focal lymphocytes form the outermost layer of the lesion. Fibrosis may be extensive in chronic lesions. Bacteria generally are not visible unless special stains are used. Tangles of filamentous organisms usually can be seen with silver stains such as Warthin–Starry or Dieterle, and Gram-positive filamentous or coccoid organisms may be seen with a Gram stain (4, 122, 151)

A variety of bacteria have been isolated from the lesions including *Actinomyces* (191), *Catenabacterium*, *Corynebacterium*, *Eubacterium*, *Propionibacterium*, *Enterococcus* (*Streptococcus*), and *Staphylococcus* (122, 151). Liver granulomas occurred after intravenous inoculation of turkeys with *Catenabacterium* spp. isolated from a naturally infected turkey. Chickens, peafowl, guinea pigs, rabbits, hamsters, and mice did not develop lesions (151). Granulomatous lesions were reproduced experimentally in the liver and spleen of turkeys and in the spleen of chickens by intravenous inoculation of *Eubacterium tortuosum* (4, 89), even though the organism is part of the normal cecal flora (89). Often, mucosal ulcers in the lower intestinal tract can be found in affected birds, suggesting that liver lesions develop from bacteria carried to the liver from the intestine via the bloodstream (4, 89, 122, 151).

Liver granulomas also occur in chickens and other poultry species but much less frequently. Gram-positive, filamentous bacteria morphologically and tinctorially distinct from *Eubacterium*, segmented filamentous organisms, *Actinomyces*, and *Nocardia* were present in sporadic cases of visceral granulomas in broiler chickens at processing in the United States (91). Lesions also occurred in the spleen, cecum, and mesentery of some birds. Numerous Gram-positive filamentous bacteria morphologically compatible with *Eubacterium tortuosum* were found within a granulomatous lesion in the liver of a 7-week-old quail (237).

Larvae of *Ascaridia dissimilis* also can cause hepatic foci ("white-spotted livers") in turkeys that need to be differentiated from bacterial granulomas. Only a low percentage of these hepatic foci in turkey livers at processing yielded bacteria; *E. coli* and *Salmonella* spp. were occasionally isolated (161). Exposing turkey poults to *Ascaridia dissimilis* ova reproduced the lesions (160). No causative organism initially was identified in granulomatous lesions in ceca and livers of older chickens from small flocks in Canada (155), but subsequently, larvae of the cecal worm, *Heterakis gallinarum*, were found to be associated with the lesions (179).

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24

Avian Chlamydiosis

Daisy Vanrompay

Summary

Agent, Infection, and Disease. This chapter covers current knowledge on *Chlamydia psittaci* infections as they occur in birds raised commercially for meat and egg production. *C. psittaci* causes respiratory disease, which is usually systemic and occasionally fatal. *C. psittaci* strains isolated from birds fall into 2 general categories: (1) highly virulent strains that cause acute epidemics in which 5%–30% of affected birds die and (2) less virulent strains that cause slowly progressive epidemics. Highly virulent strains have been characterized in turkeys, ducks, and more recently also in chickens.

Diagnosis. Nucleic acid amplification tests (NAATs) are currently recommended for quick, sensitive, and specific diagnosis. These include conventional and real-time polymerase chain reaction, DNA microarray-based detection, and DNA sequencing. Culture, cytological staining, immunological staining, immunohistochemistry, or antigen enzyme-linked immunosorbent assays (ELISA) can be used if NAATs are not available.

Intervention and Regulations. Commercial chlamydia vaccines for poultry are not available. Among tetracylines, which are the drugs of choice, chlortetracycline and doxycycline are most often used. Enrofloxacin (fluoroquinolone antibiotic) also can be used, although some countries decided to ban the use of this antibiotic in poultry because of the risk that it promotes drug-resistant bacteria that can be harmful to humans. In cases of avian chlamydiosis, the appropriate public health and/or animal health agencies should be consulted as necessary. In many countries, psittacosis (humans) and even chlamydiosis in poultry are notifiable diseases and must be reported within 48 hours.

Introduction

Definition and Synonyms

Avian chlamydiosis is defined as an infection with a *Chlamydia* species in birds. The taxonomy of the family *Chlamydiaceae* was recently revisited. The genus *Chlamydia* includes currently 11 recognized species, and among them *C. psittaci*, *C. avium*, *C. gallinacea* and occasionally *C. abortus* have been isolated from birds (34, 75, 81, 89).

Avian chlamydiosis is a respiratory disease, usually systemic and occasionally fatal. This chapter primarily covers current knowledge on *C. psittaci* infections as they occur in birds raised commercially for meat and egg production. It should be noted that the disease in pet birds is quite similar, and the disease characteristics, transmission, and diagnosis are essentially the same.

Chlamydia psittaci can be transmitted to humans. The disease in birds and humans originally was called psittacosis or parrot fever because it was first recognized in psittacine birds and in humans associated with psittacine birds. Today we speak of chlamydiosis in birds and psittacosis in humans.

Public Health Significance

The strains of avian chlamydiae can infect humans and should be handled with appropriate biosafety and containment procedures. Risk assessment and management are essential when performing diagnosis of avian chlamydiosis. Adequate information, communication, and health surveillance by an occupational physician are recommended (19, 82).

Most infections occur through inhalation of infectious aerosols. Although the disease from psittacine birds is best known, the infection in poultry is of particular

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concern because transmission to humans is common during handling and slaughter of the birds (20, 21, 42, 46, 48, 49). Postmortem examinations of infected birds and handling of cultures should be performed in certified Class II laminar flow hoods whenever possible or with proper protective equipment. Appropriate zoonotic agent decontamination procedures should be followed because human infection can result from transient exposures. The incubation period is usually 5–14 days; however, longer incubation periods are known. Human infections vary from inapparent to severe systemic disease with interstitial pneumonia and encephalitis. The disease is rarely fatal in properly treated patients; therefore, awareness of the danger and early diagnosis are important. Infected humans typically develop headache, chills, malaise, and myalgia, with or without signs of respiratory involvement. Pulmonary involvement is common. Auscultatory findings, however, may appear to be normal or to underestimate the extent of involvement. Diagnosis can be difficult and in the past was usually established through testing paired sera for antibodies to chlamydia by the complement fixation test (CFT). However, some patients hospitalized for psittacosis remain seronegative. Thus, serology is increasingly being replaced by nucleic acid amplification techniques (NAATs), which also allow bird source tracing. In humans, tetracycline, doxycycline, or azithromycin are usually the drugs of choice unless contraindicated. The length of treatment will vary with the drug, but should be continued for at least 14 days for tetracycline. Secondary spread among humans rarely occurs (124). Because the disease is rarely fatal in properly treated patients, awareness of the danger and early diagnosis are important.

History

The first description of a psittacosis outbreak dates from 1879 and was described by Jacob Ritter, linking the disease to pet parrots and finches (72). Historical aspects of chlamydia-related diseases in animals and humans are reviewed by Pospischil (69).

Etiology

Classification

The members of the family *Chlamydiaceae*, order *Chlamydiales* are obligate, intracellular Gram-negative bacteria. In 1999, Everett et al. (25) proposed a reassignment from the single genus *Chlamydia* into 2 genera, *Chlamydia* and *Chlamydophila*, based on clustering analyses of the 16S rRNA and 23S rRNA genes (Table 24.1). However, recent comparative genome analyses are consistent with the conclusion that host-divergent strains of chlamydia are biologically and ecologically closely related (45, 85). The previous taxonomic separation of the genus based on ribosomal sequences is not consistent with the natural history of the organism as revealed by genome comparisons. Consequently, the taxonomy of the family *Chlamydiaceae* was recently revisited. The genus *Chlamydia* includes currently 11 recognized species, namely *C. abortus* (sheep, goats, cattle), *C. caviae* (guinea-pigs), *C. felis* (cats), *C. muridarum* (mouse, hamster), *C. psittaci* (birds and others), *C. pecorum* (sheep, cattle), *C. pneumonia* (human and others), *C. suis* (swine), *C. trachomatis* (human), and 2 recently established species isolated from birds, *C. avium* and *C. gallinacea* (75) (Table 24.1).

Morphology

The 4 morphologically distinct forms of chlamydia are termed elementary body (EB), reticulate body (RB), intermediate body (IB), and the persistent aberrant body. The EB (Figure 24.1) is a small, electron-dense, spherical body, about 0.2–0.3 mm in diameter. The EB is the infectious form of the organism, which attaches to the target epithelial cell and gains entry. The EBs have a highly electron-dense nucleoid located at the periphery of the EB and clearly separated from an electron-dense cytoplasm. Following entry into the host cell, the EB expands in size to form the RB, which is the intracellular, metabolically active form. The RB measures approximately 0.5–2.0 mm in diameter (Figure 24.2). The RB divides by binary fission and thereafter matures into new EBs. During this maturation, morphologically intermediate forms (IB), measuring about 0.3–1.0 mm in diameter, can be observed. The IB has a central electron-dense core with radially arranged individual nucleoid fibers surrounding the core. Cytoplasmic granules are tightly packed at the periphery of the IB and are separated from the core by a translucent zone.

Chlamydiaceae also can engage in a long-term relationship with the host cell, a phenomenon known as persistence, in which no visible growth of the chlamydial organisms can be observed. The normal developmental cycle can be interrupted *in vitro* by a number of conditions and agents, such as antibiotics, nutrient deprivation, or immune factors – interferon-gamma (IFN-γ) in particular. This is generally accompanied by the development of relatively small inclusions, enlarged pleiotrophic RBs, which are named aberrant bodies, and inhomogeneity of the inclusions. Aberrant bodies accumulate chromosomes, but genes for cell division are no longer expressed. Once the stress-inducing factor is removed, aberrant bodies revert to normal RBs, complete the developmental cycle, and generate infectious EBs.

Table 24.1 *Chlamydiaceae* taxonomy.

Chlamydial Taxonomy in the Late 1990s			Chlamydial Taxonomy used in the 21st Century (Everett et al. 1999) (25)				Chlamydial Taxonomy used in the 21st century (Stephens et al. 2009) (85)	
Order	*Chlamydiales*		*Chlamydiales*				*Chlamydiales*	
Family	*Chlamydiaceae*		*Chlamydiaceae*, Simkaniaceae, Parachlamydiaceae, Waddliaceae				*Chlamydiaceae*, Simkaniaceae, Parachlamydiaceae, Waddliaceae	
Genus	*Chlamydia*		*Chlamydia*		*Chlamydophila*		*Chlamydia*	
Species	*C. trachomatis*	Trachoma biovar	*C. trachomatis*	Trachoma biovar			*C. trachomatis*	Trachoma biovar
		LGV biovar		LGV biovar				LGV biovar
		Murine biovar	*C. muridarum*				*C. muridarum*	
		Porcine biovar	***C. suis***				***C. suis***	
	C. pneumoniae	Human biovar			***C. pneumoniae***	TWAR biovar	***C. pneumoniae***	TWAR biovar
		Koala biovar				Koala biovar		Koala biovar
		Equine biovar				Equine biovar		Equine biovar
	C. psittaci	Avian subtype			***C. psittaci***		***C. psittaci***	
		Abortion subtype			***C. abortus***		***C. abortus***	
		Feline subtype			*C. felis*		*C. felis*	
		Guinea-pig subtype			*C. caviae*		*C. caviae*	
	C. pecorum				***C. pecorum***		***C. pecorum***	

LGV, lymphogranuloma venereum; TWAR, Taiwan acute respiratory agent.

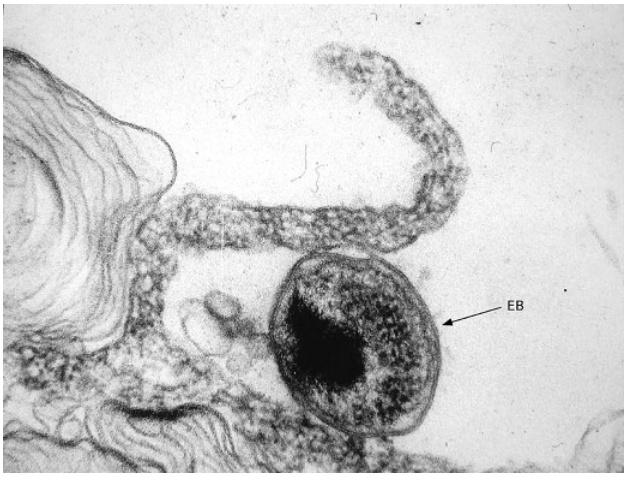


Figure 24.1 Buffalo green monkey (BGM) cell culture, 1 hour after inoculation with the *Chlamydia psittaci* Texas Turkey genotype D strain showing an elementary body (EB) attached to the side of a host cell microvillus.

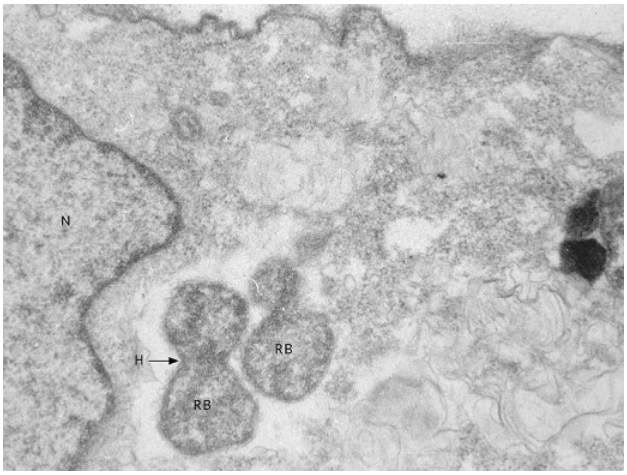


Figure 24.2 Buffalo green monkey (BGM) cell culture, 18 hours after inoculation with a *Chlamydia psittaci* genotype B strain (89/1326). Note the vacuole near the nucleus (N) with an early and late stage of division of a reticulate body (RB). Note the "hourglass" profile (arrow H).

Antigenic Structure

The cysteine-rich major outer membrane protein (MOMP) is well studied. It has a molecular weight of 40 kDa and represents approximately 60% of the weight of the outer membrane. The MOMP of *C. psittaci* is an immunodominant protein, and there is considerable evidence that antibodies to surface-accessible epitopes of MOMP have a protective role in immunity to chlamydial infection (23). The outer membrane protein A (*ompA*) gene (formerly referred to as *omp1* gene) encodes the MOMP. The *ompA* gene contains 5 conserved- and 4 variable-sequence regions, VS1–VS4, which encode for the variable-protein domains VDI–VDIV. VDI, VDII, and VDIV especially protrude from the *C. psittaci* membrane. Epitope mapping has shown the presence of genus- and species-specific antigenic determinants within the conserved regions. However, species-specific antigenic determinants also have been found in the most conserved parts of VDIV. Serovar-specific antigenic determinants are located within VDI and VDII. Monoclonal antibodies to the highly immunoaccessible serovar-specific epitopes on the MOMP can passively neutralize chlamydial pathogenicity and infectivity. Monoclonal antibodies to genus-, species-, or serovar-specific epitopes on the MOMP are excellent tools for specific chlamydial diagnosis.

A chlamydial cysteine-rich heat shock protein 60 (hsp60 or GroEL) has been described that is cross-reactive with other Gram-negative bacteria such as *Escherichia coli*, *Neisseria gonorrhoeae*, and *Coxiella burnetii* (134). Thus, the presence of cross-reactive epitopes on the chlamydial outer membrane should be kept in mind when choosing or interpreting a specific diagnostic test.

Other chlamydia proteins under study are OmcA (EnvA or Omp3), OmcB (EnvB or Omp2), Hc1, RpoB', RpoB, PorB (OmpB), Omp85, elongation factor Tu (EF-Tu/TufA), DnaK (hsp70), OprB, heat shock proteins (10, 44, 58, 59, 61, 86, 93), and especially the recently discovered polymorphic outer membrane proteins (Pmps).

Whole-genome sequencing has revealed the polymorphic membrane protein (Pmp) gene family. This is the largest protein family of *Chlamydia* species and it is a unique feature of the genus (40, 98, 103). Grouping of those proteins in one family is based on the conserved motifs FxxN and GGA (with I, L or V in the fourth position). The Pmps have been identified as autotransporter (type V secretion system) proteins, based on their cleavable N-terminal signal sequence (type II secretion) for translocation across the inner membrane, a central passenger domain which is responsible for the protein's function and a C-terminal transporter domain that forms a β-barrel and with a phenylalanine at the end, which is suggestive for outer membrane localization, for translocation across the outer membrane (reviewed in 120). The Pmp may be involved in antigenic variation and contribute to immune evasion in the infected host. Recently, Van Lent et al. (104) studied the expression all 17 *C. psittaci pmp* coding sequences of the Cal-10 strain during both normal and persistent culture conditions. They also used immunofluorescence staining and immunoelectron microscopy. PmpA and PmpH emerged as important players in *C. psittaci* pathogenesis by virtue of their unique expression properties, both at the transcript and protein level.

The chlamydial lipopolysaccharide (LPS) also is an essential constituent of the outer membrane and, like the MOMP, represents one of the major surface-exposed

antigens of chlamydiae in both the EB and the RB. It has a molecular weight of 10 kDa and is chemically and serologically related to the LPS of Gram-negative Enterobacteriaceae. In fact, the chlamydial LPS contains several antigenic determinants cross-reacting with the LPS of enterobacterial Re mutants of *Salmonella* species and *Acinetobacter calcoaceticus* (11, 62). However, the chlamydial LPS contains in its saccharide moiety a trisaccharide of 3-deoxy-D-manno-2-octulosonic acid (Kdo) of the sequence αKdo(2→8)-αKdo-(2→4)-αKdo. This antigenic epitope is shared only by all members of the genus *Chlamydia* and, thus, represents a *Chlamydiaceae*-specific antigen useful for specific diagnosis (12).

Strain Classification

Antigenicity

All *Chlamydiaceae* are recognized by monoclonal antibodies (mAbs) that detect the LPS αKdo(2→8)-αKdo-(2→4)-αKdo. *Chlamydia* species have a common antigenic epitope in variable segment 4 of the MOMP: NPTI, TLNPTI, LNPTIA, or LNPTI. *C. psittaci* strains are recognized by serovar-specific monoclonal antibodies. The 8 known *C. psittaci* serovars (A–F, M56, and WC) can be distinguished by use of a panel of serovar-specific mAbs in a micro-immunofluorescence test (4, 117). However, serotyping is currently seldom performed because the serovar-specific mAbs are not provided by a commercial supplier and because serotyping appears less discriminatory compared with the newly developed molecular characterization methods (28).

Genetic and Molecular

Chlamydia species can be distinguished by analysis of: (1) full-length 16S and 23S rDNAs, (2) the 16S-23S intergenic spacer (*rrn* spacer), (3) signature sequences in the 16S and 23S ribosomal genes (25, 26), (4) the *Chlamydiaceae* RNase P RNA gene (*rnpB*) encoding a ribonucleoprotein complex that removes 5' leader sequences from tRNA precursors during tRNA biosynthesis (38), or (5) the outer membrane protein A (*ompA*) gene encoding the chlamydia major outer membrane protein (MOMP). Polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP), nested (multiplex) PCR, TaqMan-based real-time PCR, microarrays, PCR followed by high resolution melt (HRM) curve analysis of the amplified 16S rRNA gene, as well as gene sequencing has been described for identifying *Chlamydia* species in livestock (26, 29, 57, 73, 78–80, 109).

OmpA genotyping by real-time PCR using genotype-specific probes is very often used. It allows the detection of an additional variant described as the avian *C. psittaci* genotype E/B (28). A few years ago, a genotyping microarray was introduced, allowing the identification of all currently known avian and mammalian *C. psittaci* genotypes (98). Genotyping is very convenient because it is a rapid, powerful technique that can be used directly on clinical samples in any veterinary clinical laboratory.

Some avian *ompA* genotypes appear to occur more often in a specific order of birds. Genotype A, for instance, is endemic among psittacine birds (*Psittacidae*) but it also has been found in turkeys, ducks, pigeons, and *Passeriformes*. Genotype B is endemic in pigeons (*Columbiformes*) but also can infect chickens, turkeys, ducks, *Psittacidae*, and *Passeriformes* (4). Waterfowl (*Anseriformes*), such as ducks and geese, most frequently seem to be infected with genotype C. Genotype C also has been detected in chickens, ducks, and pigeons (22, 42, 136). Genotype D strains are most often associated with turkeys, but they can also infect pigeons. More recently, genotype D has been detected in chickens (21). Genotype E, also known as Cal-10, MP, or MN, was first isolated during an outbreak of pneumonia in humans during the early 1930s. Later on, genotype E isolates were obtained from a variety of bird species including turkeys, pigeons, ducks, ostriches, and rheas. Genotype F is represented by the psittacine isolates VS225, Prk Daruma, 84/2334 (110), and 10433-MA, but has also been isolated on a Belgian turkey farm (108). Genotype E/B is often found in ducks, but it has also been detected in parrots (35), turkeys (100), and pigeons (30). The mammalian M56 and WC genotypes were isolated during an outbreak in muskrats and hares and during an outbreak of enteritis in cattle, respectively. Subgroups for 3 of the more heterogeneous genotypes have been introduced, i.e. A-VS1, A-6BC, A-8455, EB-E30, EB-859, EB-KKCP, D-NJ1, D-9 N, and provisional genotypes to cover the strains that were previously nontypeable have been suggested (77). All genotypes should be considered to be readily transmissible to humans.

Pannekoek et al. (66) used multilocus sequence typing (MLST) for studying the population structure of *C. psittaci* and *C. abortus* because Van Loock et al. (110) showed that *ompA* sequencing and even sequencing of the *rrn* spacer (25, 26) cannot always distinguish *C. psittaci* from *C. abortus*. The obtained MLST scheme was based on the partial sequences of 7 housekeeping genes, *enoA*, *fumC*, *gatA*, *gidA*, *hemN*, *hflX*, and *oppA*, representative for the whole genome sequence. MLST of *C. psittaci* strains resulted in 11 unique sequence types. MLST was extremely useful for distinguishing the phylogenetic highly related species *C. psittaci* and *C. abortus*. Interestingly, according to MLST, the *ompA* genotype F strain 84/2334 appears to be a *C. abortus* strain instead of a *C. psittaci* strain (66).

According to Wang et al. (125), high-resolution genotyping within one chlamydia species can be achieved by multilocus variable-number tandem-repeat (VNTR)

analysis (MLVA) in combination with *ompA* sequencing (MLVA-*ompA*). This method is highly accurate for distinguishing closely related strains within one *Chlamydia* species. VNTR analysis was used for exploring the diversity of *C. psittaci*. For *C. psittaci*, 20 selected genetic loci were initially tested on 9 avian reference strains including representatives of all major *ompA* genotypes (A to F and E/B). Thereafter, 8 loci were retained for a more complete study performed on more than 150 *C. psittaci* isolates from different bird species and geographical origins. The MLVA system provides an additional level of discrimination within the *C. psittaci* species, with 20 distinct patterns identified to date (50). MLVA could provide the high resolution needed for local epidemiology and accurate contact tracing in cases in which zoonosis is contracted from poultry. However, current MLVA (and MLST) methods are still easier to perform on culturable samples or clinical samples of *C. psittaci*-infected poultry with a high bacterial DNA load.

Conserved synteny, i.e. sequence and gene order conservation, in a genome of reduced size is recognized as a hallmark of the genus *Chlamydia* (31). Comparative genomics of *C. psittaci* has already revealed a number of characteristic features (15, 71, 105, 128, 136).

Pathogenicity

Chlamydia psittaci strains isolated from birds fall into 2 general categories: (1) highly virulent strains that cause acute epidemics in which 5%–30% of affected birds die and (2) less virulent strains that cause slowly progressive epidemics. Strains of both high and low virulence appear to have equal ability to spread rapidly through a flock, as evidenced by serologic test results. Highly virulent *C. psittaci* strains have been isolated from European turkeys (118), ducks (48, 96), pigeons, and more recently chickens (49, 132). They also appear in clinically normal wild birds. So far, genotypes B, C, F and E/B have been found in chickens (22, 27, 136, 137).

Highly virulent strains cause rapidly fatal disease in natural and experimental hosts with lesions characterized by extensive vascular congestion and inflammation of vital organs. Highly virulent strains have a broad spectrum of pathogenicity for laboratory animals and can cause serious human infections (some fatal) in poultry handlers and laboratory research workers. Strains of low virulence cause slowly progressive epidemics with a mortality rate of less than 5% when uncomplicated by secondary bacterial or parasitic infection. Strains of this category are often isolated from pigeons and are also found in ducks, turkeys, chickens, sparrows, and other wild birds. The turkey isolates from outbreaks with low mortality have been of genotype B or E. Birds infected with these strains usually do not develop the severe vascular damage typical in birds infected with the virulent strains, nor do they have the severe clinical signs (94).

Virulence Factors

The initial event in the infectious process begins with attachment of *C. psittaci* EBs to microvilli at the apical surface of a susceptible columnar epithelial cell (39) (Figure 24.1). The EB travels down the microvillus and locates in indentions of the eukaryotic plasma membrane, some of which resemble coated pits. The bases of micropilli represent areas of active transport of extracellular materials into the cells and, therefore, might assist rapid and efficient entry of EBs. After 1–3 hours, the EBs are internalized in invaginations of the plasma membrane. Uptake of *C. psittaci* is an endocytic mechanism involving microfilament-dependent and/or independent processes. The *C. psittaci* containing endocytic vesicles or vacuoles escape interaction with lysosomes and proceed in about 8–12 hours to the nuclear area, where EBs are converted to RBs. Conversion to RBs primarily involves reduction of disulfide bond cross-linking among the outer membrane proteins altering the EB cell wall. Synthesis of DNA, RNA, and proteins is initiated, permitting growth and binary fission of the RBs. Binary fission is characterized by the appearance of typical "hourglass" profiles inside the vacuole (Figure 24.2). The enlarging vacuole also is termed an "inclusion."

Chlamydia psittaci microorganisms do not always remain within the inclusion throughout their intracellular development. In some cases, and in apparent correlation with high virulence of strains, the inclusion membrane seems to degrade during the active multiplication, liberating the bacteria into the cytoplasm of the host cell (111). About 30 hours after internalization of the EB, the first RBs are reorganized into newly formed EBs. At about 48–50 hours, the developing chlamydial inclusion may contain anywhere from 100 to 500 progeny, depending on the characteristics of the *C. psittaci* strain (Figure 24.3). With most *C. psittaci* strains, the host cell has undergone severe degenerative changes, and microorganisms are released by lysis (Figure 24.4). Exocytosis of the inclusion, followed by a "healing" or closing of the open-cavern structures where the inclusion had existed, has been reported (111). Persistent infections may occur with nonreplicating RBs, the so-called aberrant bodies, remaining inside the host cell cytoplasm.

Long, unique, rosette-like structures and projections have been observed on the surface of both EBs and RBs of *C. psittaci* strain Mn (56). Shortly after the first description of a type III secretion system (T3SS) in *C. caviae* (GPIC strain) (41), Bavoil and Hsia (5) speculated that Matsumoto's projections are in fact functional T3SSs, injecting chlamydial virulence proteins into the host cell cytoplasm. *C. psittaci* strains also contain a T3SS (8). Beeckman et al. (8) identified a T3SS in a viru-

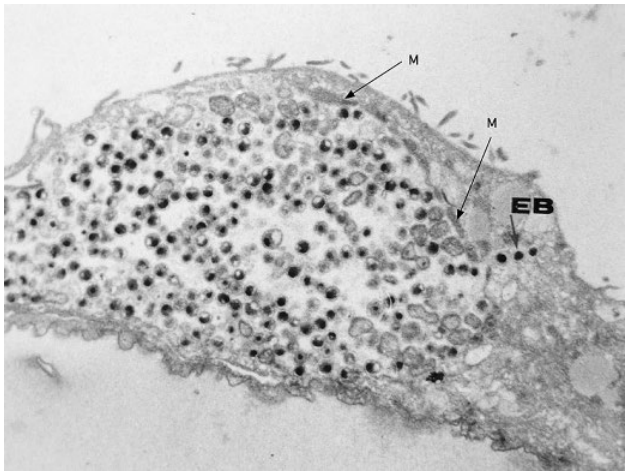


Figure 24.3 BGM cell culture, 52 hours after inoculation with a *Chlamydia psittaci* genotype D strain (92/1293), isolated from diseased turkeys. Note the large inclusion and the elementary bodies (EB) apparently "escaping" from the inclusion. Also notice the mitochondria (M) lining the inclusion.

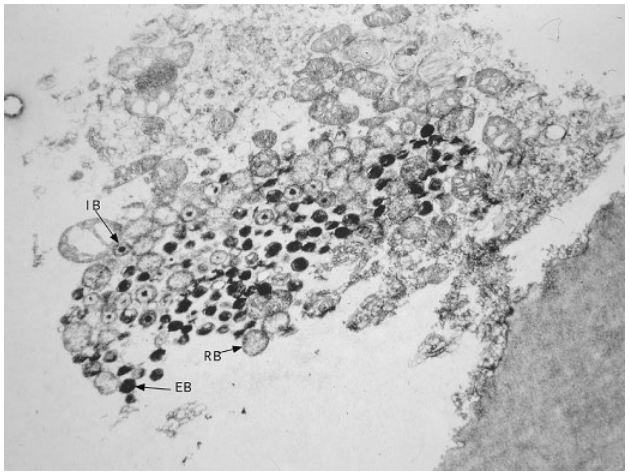


Figure 24.4 BGM microcarrier culture, 50 hours after inoculation with the *Chlamydia psittaci* Texas Turkey genotype D strain showing lysis of an infected BGM cell. The cell is sloughing off the microcarrier. Note the presence of elementary bodies (EB), reticulate bodies (RB), and intermediate bodies (IB).

lent *C. psittaci* genotype D strain isolated from turkeys and studied its possible role in virulence.

Other virulence factors under study are the Pmps (93). The Pmps belong to the family of autotransporter proteins (type V secretion system). Most autotransporter proteins contribute to the virulence of many Gram-negative pathogens. Specific roles in pathogenesis have been described, including adhesion, host and tissue tropism, and antigenic variation (immune evasion) (6, 58, 92, 126). Additional potential functions of the Pmp proteins still need to be examined.

Pathobiology and Epidemiology

Incidence and Distribution

Avian chlamydiosis occurs worldwide, with the incidence and distribution varying greatly with the species of bird and the genotype of the chlamydial organism. Antibiotics have been used extensively to control the spread of the disease in birds and to reduce the risk to humans.

The disease pattern in turkeys has changed. Historically, most outbreaks were explosive and occurred in free-ranging birds. These severe respiratory disease outbreaks were attributed to genotype D. Chlamydia was thought to have been introduced from the outside. Today, genotype D and sometimes genotype A are found in confinement turkeys when death losses are high; genotype B is less virulent (4, 74). Introduction through vertical transmission (55, 127) or through infected hatchlings (20) may occur. Studies on turkeys in Belgium and France showed that strains (genotypes A, B, D, E, E/B, and F) of high and low virulence are widely distributed in commercial turkeys and may be endemic. Chlamydia is part of the turkey respiratory disease complex (108). Infection of turkey broilers with *C. psittaci* also seems to predispose animals to more severe clinical outcomes of a simultaneous or subsequent infection with *E. coli*, avian metapneumovirus (aMPV), and/or *Ornithobacterium rhinotracheale* (106–108).

Over the last decade, *C. psittaci* infections in ducks seem to be reported more often in China and Europe (14, 16, 32, 52, 97, 131, 132) than in the United States. In Europe, the number of outbreaks in ducks seems to be increasing as well as the number of zoonotic case reports linked to handling ducks (42, 48, 123). This could be caused by reduced antibiotic use in poultry or by the occurrence of more virulent *C. psittaci* strains. European isolates have been characterized as genotype C and genotype E/B (28, 42, 48). In China, genotypes A and C have been found in ducks (83, 131).

Chlamydia psittaci outbreaks on chicken farms and zoonotic transmissions linked to contact with *C. psittaci*-infected chickens have also appeared to occur more frequently over the last decade (17, 20, 21, 27, 33, 46, 49, 73, 131, 132). In one study by Verminnen at al. (122) the author investigated the occurrence of *C. psittaci* by performing a retrospective study of 300 serum samples collected in 2005 from 10 randomly selected chicken breeder, broiler, and layer farms in Belgium. Sera were examined using a recombinant MOMP-based enzyme-linked immunosorbent assay (ELISA). Seropositive results were obtained from 98%, 95%, and 95% of layers, broilers, and breeders, respectively (21), and seropositive birds were found on all farms. Highly virulent genotype A and D strains, as well as genotypes B, C, F, and E/B

have been found in chickens in Belgium, China, France, and Germany (27, 46, 49, 132, 135, 137).

In 2008, 3 cases of atypical pneumonia in individuals working at a French slaughterhouse processing guinea fowl, ducks, and especially chickens prompted an epidemiologic survey of the 10 farms that had supplied the birds. Using a *Chlamydiaceae*-specific real-time PCR assay, chlamydial agents were detected in 14 of the 25 investigated flocks. In one duck flock studied, 20% of the tested animals were positive. Additionally, 12 of 18 (67%) and 1 of 6 (17%) of the chicken and guinea fowl flocks examined were PCR positive, respectively. Positivity for the chicken flocks ranged from 10% to 100%. For the positive guinea fowl flock, 12.5% of the tested animals were positive. Rather unexpectedly, *C. psittaci* was identified only in the positive duck flock, whereas ArrayTube DNA microarray testing indicated the presence of a new chlamydia agent in all the other French poultry flocks that were examined. Further studies on the agent found in chickens revealed the presence of a new member of the family *Chlamydiaceae* namely *C. gallinacea* sp. nov. (49, 75).

Data on *C. psittaci* infections in meat-type pigeons are primarily published in China. An indirect haemagglutination assay (detects *Chlamydiaceae*) was used to examine the seroprevalence of *C. psittaci* infections in Guangdong. Seroprevalence was 17% (34/200) in meat-type pigeons obtained from 7 commercial flocks (53). Occupationally contracted psittacosis was reported after contact with meat-type pigeons in Beijing. Employees of pigeon farms in Beijing were diagnosed with psittacosis by positive CFT and recovered after treatment with erythromycin (131).

Natural and Experimental Hosts

Chlamydiae or chlamydial antibodies have been found in at least 465 bird species (43). Common reservoirs of chlamydiae include wild and feral birds such as sea gulls, ducks, Canada geese, herons, egrets, pigeons, blackbirds, grackles, house sparrows, and killdeer, all of which freely intermingle with domestic birds (51). Highly virulent strains of *C. psittaci* can be carried by and excreted in large numbers without any apparent effect on these hosts.

Experimental hosts of avian chlamydiae can include virtually any species of bird. Mammalian laboratory hosts used for avian chlamydiae are principally mice and occasionally guinea pigs. Mice and guinea pigs are the natural hosts for *C. muridarum* and *C. caviae*, respectively. Investigators using these animals should determine the chlamydial status of the breeding stock. Rabbits are refractory to clinical disease caused by avian chlamydiae, but they may be used to produce polyclonal antibodies.

Younger domestic birds generally are more susceptible than older birds to infection, clinical disease, and mortality. However, maternal antibodies might protect against respiratory disease outbreaks on the farm. Infection in old turkeys, such as spent breeder hens, can go unnoticed unless birds are subjected to stressful conditions such as shipment to market on crowded trucks. Turkey toms may have a higher mortality rate than turkey hens.

Transmission, Carriers, and Vectors

Transmission of *C. psittaci* primarily occurs from one infected bird to another susceptible bird in close proximity. The agent is excreted in feces and nasal discharges. Fecal shedding occurs intermittently and can be activated through stress caused by nutritional deficiencies, prolonged transport, overcrowding, chilling, breeding, egg laying, treatment, or handling. Bacterial excretion periods during natural infection can vary depending on virulence of the strain, infection dose, and host immune status. However, shedding may occur for several months. Transmission of chlamydiae occurs primarily through inhalation of contaminated material and, sometimes, ingestion.

Large numbers of *C. psittaci* cells can be found in respiratory tract exudate and fecal material of infected birds. The importance of the respiratory exudate in transmission has become more apparent. In turkeys, the lateral nasal glands become infected early and remain infected for more than 60 days. Choanal/oropharyngeal swabs are more consistent for isolation of the agent than fecal swabs, especially during early stages of infection. Direct aerosol transmission through aerosolization of respiratory exudate must be considered as the primary method of transmission

Avian species, including domestic poultry sharing aquatic or moist soil habitats with wild infected aquatic birds, may become infected via contaminated water. Granivorous birds, like pigeons, doves, pheasants, and house sparrows, may become infected by dust inhalation in barnyards and grain storage sites contaminated by feces. The consumption of infected carcasses may transmit *C. psittaci* to host species that are predators or scavengers of other birds.

Transmission of *C. psittaci* in the nest is possible. In many species, such as *Columbiformes*, cormorants, egrets, and herons, transmission from parent to young may occur through feeding by regurgitation, whereas contamination of the nesting site with infective exudates or feces may be important in other species, such as snow geese, gulls, and shorebirds. Furthermore, *C. psittaci* can be transmitted from bird to bird by bloodsucking ectoparasites such as lice, mites, and flies or, less commonly, through bites or wounds. Transmission of *C. psittaci* by arthropod vectors may be facilitated in the nest.

Mites from turkey nests can contain chlamydiae (23) and during an epidemic in turkeys in South Carolina, simulid flies were suspected as a possible method of transfer (64). Transmission through insects is indeed not unlikely because a recent study by Pilloux et al. (68) demonstrated a high prevalence and variety of *Chlamydiales* DNA within *Ixodes ricinus* ticks.

Vertical transmission has been demonstrated in turkeys, chickens, ducks, parakeets, seagulls, and snow geese, although the frequency appears to be fairly low (55, 127). However, it could serve as a route to introduce chlamydiae into a poultry flock. Recently, experimental evidence was presented for the transmission of *C. psittaci* in poultry by eggshell penetration (1).

Chlamydia psittaci can be introduced into poultry through the wild bird population. Contaminated feed, barn bedding or equipment also can be a source of infection, and feed should therefore be protected from wild birds. Careful cleaning of equipment being used in several barns during one and the same production round is extremely important because *C. psittaci* can survive in feces and bedding for up to 30 days (34).

Incubation Period, Clinical Signs, Morbidity and Mortality, Pathology, and Pathogenesis

Turkeys

Vanrompay et al. (113) used immunodetection to study the pathogenesis of *C. psittaci* genotype A, B, and D strains in specific pathogen free (SPF) turkeys. Use of immunodetection allowed precise determination of tissue and cell tropism. In this study, turkeys were aerosol infected, because it represents the natural route of infection (65). From this study, the following pathogenic sequence of events can be deduced for all 3 genotypes investigated. In turkeys infected by aerosol, the primary site of replication is the upper respiratory tract, where epithelial cells become infected. Subsequently, epithelial cells of the lower respiratory tract and macrophages throughout the respiratory tract become infected. Then, intense replication occurs in the respiratory tract. At the same time, chlamydiae can be demonstrated in plasma and monocytes, indicating septicemia, and chlamydiae appear in epithelial cells and macrophages of various tissues throughout the body.

In turkeys, an experimental infection with a genotype B strain induced much milder clinical signs and lesions than infection with genotype A or D strains (115). The genotype B strain had a longer incubation period, took longer to reach maximum titers in the tissues, and had shorter periods during which the organism was found in the tissues.

The incubation period of chlamydiosis in naturally infected birds varies, depending upon the number of chlamydiae inhaled, the virulence or pathogenicity of the infecting strain for that host species, and host immunogenetics. Experimentally, definitive disease signs in young turkeys receiving a virulent strain may be evident in 5–10 days. In birds naturally exposed to smaller doses or in older birds, the period may be longer. Strains of lower virulence, which cause less severe signs, may have longer incubation periods. Clinical signs may not be noticeable until 2–8 weeks after exposure.

Signs of chlamydiosis in turkeys infected with virulent strains are cachexia, anorexia, elevated body temperature, conjunctivitis, and respiratory distress. Diseased birds excrete yellow-green, gelatinous droppings. Egg production of severely affected hens declines rapidly to 10%–20% and may temporarily cease or remain at a very low rate until recovery is complete. Disease signs in a flock infected with strains of low virulence are usually anorexia and loose, green droppings in some birds, with less effect on egg production.

At the peak of disease outbreak in a flock infected with a virulent strain, 50%–80% of the birds will show clinical signs, whereas morbidity from less virulent strains is only 5%–20%. Mortality caused by virulent chlamydia ranges from 10% to 30% and is only 1% to 4% with less virulent strains.

The less virulent strains cause gross lesions, which are similar to those caused by virulent strains, only less severe and extensive. In overwhelming infections with virulent strains, lungs show diffuse congestion, and the pleural cavity may contain fibrinous exudate. In fatal cases, a dark transudate may fill the thoracic cavity. The pericardial membrane is thickened, congested, and coated with fibrinous exudate. The heart may be enlarged, and its surface may be covered with thick fibrin plaques or encrusted with yellowish, flaky exudate (Figure 24.5). The liver is enlarged and discolored and may be coated with thick fibrin. Air sacs are thickened and heavily coated with fibrinous exudate (Figure 24.6). The spleen is enlarged, dark, and soft and may be covered with gray-white spots representing areas of focal cellular proliferation. The peritoneal serosa and mesentery show vascular congestion and may be coated with foamy, white, fibrinous exudate. All of these exudates contain large numbers of mononuclear cells in which numerous microcolonies of chlamydial RBs may be seen. Fibrinous exudates, found on all organs and tissues of the thoracic and peritoneal cavities, reflect vascular damage as well as increasing inflammatory response caused by the continued multiplication of the organisms. In birds that survive infection with a strain of low virulence, the lungs may not be seriously affected. However, multiplication of organisms on the epicardium may result in the formation of 1 or more fibrin plaques.

Vanrompay et al. (112) examined histopathologic changes in 4 groups of 20 SPF turkeys kept in isolation units and inoculated by the natural route of infection

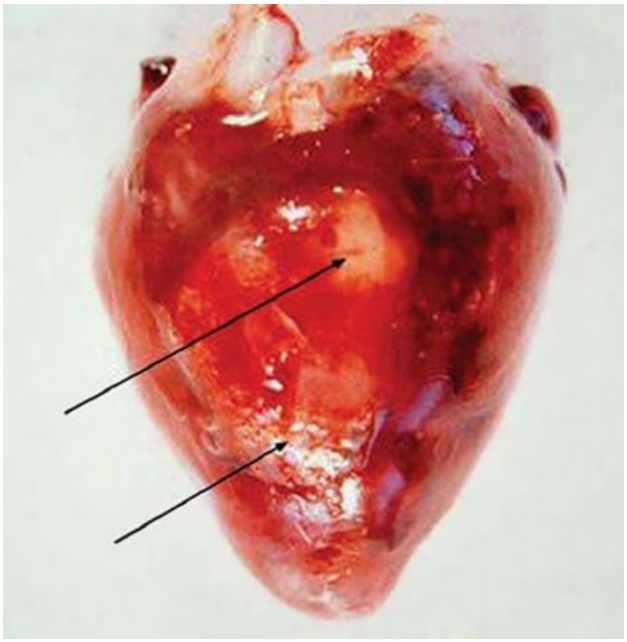


Figure 24.5 Turkey experimentally infected (aerosol) with a *Chlamydia psittaci* genotype A strain (84/55) isolated from the lungs of a budgerigar. Note the presence of serous fluid together with fibrin in the pericardial sac (arrows).

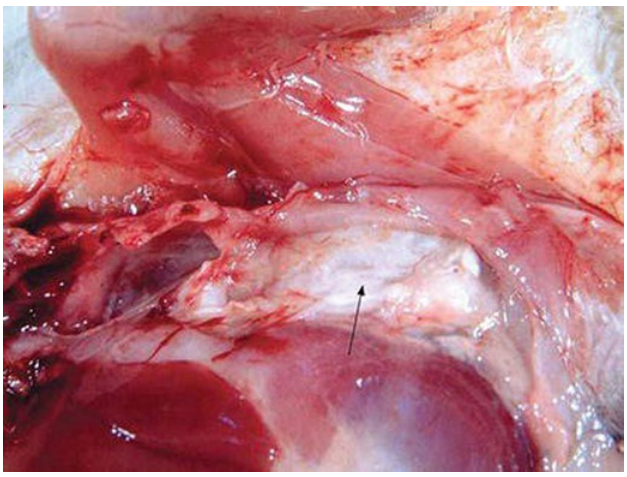


Figure 24.6 Turkey experimentally infected (aerosol) with a *Chlamydia psittaci* genotype A strain (84/55). Note the thickened abdominal airsac totally covered with fibrin cloths (arrow).

(aerosol). Turkeys were experimentally infected with strain 84/55 (*C. psittaci* genotype A), isolated from a parakeet, strain 92.1293 from a turkey (*C. psittaci* genotype D), the Texas Turkey strain (*C. psittaci* genotype D), or strain 89/1326 (*C. psittaci* genotype B) from a pigeon. All 4 strains proved to be pathogenic for SPF turkeys. Turkeys showed conjunctivitis, sinusitis, rhinitis, keratitis, pericarditis (Figure 24.5), pneumonia, airsacculitis (Figure 24.6), hepatosplenomegaly, enteritis, congestion

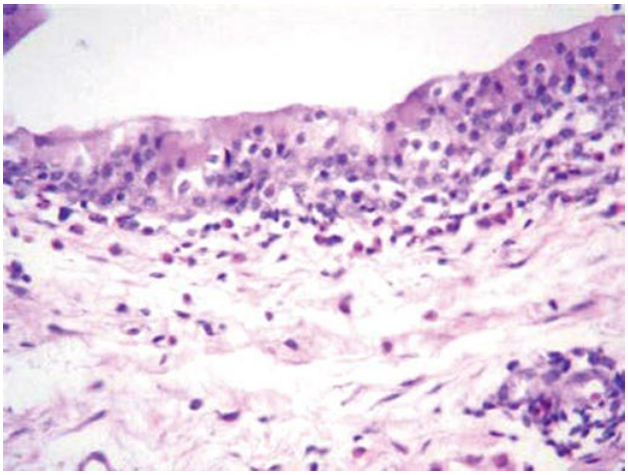


Figure 24.7 Hematoxylin and eosin staining of experimentally infected turkeys. Conjunctiva with infiltration of lymphocytes and heterophils together with epithelial vacuolization and hyperplasia. ×172.

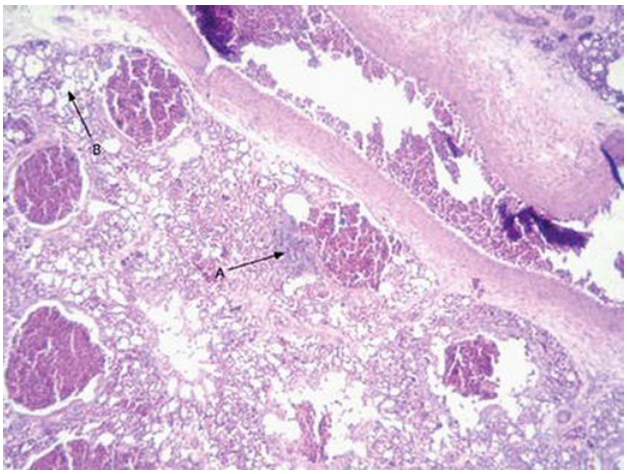


Figure 24.8 Hematoxylin and eosin staining of experimentally infected turkeys. Congested lung with infiltration of lymphocytes (arrow A) and dilated bronchi and parabronchi (arrow B). ×69.

of the kidneys, and congestion of the ovaries or testes. There were epithelial erosions and fibrin deposit in the conjunctivae (Figure 24.7), corneal ulceration, bronchopneumonia (Figure 24.8), fibrinous necrotizing airsacculitis (Figure 24.9), fibrinous pericarditis, interstitial nephritis, peritonitis, and catarrhal enteritis. The type and distribution of the lesions was similar for genotypes A and D. However, the lesions produced after genotype A infection appeared more severe. For genotype B, in comparison with both other genotypes, no lesions were observed in the small intestine, pancreas, ovary, and testis.

Controlled dual infections in SPF turkeys demonstrated the pathogenic interplay between *C. psittaci*, aMPV, and *E. coli*. *E. coli* is a predisposing factor for the

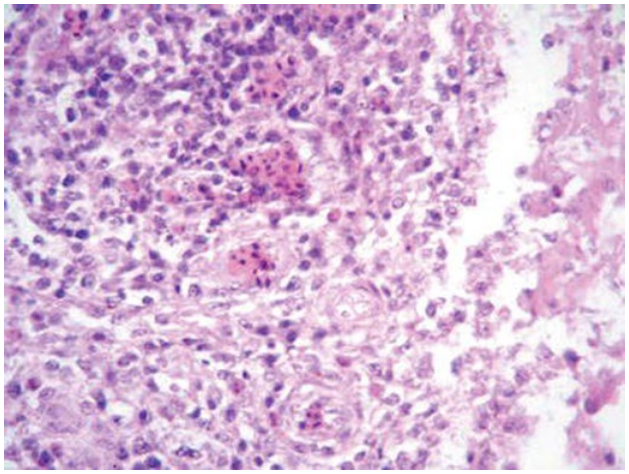


Figure 24.9 Hematoxylin and eosin staining of experimentally infected turkeys. Fibrinous necrotizing airsacculitis. ×172.

outcome of a *C. psittaci* infection. It can increase the severity of a *C. psittaci* infection and can reactivate a latent *C. psittaci* infection (106). An aMPV infection during the acute phase of a *C. psittaci* infection aggravates the severity of clinical signs, macroscopic lesions, pharyngeal aMPV excretion, and histological tracheal lesions. However, no clear interaction was established after an aMPV infection in latently *C. psittaci*-infected turkeys (107).

Chickens

Epidemiologic evidence formerly indicated that chickens are relatively resistant to disease caused by *C. psittaci*. Acute infection progressing to disease and mortality only occurred in young birds, and the incidence of actual epidemics was very low. Most natural infections in chickens were believed to be inapparent and transient. However, *C. psittaci* strains isolated from turkeys caused similar pathology and mortality in chickens as in turkeys (88, 90).

Recently, highly virulent *C. psittaci* strains have been isolated from the lungs of diseased chickens raised in Belgium, France, Germany, and China (27, 131, 132, 137). The strains obtained from Belgian and French farms belonged to genotypes D and B and could successfully be used to reproduce the disease in experimentally infected SPF chickens (132). Chickens showed conjunctivitis, rhinitis, pneumonia, fibrinous airsacculitis, fibrinous pericarditis, and hepatosplenomegaly. Histopathological lesions (133) and mortality was observed.

Ducks and Geese

Chlamydiosis in domestic ducks is important both economically and as a public health hazard. Over the last decade, outbreaks have primarily been reported in China and Europe (14, 32, 33, 48, 49, 52, 131). Chlamydiosis in ducks is usually a severe, debilitating, often fatal disease in which young ducks develop trembling, imbalanced gait, and cachexia. They become anorexic with green, watery intestinal contents and develop a serous to purulent discharge from the eyes and nostrils causing the feathers on the head to become encrusted with exudate. As the disease progresses, the ducks become emaciated and die in convulsions. Morbidity ranges from 10% to 80%, and mortality varies from 0% to 30% depending on age and the presence of concurrent infections. Recently, severe outbreaks associated with human disease were reported in France (48, 49).

Incidental to studies of chlamydiosis in ducks, several investigators have observed *C. psittaci* antibodies or the disease in geese and have isolated *C. psittaci* from diseased tissues (2). Clinical disease and necropsy findings were similar to those in ducks.

Pigeons

Signs of uncomplicated chlamydiosis in meat pigeons are variable, but those that develop acute disease are anorexic, unthrifty, and diarrhetic (2, 137). Some develop conjunctivitis, swollen eyelids, and rhinitis. Respiratory difficulty is accompanied by rattling sounds. As disease progresses, birds become weak and emaciated. Mortality occurs. Recovered birds become asymptomatic carriers. Some birds progress through an infection showing no signs or, at the most, transient diarrhea before becoming carriers. Salmonellosis or trichomoniasis exacerbates the illness in chlamydia-infected carrier birds, inducing signs and lesions of acute disease. Gross lesions of uncomplicated chlamydiosis in pigeons are fibrinous exudates on thickened air sacs, the peritoneal serosa, and occasionally the epicardium. The liver is usually swollen, soft, and discolored. The spleen may be enlarged, soft, and dark. Greater than normal amounts of urates are seen in cloacal contents if catarrhal enteritis occurs. In less severe infections, only the liver or air sacs are involved. Some heavily infected shedders have no lesions.

Pheasants, Quail, Guinea Fowl, Peacocks, and Partridges

Chlamydiosis has been reported in farm-raised pheasants, quail, peacocks, guinea fowl and partridges from all over the world (24, 42, 131). The clinical signs and lesions are similar to those seen in other birds (91). Morbidity and mortality can be very high, especially in young birds. Only a few human infections have been reported during the last decade (42, 49, 130, 131)

Immunity

Natural immunity to chlamydia is generally poor and short-lived. As birds become older, however, they become more resistant to clinical disease, even though infection may occur.

The immune response to chlamydia is a coordinated event in which innate immune cells, B cells, and T cells, act in concert and each of these immune effectors have roles in recognizing different stages of the infection. To date, *C. psittaci* vaccine studies suggest that the ideal, efficacious chlamydia vaccine should induce $CD4^+$ T helper 1 (Th1), and $CD8^+$ cytotoxic T cell responses (70). Humoral immune responses, albeit not considered as crucial, seem to contribute significantly to protection (121).

Less is known about innate immune detection of *C. psittaci*. Beeckman et al. (7) determined the cytokine responses following *C. psittaci* infection of avian monocytes/macrophages. High IL-10 and no TGF-β responses were observed at 4 hours post inoculation. This could induce macrophage deactivation and NF-κB suppression, and thereby could dampen innate immunity and promote *C. psittaci* survival in macrophages.

Diagnosis

The preferred method for the identification of avian chlamydiosis is no longer isolation and identification of the organism. Because of the time involved, the need for high-quality samples, the fact that some strains will never grow *in vitro*, and the hazard to laboratory personnel (BSL3 laboratory required), NAATs are currently recommended for quick, sensitive, and specific diagnosis. These include conventional and real-time PCR, DNA microarray-based detection and DNA sequencing. Culture, cytological staining, immunological staining, immunohistochemistry, or antigen ELISA can be used if NAATs are not available.

Specimen Collection and Storage of Samples

The following samples should be preferably collected: pharyngeal/choanal slit swabs in live birds experiencing respiratory disease (3, 112) and/or conjunctival swabs if indicated by the presence of conjunctivitis. Cloacal swabs or fresh feces are less optimal because chlamydial shedding is intermittent. In dead birds, lungs and thickened exudate-coated air sacs are especially suitable but spleen, liver, and free exudates can also be sampled.

Specimens should be collected aseptically if culturing chlamydiae is desired as contaminating bacteria can interfere with the isolation of chlamydiae. Proper handling of clinical samples is necessary to prevent loss of infectivity if culture is to be used. If specimens are used to inoculate cell cultures or embryonated eggs immediately, most diluents will be adequate; however, if the specimen is to be shipped and/or stored before analysis, a special chlamydia transport medium should be used (84, 119). Samples in chlamydia transport medium can be stored for 1 or 2 days at 4 °C prior to analysis. However, longer preservation needs to be performed at −80 °C. Chlamydiae in tissue specimens or yolk-sac suspension can be preserved almost indefinitely by storage at −80 °C.

Culture

Preparation of Inoculum

Prior to inoculation, samples must be treated properly. The processing of samples is similar for inoculation of cell cultures or embryonating eggs. Penicillin and tetracyclines should be avoided because they inhibit the growth of *C. psittaci*.

A standard procedure is to prepare 20% tissue suspensions in phosphate-buffered saline (PBS). The suspensions are centrifuged (2,790 × *g*, 4 °C) for 10 minutes. The supernatants are collected and 10 µL/mL streptomycin sulfate (streptomycin sulfate 1% w/v) and 20 µL/mL vancomycin (vancomycin 0.5 w/v) are added. If needed, 0.1% amphotericin B can be added. After 1 hour of incubation at room temperature, the suspensions are centrifuged for 30 minutes (2,790 × *g*, 4 °C) and supernatants are immediately used for inoculation or stored at −80 °C until use. Swabs in chlamydia transport medium are shaken for 1 hour at 4 °C on a rocking platform, centrifuged (2,790 × *g*, 4 °C), and the supernatant immediately used or stored at −80 °C until use. Fecal samples, although not preferably used for diagnosis because of the intermittent chlamydia shedding and the risk for false negatives, are processed as follows. A 20% suspension is made in PBS, and the suspension is shaken for 1 hour (4 °C) on a rocking platform. Then, the suspension is sonicated for 10 minutes using an ultrasonic water bath. The supernatant is collected, and 10 µL/mL streptomycin sulfate (streptomycin sulfate 1% w/v), 20 µL/mL vancomycin (vancomycin 0.5 w/v), and 0.1% amphotericin B are added. The suspension is incubated at room temperature (1 hour) and subsequently centrifuged (2,790 × *g*, 4 °C) for 30 minutes. The supernatant is collected and thereafter ultracentrifuged (45,000 × *g*, 4 °C) for 45 minutes. The supernatant is discarded and the remaining chlamydia pellet is resuspended in diluent and immediately used for inoculation or stored at −80 °C until use.

Cell cultures are the most common and convenient method for the isolation of *C. psittaci*. The most commonly used cell lines are Buffalo green monkey (BGM), Vero, McCoy, HeLa, and L-929, although a number of other cell cultures, such as chicken embryo fibroblasts, can be used. A study showed BGM to be the most sensitive, with Vero and L-929 listed as satisfactory (136). Standard cell culture medium is used, containing 5%–10% fetal calf serum and antibiotics like vancomycin, streptomycin, and amphotericin B because they do not inhibit the growth of *C. psittaci*. Cell culture harvest can

be frozen at –80 °C in sucrose phosphate glutamate (SPG) buffer (1/1; SPG/culture medium).

The culture equipment must be suitable for: (1) preferably identification by immunofluorescence staining, (2) centrifugation (500–1,500 × *g* for 60–90 minutes) of the inoculum onto the monolayer at 37 °C to enhance infectivity (eventually in the presence of diethylaminoethyl (DEAE), (3) possible blind passages at 3 or 6 days postinoculation to increase sensitivity of isolation, (4) examination of the sample 2–3 times during passage, and (5) protection of humans against possible infection. Small, flat-bottomed vials (1-dram shell vials) or bottles with 12-mm diameter glass coverslips meet these requirements and are often used because the cell culture monolayer can be grown directly on the coverslip. Several vials are inoculated with each sample to permit fixing and staining at various times and to permit passages of negative samples after 6 days of incubation.

Chlamydiae can be isolated from cells that are replicating normally. Most diagnosticians, however, prefer to use nonreplicating host cells for 2 reasons: (1) to provide increased nutrients for the replication of chlamydiae, and (2) because nonreplicating cells can be maintained for longer periods for observation. Host-cell replication is suppressed most commonly using cycloheximide (0.5–2.0 mg/mL). Incubation is usually at 37 °C–39 °C, depending on the cell culture used. Disruption of the monolayer by freeze–thawing should be avoided because it can destroy *C. psittaci*.

Some laboratories still use chicken embryos for primary isolation of chlamydiae. The standard procedure is to inject up to 0.3 mL of inoculum into the yolk sacs of 6-day-old embryos (136). Replication of chlamydia usually will cause the death of the embryo within 5–12 days after inoculation. If no deaths occur, 2 additional blind passages are usually made before calling the sample negative. Chlamydia infection typically causes vascular congestion of the yolk-sac membranes, which are harvested and homogenized as a 20% yolk-sac suspension. This suspension can be frozen (–80 °C) to preserve the strain or inoculated into other eggs if needed or into cell culture monolayers. *C. psittaci* is usually identified by immunofluorescence staining of yolk-sac impression smears.

Staining Cell Monolayers or Yolk-Sac Impression Smears

The preferred method for fixing of the monolayer is to remove the medium, wash once with PBS, and fix with cold acetone for 10 minutes (–20 °C). If the cell culture vessel is plastic, the monolayer can be fixed with a mixture of 50% acetone and 50% methyl alcohol or with 100% methyl alcohol. The preferred method for staining is the fluorescence method. With the fluorescence method, the fluorescein-conjugated anti-chlamydia serum is applied to the glass slide and incubated for a minimum of 30 minutes at 37 °C. The slides are then washed with PBS and deionized water, air dried, and mounted for use by a fluorescence microscope. Commercially fluorescently labeled mAbs are available.

Direct Visualization: Cytological Staining Techniques

Chlamydiae can be detected in smears of cloacal and/or conjunctival swabs and in impression smears of tissues (lung, liver, spleen, kidney, and airsacs if enough material is available) by cytological staining such as Giemsa, Giménez, modified Giménez, Ziehl–Neelsen, and Macchiavello stains (13). The modified Giménez technique is most often used (2). However, none of the stains specifically detects chlamydia. They are all less sensitive than antibody-based antigen detection methods or specific NAATs. Therefore, use of cytological staining is losing popularity.

Antigen Detection

Immunological Staining Techniques

Immunofluorescence staining can be used to detect chlamydiae in smears of cloacal and/or conjunctival swabs, in impression smears of tissues, and in frozen tissue sections. Most commercial staining kits use fluorescein isothiocyanate conjugated anti-LPS or anti-MOMP mAbs and most of them are originally developed for detection of *C. trachomatis* in human specimens.

Immunohistochemical staining is used to detect chlamydiae in paraffin sections and to show the association of chlamydial agents and pathological lesions in tissues. Commercial anti-*Chlamydia* antibodies for immunohistochemistry are available and most of them detect the chlamydial LPS.

Antigen Enzyme-Linked Immunosorbent Assays

The ELISA has been extensively promoted in kit format for use in the diagnosis of human chlamydiosis. These test kits detect the LPS antigen (group reactive) and will detect all species of *Chlamydiaceae*. In the past, a number of these kits have been tested for use in detecting chlamydiae in birds (116), but none of the kits has been licensed for detection of *C. psittaci*. One problem with some of these tests is that the chlamydial LPS shares some epitopes with other Gram-negative bacteria, and these epitopes can cross-react, resulting in a high number of false-positive results. This problem has been reduced or eliminated in more recently developed kits by careful selection of the mAbs used. These kits, however, still lack sensitivity because a few hundred organisms are still needed to give a positive reaction.

Detection of Nucleic Acids

Reagents designed to stabilize the DNA should be considered when a delay in processing the sample is anticipated (18). DNA samples can be prepared using inexpensive reagents or commercially available kits.

In the last decade, real-time PCR has become the preferred method in diagnostic laboratories for its rapidity, high throughput, potential for quantification, and ease of standardization. Several real-time PCRs for the detection of *C. psittaci* have been developed (29, 37, 67). This technology requires a fluorescent-labelled probe and special equipment, which increases costs. Its sensitivity is lower than that of the nested PCR (78, 109) but contamination problems, caused by post-PCR carry over of DNA of a previous amplification round and labor, are reduced because it is based on one reaction in a closed system. *Omp*A-based real-time PCR protocols were developed to differentiate between genotypes of *C. psittaci* (29, 36) and to distinguish *C. psittaci* from *C. abortus* (63). The PCR developed by Heddema et al. (36) is also validated on a large number of human psittacosis samples and thus is helpful to trace infection sources of zoonotic transmission. Real-time PCR protocols are available for the specific detection of *C. avium* (139) and *C. gallinacea* (47).

DNA microarray technology has also been used in the diagnosis of chlamydial infections in animals (78). The assay for detection and identification of *Chlamydiaceae* spp. is based on PCR amplification of the 23S rRNA gene and subsequent identification of *Chlamydia* species occurring in animals, including *C. psittaci*, *C. avium,* and *C. gallinacea,* by hybridization with species-specific probes. An extended version of the *Chlamydiaceae* DNA microarray allows for *omp*A-based genotyping of *C. psittaci* strains (77).

Serology

Serology alone is not particularly useful in diagnosing a current chlamydial infection in birds because of the high prevalence of this infection in birds and the long-term (up to several months) persistence of anti-chlamydial antibodies. In most bird species, there is a high background rate of anti-chlamydial antibodies in birds. Thus, to determine if a single bird is infected, serology should always be used in conjunction with antigen or gene detection, or paired sera should be examined. However, obligatory examination of paired sera removes serology from immediate clinical relevance. A positive test is evidence that the bird was infected by the bacterium but does not necessarily indicate an active infection. False negative results can occur in birds with acute infections that are sampled before seroconversion. Treatment with antibiotics also may delay and/or diminish the antibody response. The main serological method used for detecting chlamydial antibodies is the CFT. However, the CFT is more often being replaced by highly sensitive and specific ELISAs based on the use of recombinant proteins (122) or peptide antigens (76). ELISAs can detect avian IgM, IgG, and IgA as long as the correct isotype-specific conjugate is used.

Differential Diagnosis

The signs of chlamydiosis in birds are nonspecific and resemble those observed in many other diseases. Suspected chlamydiosis may have to be differentiated from pasteurellosis, particularly in turkeys, in which some signs and lesions may be similar. Pasteurellosis can be ruled out by appropriate culture procedures. Because of some similar signs and lesions, *O. rhinotracheale* infections and mycoplasmosis may need to be ruled out in turkeys and chickens suspected of having chlamydiosis. This can be accomplished by culturing and serologic testing. Colibacillosis may mimic chlamydiosis to some extent, but it can be excluded by the use of appropriate coliform culturing procedures. Avian influenza virus, paramyxoviruses, herpes viruses or aMPV may have to be ruled out in suspected chlamydiosis by virus isolation and serologic testing.

Intervention Strategies

Management Procedures

Ideally birds should be reared in confinement without contact with contaminated equipment or premises. Contact with potential reservoirs or vectors such as pet birds, rodents, arthropods, and wild and feral birds also should be prevented. General sanitation must be practiced diligently. Movement of people should be restricted so that visitors do not have free access to premises holding birds. This is easier to accomplish if birds are confined in houses and if the "all-in-all-out" principle is used on the farm.

A sensitive technique for *C. psittaci* bioaerosol monitoring is available. The air collection medium used (ChlamyTrap) can be examined by PCR or culture. The technique could be used for monitoring the infection pressure in the poultry industries (101).

Susceptibility to Chemical and Physical Agents

The survival of microorganisms in aerosols depends on 5 different factors: relative humidity, temperature, level of oxygen, presence of ultraviolet radiation, and constituents

of the aerosol and of air (95). The degree to which these factors influence the survival of microorganisms in aerosols depends strongly on the type of microorganism and the time it has to spend in the aerosol. In general, the following rule applies: Gram-negative bacteria survive best at low temperatures and relative humidity. Thus, *C. psittaci* remains viable at low temperatures and is resistant to desiccation. The bacterium is highly susceptible to repeated freeze–thawing cycles and is destroyed within 3 minutes when exposed to ultraviolet light.

Chlamydiae are highly susceptible to chemicals that affect their lipid content or the integrity of their cell walls. Even in a milieu of tissue debris they are inactivated rapidly by surface-active compounds, such as quaternary ammonium compounds and lipid solvents (104). Infectivity is destroyed within minutes by exposure to common disinfectants such as benzalkonium chloride, alcoholic iodine solution, 70% ethanol, 3% hydrogen peroxide, and silver nitrate, but they are resistant to cresol compounds and lime. Dilute suspensions (20%) of infectious tissue homogenates are inactivated by incubation for 5 minutes at 56 °C, 48 hours at 37 °C, 12 days at 22 °C, and 50 days at 4 °C (88).

Vaccination

Commercial chlamydia vaccines for poultry are not available. Protective immunity to *Chlamydiaceae* is believed to be effected primarily through the action of $CD4^+$ Th1 lymphocytes, $CD8^+$ T lymphocytes, mononuclear phagocytes, and cytokines secreted by these cells. In addition, the role of antibodies is not to be underestimated. A protective chlamydial antigen that has been unambiguously identified is the MOMP.

This paragraph summarizes knowledge on *C. psittaci* vaccination experiments in poultry performed during the last decade. In light of current knowledge on protective chlamydial immunity, plasmid DNA expressing the MOMP of *C. psittaci* has been tested for its ability to raise a protective immune response in SPF turkeys against challenge with *C. psittaci* strains (121). Effective priming of T cell memory and significant reduction in clinical signs, lesions, bacterial excretion, and *C. psittaci* replication in tissues was observed. Zhou et al. (138) used a human adenovirus serotype 5 (AdEasyTM-1), which was rendered replication defective by the deletion of the E1 and E3 genes, to obtain a recombinant adenovirus containing the MOMP gene (rAd-MOMP) of a Chinese *C. psittaci* strain of chicken origin. Low mean serum antibody responses (determined by indirect haemaglutination assay) and extremely low mean stimulation indexes in the T cell proliferation assay were observed. The vaccine seemed to induce protection in SPF chickens based on the observed differences in pathology. Unfortunately, chlamydia excretion and replication in tissues was not examined in this study. More recently, Liu et al. (54), evaluated a recombinant herpes virus of turkey (HVT)-delivered vaccine against *C. psittaci* and Marek disease, expressing *C. psittaci* PmpD in SPF chickens. Postchallenge with *C. psittaci* CB7 strain, a significant decrease in respiratory distress, lesions, and chlamydia load was found in the vaccinated group compared with the nonvaccinated controls.

Prevention by Means of Ovotransferrin

Ovotransferrin (natural antimicrobial protein) was successfully used to reduce clinical signs, lesions, excretion, and chlamydia replication in experimentally infected SPF turkeys (102). Also, *C. psittaci* infection pressure on a turkey broiler farm was significantly reduced by administering ovotransferrin aerosols. Ovotransferrin significantly reduced respiratory disease, mortality, and antibiotic use on the farm (99).

Treatment

Chlamydiosis treatment for poultry has not changed over the years (113). The drug of choice varies from country to country. Among tetracyclines, which are the drugs of choice, chlortetracycline and doxycycline are most often used. Enrofloxacin (fluoroquinolone antibiotic) also can be used, although some countries decided to ban the use of this antibiotic in poultry because of the risk that it promotes drug-resistant bacteria that can be harmful to humans.

Turkeys can be treated with chlortetracycline (CTC) at a concentration of 400 g/ton of pelleted feed. Care must be taken so that heat generated during pelleting does not destroy CTC and lower the active concentration below an effective level. The CTC-medicated feed must be given for 2 weeks and then replaced by nonmedicated feed for 2 days prior to the birds being slaughtered for human consumption. Calcium supplements should not be added to CTC-medicated pellets because calcium ions chelate CTC and diminish its effectiveness. Turkeys also can be treated with doxycycline hyclate formulations for drinking water (doxycycline hyclate 500 mg/g, dose of 20 mg/kg bodyweight/day) for 5 days or enrofloxacin (100 mg/mL) at a daily dose of 10 mg of enrofloxacin/kg of bodyweight, in water, for 3–5 days and in case of mixed or chronic infections for 5–10 days. Medicated drinking water should be replaced every 24 hours. It is recommended that all turkeys on the infected premises be treated.

Essentially the same treatment methods are used to treat other fowl infected with *C. psittaci*. In other birds, salmonellosis may often be a complicating factor so it may be necessary to use a combination of antibiotics. Treatment may not be effective in eliminating the carrier

state. Additional periods of treatment may be needed, especially when birds are kept for several weeks.

State Regulations

Because regulations may vary from country to country, the appropriate public health and/or animal health agencies should be consulted as necessary. In many countries, psittacosis (humans) and even chlamydiosis in poultry are notifiable diseases and must be reported within 48 hours.

According to United States Department of Agriculture (USDA) regulations, movement of poultry, carcasses, or offal from any premise is prohibited where the existence of chlamydiosis has been confirmed by isolation of a chlamydial agent. The Animal and Plant Health Inspection Service (APHIS) of the USDA and the United States Department of Health and Human Services (US HHS) forbid interstate movement of birds from infected flocks, but there is no restriction on movement of eggs from such flocks, which is not wise considering the possibility of vertical and horizontal transmission of *C. psittaci* through eggs. The European legislation is similar and also rather vague on movement of eggs when *C. psittaci* is suspected or even proven. It states that the competent authority shall ensure that the measures necessary to avoid any spread of disease are taken, in accordance with the requirements of Union legislation governing measures to be taken against the disease in question and on trade in animals.

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Section IV

Fungal Diseases

Chapter 25 Fungal Infections 1111

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Fungal Infections

Pascal Arné and Margie D. Lee

Summary

Agent, Infection, and Disease. Aspergillosis is the most common fungal disease in birds. Zygomycosis has similar signs and gross lesions. Aspergillosis, zygomycosis, and ochroconosis are noncontagious infections caused by inhalation of spores and produce both acute and chronic disease depending on the bird's age. Candidiasis (crop mycosis) is a yeast infection of the digestive tract resulting from a microflora imbalance. Ringworm (favus) is a fungal infection of the skin surface that is uncommonly seen in commercial poultry.

Diagnosis. Diagnosis of fungal diseases, particularly in chronic cases, can be a challenge. It involves a combination of clinical signs, gross and microscopic pathology, and culture to verify the agent. Granulomas are common in aspergillosis, ochroconosis, and zygomycosis; histopathologically fungal elements can be seen in lesions. Thickening of the crop mucosa with white rugose lesions is common in candidiasis; abundant yeast can be seen with cytology. Favus is associated with hyperkeratosis of the skin and fungal structures may be seen in cytology. Confirmation of the etiologic agent is performed by diagnostic microbiology.

Intervention. With the exception of candidiasis, spore contamination of the environment increases the prevalence of mycotic diseases. Reducing spore load by sanitation of the hatching or rearing premises, removal of moldy feed and bedding, and good air quality will help reduce fungal disease problems. Culling affected animals is recommended in commercial flocks because antifungal therapeutics have not been shown to be effective. Candidiasis can be treated with antifungals in the drinking water.

Introduction

Mycotic infections are relatively common in avian species. Medically important fungi are opportunistic rather than obligate parasites (44). They belong to the Fungi kingdom (Eumycota) which includes a diversity of organisms including filamentous microscopic structures, yeast-like, and macroscopic forms. Until recently Fungi were divided into 4 phyla based on their sexual structures (Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota). Molecular sequencing data has clearly shown that both "zygomycetes" and "chytrids" are not monophyletic assemblages (11, 44). Characteristically, fungi are heterotrophic eukaryotes with unicellular or multicellular structures surrounded by a rigid cell wall usually comprised of glucans and cellulose. Ergosterol represents the principal sterol in the cell membranes. They obtain their nutrients by extracellular digestion using enzymes and secreted acids to release simple molecules from complex substrates. Therefore, they fulfill important roles in diverse ecosystems by recycling carbon and nitrogen from complex organic sources.

Fungi reproduce by asexual, parasexual, or sexual means (11) and historically were classified according to the morphologic characteristics of their sexual (teleomorphs) and asexual forms (anamorphs). For a given pleomorphic species, mycologists used to give the anamorph (e.g., *Aspergillus fumigatus*) a distinct name from their teleomorph form (e.g., *Neosartorya fumigata*). According to the newly adopted International Code of Nomenclature for algae, fungi, and plants (ICN), dimorphic fungi now bear a single name ("one fungus, one name policy") instead of the previous dual nomenclature (78). In the genus *Aspergillus*, many species are able to reproduce sexually but for many of them the sexual state is still unknown (previously classified as fungi imperfecti or Deuteromycetes). The production of meiotic ascospores

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by teleomorphs and cumulative molecular phylogenetic analyses have led some taxonomists to suggest that all *Aspergillus* species, including anamorphs, could be placed within the phylum Ascomycota (9, 24).

Mycotic diseases are relatively uncommon but are often devastating to the infected host. Most fungi are decomposers subsisting primarily on plant materials and other organic debris but can attack living hosts under certain conditions. Most clinically important fungi, other than yeasts, produce large amounts of airborne spores for dissemination. Except for the dermatophytoses, which affect the integument, animals are dead-end hosts for fungal infections, because they are not contagious (44).

Histoplasmosis and cryptococcosis are rare fungal infections of poultry but are notable as public health hazards. Information on these can be found in previous editions of this book (44).

This chapter does not address mycotoxicosis (see Chapter 31) following ingestion of secondary metabolites of fungal origin.

Aspergillosis

Introduction

Definition and Synonyms

Invasive and noninvasive infections of vertebrates caused by opportunistic pathogens of the genus *Aspergillus* are collectively named aspergillosis (93). Manifestations of the disease depend on which organs or systems are involved and whether the infection is localized or disseminated. Aspergillosis is the most important fungal respiratory infection in birds and a major cause of mortality in free-living, captive, and domestic birds worldwide (15). In young poultry, the disease is referred to as "brooder pneumonia" because it is most commonly diagnosed in neonatal poultry associated with infection at around hatching. Other synonyms for avian aspergillosis include fungal or mycotic pneumonia, pneumomycosis, bronchomycosis, and colloquialisms such as "asper" and "air sac." Less common manifestations relate to infections of the eye, brain, skin, joints, bones, and viscera (44).

Economic Significance

Besides direct losses related to mortality, feed conversion and growth rate in recovering birds remain poor. Productivity losses in growing turkey flocks are especially significant because aspergillosis tends to occur late in the rearing cycle and the air sac lesions caused by the infection result in condemnation at slaughter inspection (3). In Iowa, between 1985 and 1994, aspergillosis was reported in a yearly average of 8.3% of flocks ranging in age from 13 to 18 weeks (66). In a recent 2-year Chinese survey by 8 representative veterinary clinics (111), aspergillosis was identified as 1 of the 5 most prevalent diseases in meat ducks (8%), geese (3.6%) and other birds such as quails, or pigeons (6%).

Public Health Significance

Aspergillosis is not a zoonotic or contagious disease (44). According to their immunological status, inhalation of *Aspergillus* spores may cause multiple diseases in humans including invasive pulmonary aspergillosis in immunocompromised patients but also different forms of hypersensitivity diseases such as allergic asthma or pneumonitis (93). Therefore, care should be taken to avoid heavy environmental exposure to spores. Protective face masks should preferably be used when removing moldy hay, feed, and litter from poultry barns and when performing necropsies on heavily affected animals (15). Sporulating cultures should be handled with care in the laboratory.

History

Molds, likely belonging to the genus *Aspergillus*, were described in a Greater Scaup (*Aythya marila*) and in a captive flamingo in the early 1800s. The first description of *Aspergillus* in a lesion was in 1842 when Rayer and Montagne identified *A. candidus* from the air sac of a bullfinch. In 1863 Fresenius introduced the term aspergillosis when investigating the infection of the air sacs of a great bustard (*Otis tarda*) by the mold he described as *Aspergillus fumigatus*. Since then, these fungal agents have been involved in sporadic cases but also in die-offs either in free-ranging or captive birds and have provoked significant economic losses in the poultry industry (3, 15, 25).

Etiology

Classification

The genus *Aspergillus* includes approximately 250 species (2) but only a few well-known species are considered to be important opportunistic pathogens in vertebrates and invertebrates (93). *A. fumigatus* is the principal agent causing aspergillosis in poultry because the spores are very small and easily inhaled. Isolation of *A. flavus* is less common but not rare in heavily contaminated environments

with high spore load (5, 16, 53). Other species like *A. niger*, *A. nidulans*, *A. terreus*, and *A. amstelodami* (3, 25) may also be isolated from avian cases of aspergillosis when the environment is heavily contaminated with fungi. Mixed infections are possible (74).

Based on the analysis of DNA sequences, new cryptic species within the most frequent pathogens have been recently described. These sibling species which are morphologically or biochemically similar and otherwise indistinguishable by classical methods and/or internal transcribed spacer rDNA region (ITS) sequencing are regrouped in sections or species complexes. One of the main concerns about the emergence of the cryptic species, which may account for 10%–14% of the total *Aspergillus* clinical strains in human patients, is that they can be more resistant to the antifungal drugs. Therefore, molecular identification is currently recommended for the correct identification of species within the "*A. fumigatus* complex" group. Sequencing of genes (multilocus sequence typing), such as actin, calmodulin, rodlet A, and/or β-tubulin, has been used to distinguish *A. fumigatus* from related species (2). The occurrence of cryptic species has not been investigated yet in the veterinary field.

A complete key to aspergilli will list: phylum *Ascomycota*, order Moniliales, family Moniliaceae, genus *Aspergillus*, section *Fumigati*/*Flavi*, *A. fumigatus, A. flavus* (2, 25).

Aspergillus fumigatus Fresenius 1863

Colony Morphology

Aspergillus fumigatus grows rapidly on Sabouraud dextrose agar (SDA), Czapek yeast autolysate agar (CYA), malt extract agar (MEA) or potato dextrose agar (PDA) at 25 °C–37 °C. Growth is inhibited by cycloheximide and some isolates may not grow well on blood agar because it lacks sufficient carbohydrate. Colonies develop a diameter of approximately 3–4 cm in 7 days. The flat colonies are initially white and turn blue-green as conidia (spores) begin to mature, especially near the center of the colony. As the colony matures, conidial masses become gray-green, but the colony edge remains white. The colony surface varies slightly among isolates; it is smooth and velvety to slightly downy to powdery floccose or folded. The colony reverse is usually colorless or yellowish. This description represents typical characteristics of clinical isolates, but variations occur in colony color, morphology, and growth rate (92, 96).

Microscopic Morphology and Staining of Cultures

Hyphae measure 3–5 μm in diameter, have parallel sides, are frequently septate, and branch dichotomously. Spore-bearing branches (conidiophores) of *A. fumigatus* are smooth-walled, colorless to light green near the vesicle, up to 200–400 μm in length, and 5–11 μm in diameter. The conidiophore gradually enlarges distally to form a flask-shaped vesicle. The vesicle is 15–30 μm in diameter with a single (uniseriate) layer of conidiogenous cells called phialides which are concentrated on the upper surface and upwardly directed. Phialides are 5–9 μm in length. Conidial heads are predominantly columnar with unbranched parallel chains of conidia (asexual spores) grouped tightly together, but this feature is frequently disturbed when slide mounts are made (Figure 25.1). Conidial chains may attain a length of up to 400 μm. Conidia, green in mass, are finely roughened, globose to subglobose, with a diameter of 2–3 μm (25, 96).

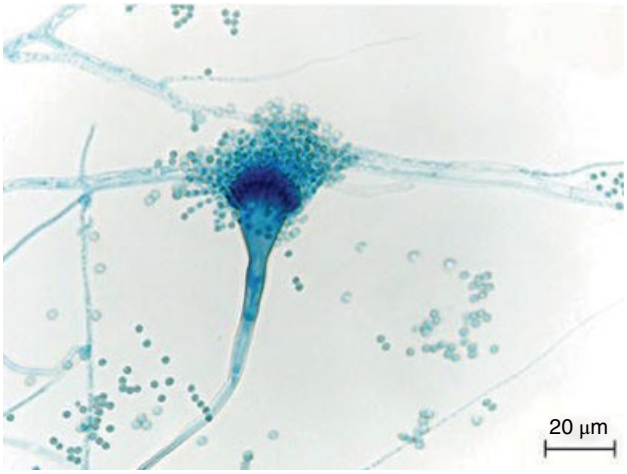


Figure 25.1 *Aspergillus fumigatus*. Conidiophore with flask-shaped vesicle, phialides, and chains of conidia. Lactophenol cotton blue, ×450. (M.J. Dykstra)

Visualization of specimens from slide cultures or slide mounts made with clear tape is improved by using methylene blue or lactophenol cotton blue stains (25, 96). Cell walls of fungal elements (septate, acute-angle, or dichotomous branching hyphae) in embedded tissue sections appear black or dark brown with Gomori methenamine silver (GMS) staining and pink to red purple with periodic acid-Schiff (PAS) staining (33).

Aspergillus flavus Link 1809

Colony Morphology

Aspergillus flavus grows rapidly, obtaining a colony diameter of 6–7 cm in 10 days at 25 °C on SDA, CYA, or PDA; some isolates may grow slower. Colonies are initially white but turn yellow to yellow-green with a white colony edge as conidia develop. Mature colonies may become somewhat olive-green. The colony may be furrowed radially or flat. Brown to black-brown sclerotia (densely tangled mats of mycelia), which begin as white tufts of mycelium, may be present. Isolates vary considerably in color and numbers of sclerotia, if any are present. The colony reverse varies from colorless, yellowish to pinkish drab to brown in sclerotial strains. Conidial heads of *A. flavus* are radiate with the chains of conidia

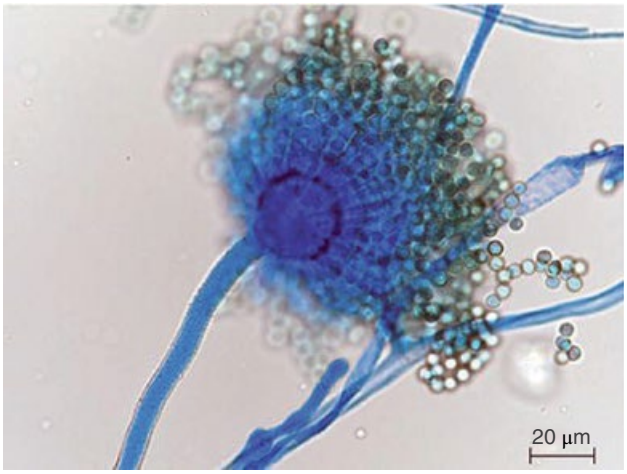


Figure 25.2 *Aspergillus flavus*. Conidiophore with globose vesicle, phialides, and radiate chains of conidia. Lactophenol cotton blue, ×410. (M.J. Dykstra). Note size of spores compared to *A. fumigatus*.

splitting to form loose columns, though this feature is frequently not evident in slide mounts (Figure 25.2) (25, 96).

Microscopic Morphology

Conidiophores (400–800 µm long and 8–17 µm in diameter) of *A. flavus* are thick-walled, rough (especially near the vesicle), and colorless. Vesicles, although more elongated when young, are globose to subglobose (20–45 µm in diameter) with a row of cells (metulae) bearing phialides (biseriate) over the entire surface of the vesicle. Phialides bear chains of globose to subglobose, smooth to finely echinulate conidia with a diameter of 3–6 µm (visibly larger than *A. fumigatus* spores). Some isolates are uniseriate, bearing a single layer of phialides directly on the vesicles (25, 96).

Growth Requirements

Aspergillus spp. have a very simple biological cycle. Propagation and dissemination of the opportunistic pathogens occur as the result of their saprophytic lifestyle. They are fundamentally grass eaters that recycle carbon and nitrogen from decaying vegetation in soil, compost heaps, litter, seeds, grains, and even from feathers (3). These filamentous fungi are equipped to survive and propagate successfully under a wide range of environmental conditions. If the optimum temperature for rapid culture of *A. fumigatus* is 38 °C, growth remains possible over a wide range of temperatures (12 °C–65 °C), pH (2.1–8.8) and relative humidity levels (11%–96%) (49, 69). Growth on CYA at 45 °C–50 °C is informative for *Fumigati* (92).

Spores of *Aspergillus* species have great longevity, high tolerance to heat and ability to germinate at low water activity (0.640 water activity) (70). An oxygen tension as low as 0.5% will support growth and sporulation (35).

Biochemical Properties

Identification of fungi, including aspergilli, at least at the species "complex level," is based primarily on colony and microscopic morphology, and growth characteristics. Biochemical criteria are infrequently used for species identification (25). Isolates of *Aspergillus* species usually produce a diverse range of secondary metabolites (extrolites) on standard growth media that are characteristic. For example, isolates of *Aspergillus* section *Flavi* nearly all produce kojic acid. But some important extrolites such as ochratoxin A are produced by species in different sections (92).

Susceptibility to Chemical and Physical Agents

Some species of *Aspergillus* are quite resistant to chemical agents and can grow in sanitizing fluids, sulfuric acid, copper sulfate plating baths, and formalinized tissues in museum specimens (90). Phenolic disinfectants are commonly used fungicidal agents. Commercial preparations of enilconazole have been used to control aspergilli in the poultry house environment (81). Certain oils derived from spices, such as cinnamaldehyde, inhibit *Aspergillus* growth (52).

Toxins

Aspergillus is among the most common mycotoxigenic genera. Aflatoxins, along with other mycotoxins, are discussed in detail in Chapter 31.

Toxins produced by pathogenic species of *Aspergillus*, particularly *A. flavus* and *A. fumigatus*, may be involved in the pathogenesis of *Aspergillus* infections in poultry, but no enhanced pathogenicity of aflatoxigenic strains of *A. flavus* for turkey poults has been found experimentally. *A. flavus* conidia caused neither mortality nor antibody production, whereas *A. fumigatus* conidia caused approximately 50% mortality and induced antibodies in turkey poults after aerosol exposure (82). Clinical signs and torticollis detected in turkey poults without CNS lesions (82, 87) indicated the possibility of toxin involvement in aspergillosis caused by *A. fumigatus*.

Gliotoxin produced by various turkey (83) or chicken (20) isolates of *A. fumigatus* has attracted the most interest in this species because of its potent immunosuppressive and cytocidal properties. Turkeys seem to be quite sensitive to oral doses of the toxin (85). Concentrations exceeding 20 µg/g have been detected in poultry feedstuffs (72). Gliotoxin in excess of 6 ppm occurred in tissues of experimentally infected turkey poults (89). Higher concentrations of the toxin have been found in the lungs of naturally infected turkeys (83). Turkey blood peripheral lymphocytes, when exposed to high levels of gliotoxin, either died or exhibited a lower lymphoblastogenic response (85).

Virulence Factors

Metabolites necessary for the pathogenicity of *A. fumigatus* have not been identified. *Aspergillus* species possess versatile features that meet their requirements to survive under different environmental conditions and make the species a ubiquitous fungal pathogen in a wide range of hosts. They make use of their saprobic lifestyle, thermotolerance, and competitiveness to ensure their survival both in their complex environmental niche and during infection. Different molecules like proteases (elastinolytic or collagenolytic enzymes) or cell wall components, secreted during mycelial growth, can also degrade or bind to host tissues, especially components of the extracellular matrix (25, 93).

However, the distinction of true virulence factors remains uncertain because either environmental or clinical isolates seem to be able to induce an aspergillosis in susceptible hosts (102). When 16 isolates of *A. fumigatus* were compared for pathogenicity by air sac inoculation of turkey poults, mortality was not influenced by the number of conidia given or the source (environmental versus clinical) of the isolates; a single environmental isolate produced no mortality (71). The analysis of the genome of *A. fumigatus* suggests that its primary ecological niche is in plants and that opportunistic infections of animal hosts are a dead end for this fungal species (3). Studies have also used embryonated eggs to assess virulence of various *A. fumigatus* strains (37).

Different genotyping methods have highlighted the remarkable genetic variability encountered within *A. fumigatus* (3). The possible effect of sexual reproduction, which have been observed in both *A. fumigatus* and *A. flavus* is still a matter of discussion (70). Recombination in teleomorphs could also promote the emergence of strains with increased virulence or exhibiting various patterns of antifungal resistance (70) which have already been evidenced in avian isolates (7, 112).

Pathobiology and Epidemiology

Incidence and Distribution

Two primary forms of aspergillosis, acute and chronic, occur in poultry. Acute aspergillosis usually affects young birds and is characterized by high morbidity and mortality. Brooder pneumonia in broiler chicks as a result of hatchery contamination can be a significant problem, especially with *in ovo* vaccination. Chronic aspergillosis occurs in adult birds, especially turkey breeders, or occasionally in captive birds in aviaries. Although chronic aspergillosis is less common, significant economic losses, resulting from reduced production, increased condemnation rate, and downgrading at the processing plant, can accrue in commercial poultry flocks when high value adult birds are concerned (25).

Aspergillosis is a greater problem in confined flocks where moldy litter or grain is present (25). When environmental conditions are favorable for fungal growth this results in copious sporulation which will expose birds either to high numbers of conidia sporadically or to continuous exposure to lower levels. Organic substrates such as beddings or feedstuffs, coupled with the humidity and temperature encountered in poultry farms, promote the rapid growth of different fungal genera such as *Aspergillus*, *Alternaria*, *Cladosporium*, *Penicillium*, or *Scopulariopis* spp. (21, 30, 61, 62, 105), which are regularly isolated in these environments. A strong correlation between litter fungal contamination and aerial mycoflora corroborates the presence of aerosolization of fungi found in litter and indicates that it may constitute the main reservoir of indoor contamination (21, 105). Alternating wet and dry conditions are ideal for the development of *Aspergillus*. The fungus multiplies during the wet period producing abundant hydrophobic propagules which are then dispersed in the atmosphere when conditions become dry. Subsequently, air contamination levels and mycoflora composition are characterized by dynamic (cyclic) variations. These fluctuations may be related to season or husbandry management (23, 30).

Therefore, 2 main groups of factors seem to play a key role in the occurrence and development of aspergillosis: external (poor hygiene, deficient air renewal, excessive stocking density) or internal stressors (immunocompetence, nutritional status, concurrent diseases) which cause impaired resistance in birds (3, 44).

Natural and Experimental Hosts

Infection by *Aspergillus* spp. has been reported in almost all domesticated avian species and production types (3): layer cockerels, pullets in cages, broiler breeders and growers of chicken or turkey poults, common duck breeders and growers (68), goslings, Japanese quails, or pigeons. Both field data and experimental results clearly demonstrated a higher susceptibility of turkeys, ducks, and quails to fungal infection when compared with chickens for example. Differences in susceptibility have been demonstrated between different turkey (48) and chicken (97) lineages following experimental inoculation of conidia. Spontaneous cases of aspergillosis occur in farms rearing great rheas, ostriches, partridges (19), or pheasants (4).

Raptors, waterfowl, and gulls represent the majority of the free-living birds reported to have aspergillosis. This disease has been described in 33 species of 13 orders of captive wild birds. Therefore, no avian species should be considered resistant to infection (15). In contrast to mammalian experimental models, immune modulation is not a necessary prerequisite in avian challenge models (3).

Age of Host Commonly Affected

Acute aspergillosis seems to be more frequent in young poultry whereas older birds may suffer from the chronic form of the disease (25).

Pulmonary Aspergillosis

Aspergillosis is primarily an infection of the respiratory tract. Infection of the lungs and the air sacs is the most common form of the disease in avian species (44).

Systemic Aspergillosis

Systemic aspergillosis in poults and caponized 5-week-old cockerels has been reported. The latter resulted from an infection after the caponizing procedure (44). Systemic aspergillosis in turkey poults caused by *A. flavus* involved the sternum (31). Intravenous inoculation of *A. fumigatus* conidia causes acute miliary hepatitis (47).

Dermatitis

Necrotic granulomatous dermatitis from which *A. fumigatus* was isolated has been described in chickens and in white Pekin mallards (16, 44). In the latter case, the presence of previous skin lesions was suspected and the ducks were suffering a concomitant pulmonary aspergillosis caused by *A. flavus*.

Omphalitis

Two outbreaks have been described in 3–9-day-old turkeys where the primary cause of omphalitis was *A. fumigatus* occurring simultaneously with brooder pneumonia (17).

Mycotic Osteomyelitis and Arthritis

Osteomyelitis of cervical and thoracic vertebrae involving *Aspergillus* spp. have been reported in 6–10-week-old pheasants (4) and in 2–3-week-old broilers (101). The induced compression of the spinal cord resulted in partial paralysis of the wings and legs. In both cases, infections were probably sequela to lung disease with hematogenous dissemination of the organism. By contrast, a spread of the fungal infection from the abdominal air sac has been suspected in young turkey poults exhibiting diffuse granulomatous bilateral arthritis of the hip joints (64).

Ophthalmitis

Ocular aspergillosis in birds caused by *Aspergillus* has been described in young red-legged partridges (19), white leghorn chicks (6), and turkey poults (1, 44). Unilateral (6, 19) or bilateral (1) infections can occur. Two forms of ocular aspergillosis were apparent among these early cases. One form involved primarily the conjunctiva and external surfaces of the eye. Clinical signs included periorbital swelling, epiphora, turbid discharge, swollen eyelids adhering together, and keratitis (23). A caseous or cheesy exudate or plaques were found in the conjunctival sac beneath the nictitating membrane. Fungus was readily isolated from cultured plaque material. The other form did not involve the cornea and occurred in birds with concurrent respiratory aspergillosis. Most pathologic changes occurred in the posterior eye involving the vitreous humor with extension into adjacent tissues. Pathogenesis of the 2 conditions was apparently quite different. Keratitis and superficial infections probably resulted from exposure of conjunctival surfaces (23) to fungal elements from environmental sources which might be promoted by excessive amounts of ammonia (6) or harmful litter material (19). Mycotic ophthalmitis involving the posterior eye most likely resulted from hematogenous dissemination of the organism from a primary respiratory infection. Although not frequent, the latter type of eye infection usually is apparent in birds with respiratory involvement. The superficial eye infection was reproduced in chickens by introducing conidia of *A. fumigatus* into the eye. The yellow caseous plaque can become adherent to the cornea in the superficial type of infection (44).

A fungal keratitis caused by *A. fumigatus* resulting from superficial invasion of the cornea has been described in 2-week-old chickens (6) and in red-legged partridges (19). In these outbreaks, there was histopathological evidence of fungal invasion of the anterior chamber and cornea. However, the lack of involvement of any intraocular structures seemed to rule out haematogenous spread from detected respiratory lesions.

Turkeys experimentally exposed to aerosols of conidia occasionally developed a cloudy eye with retinitis, iridocyclitis, and secondary involvement of the remainder of the eye (88). There was a cellular infiltration of heterophils and macrophages, and cellular debris and fungal elements were present in the chambers and retina. The pecten was severely involved with edema, heterophils, mononuclear cells, and fungal elements present. In some turkeys, the pecten contained granulomas.

Following oculonasal vaccination against Newcastle disease, mortalities increased rapidly in chickens that had contracted superficial ocular aspergillosis in the hatchery. This type of ocular involvement occurred in 5 widely separated flocks of young poults and 3 breeding flocks (44).

Encephalitis

Numerous reports describe encephalitic or meningoencephalitic aspergillosis in a variety of avian species. In turkeys, solitary abscess or multifocal necrotic foci in the cerebrum or cerebellum were found in naturally occurring aspergillosis (44) and in turkey poults experimentally exposed to aerosols of *A. fumigatus* conidia (82). A granulomatous encephalitis containing branched hyphae was characterized in turkeys presenting delayed

neurological signs (23). Malacic but not granulomatous lesions restricted to the cerebellum have been identified in broiler breeders (1).

The observation of concomitant chronic granulomatous lung lesions and acute fungal encephalitis in turkey poults suggests that the organisms within pulmonary granulomas may become activated and subsequently be spread haematogenously to the central nervous system and provoke acute mycotic encephalitis (40, 87).

Transmission, Carriers, and Vectors

Aspergillosis is not transmissible. Initial contamination of poultry farms may occur through the use of moldy litter, contaminated feedstuffs, or introduction of 1-day-old birds whose down has retained conidia in hatchery facilities. Further contamination may involve inappropriate bedding management. Constant animal movements under high stocking densities, litter refreshing, or deficient ventilation may contribute by generating a conidial aerosol. Fresh litter, especially if it has been wet previously and contaminated with *A. fumigatus*, is associated with outbreaks of aspergillosis. Dust appears to be an excellent carrier and reservoir for fungal spores (3, 23). Therefore, distribution of very dry litter may generate large amounts of dust indoors (23).

Aspergillosis can be acquired *in ovo* and outbreaks of aspergillosis in chicks often originate in hatcheries. Egg embryos are quite susceptible to infection by *A. fumigatus* during incubation. The yolk is an excellent nutritive media for fungal growth. *In ovo* vaccination by egg injection during late embryonic development could be a risk factor in hatcheries with high fungal contamination (108). Embryo contamination occurred when a petroleum jelly suspension of *A. fumigatus* conidia was applied to the surface of incubating eggs, and infections increased when the incubating eggs were dusted with *A. fumigatus* conidia. Within 8 days after the dusting application, the organism had penetrated the eggshell (44).

A mortality rate of 1%–10% was observed among 21 ranches where 210,000 chicks were involved. Infection could not be traced to hatching eggs but was readily found in incubators, hatchers, incubator rooms, and intake ducts. Signs and lesions were noted in some day-old chicks, but generally, classic lesions were observed in chicks at 5 days of age. Chicks up to 2 days old were infected easily with *A. fumigatus* spores by contaminating the forced-draft incubator with wheat seeded with *A. fumigatus*. Chicks older than 3 days were resistant to infection (44).

Incubation Period

Aspergillosis is primarily an infection of the respiratory tract. Birds placed in environments contaminated with aerosolized conidia may show significant pathology after only a short duration of exposure. Acute aspergillosis has been seen in chicks where the incubation period was as short as 2–5 days. A sudden peak in mortality has been registered in a 3-week-old turkey flock culminating 4 days after the setting up of the new litter which was considered to be the source of contamination (23).

Onset of signs did not occur before 48 hours in turkeys or chickens experimentally infected with high doses of *A. fumigatus* or *A. flavus* (56, 88, 97).

Signs

Signs, when present, can be subtle even in cases in which postmortem examination reveals severe lesions. In fact, extensive involvement of the respiratory tract can occur before symptoms are apparent. Dyspnea, hyperpnea and gasping may be observed especially in acute forms where major respiratory distress may lead to cyanosis. When these signs are associated with other respiratory diseases, such as infectious bronchitis or infectious laryngotracheitis, they often are accompanied by gurgling and rattling noises, whereas in aspergillosis there usually is no sound. Numerous nonspecific symptoms including anorexia, increased thirst, pyrexia, lethargy, ruffled feathers, dehydration, polydipsia, polyuria, stunting, or sudden death were attributed to aspergillosis. Birds which were less active than expected and reluctant to move became rapidly emaciated and developed diarrhea in the later stages. Dysphagia occurred in cases when the esophageal mucosa was involved. Head swelling and serous secretions from nasal and ocular mucosa also have been described (19). Cloudiness of the eye and blindness are not rare (3, 44).

Nervous system involvement causes ataxia, tremor, opisthotonos, lateral recumbency, seizures, convulsions, lameness, and fore or hind limb paresis. Because torticollis and/or a lack of equilibrium occur in both experimental (87) and naturally occurring *Aspergillus* infections (44), this should be considered a significant sign of avian aspergillosis.

Clinical respiratory signs which are typically associated with aspergillosis may be absent either in spontaneous outbreaks (40) or after experimental infection (97).

Morbidity and Mortality

Mortality ranged between 4.5% and 90% in spontaneous outbreaks of aspergillosis (3). In poultry farms, the mortality rate may rise slightly or increase suddenly, peak over a few days, and then return to the initial state. In chicks and poults, contaminated *in ovo* or during hatching, aspergillosis is highly fatal in the first 10 days of life. Death generally occurred within 2–5 days after the first symptoms (110) or following experimental exposure to aerosolized conidia (56, 82, 97). The disease in mature poultry is generally characterized by lower morbidity and mortality rates. However, morbidity can

be underestimated in finishing flocks and is only seen at slaughter inspection when pulmonary lesions are observed (3, 25).

A 10-minute aerosol exposure to 5×10^5 colony-forming units/g of lung of *A. fumigatus* killed 50% of 3-week-old turkeys (61). All turkey poults aerosol-exposed to 2.2×10^6 viable units of *A. fumigatus*/g of lung tissue died by day 5. Lower doses (5.2×10^5 viable units) delayed and reduced mortality indicating the importance of the challenge dose.

Pathology

Gross

Lesions of uncomplicated pulmonary aspergillosis evolve over several days and diminish in a few weeks. The primary location of lesions is the air sacs, pleura, and lungs although eye, esophagus, proventriculus, gizzard, small intestine, liver, kidney, spleen, skin, trachea, peritoneum, brain, eye, muscle, heart, bones, articulation, and yolk sac may be involved (3). Acute lesions of experimental aspergillosis in turkeys rapidly progress in severity. White miliary foci were present on air sac membranes as soon as 24 hours' post-air sac inoculation in 9–19 week-old turkeys but not before 48 hours in 5-day-old poults (27). Lung lesions consisted of congestion, parenchyma consolidation, and straw-colored gelatinous subpleural edema. Air sacs became progressively thicker and opaque with focal granulomas that increased in size and changed shape from raised domes (1 mm) to flat or umbilicated plaques (2–5 mm) that tended to coalesce. Extensive white discoloration of lungs and granulomatous pneumonia were evident by 72 hours after intra-air sac infection with *A. fumigatus* (45).

Lung lesions in experimental aerosol infection of turkey poults consisted of small white caseous nodules (approximately 1–3 mm in diameter) scattered throughout lung tissue (Figure 25.3), usually accompanied by similar sized caseous plaques on thickened air sac membranes (56, 88) (Figure 25.4). Occasionally, red-tinged ascites was present. In an outbreak of aspergillosis in chicks, yellow foci were not found but lungs were a diffuse gray-yellow (44).

In advanced cases of aspergillosis, air exposure can cause the organism to sporulate on the surface of caseous lesions and on the walls of thickened air sacs, producing visible green-gray mold growth (88).

Caseous, gelatinous, or less commonly mucoheterophilic exudate may be present in the syrinx of infected birds (Figure 25.5). Localized tracheal aspergillosis caused by *A. flavus* has been characterized by grossly visible yellow caseous plaques that adhered to the mucosal surface, occasionally occluding the trachea and reddened tracheal walls (5).

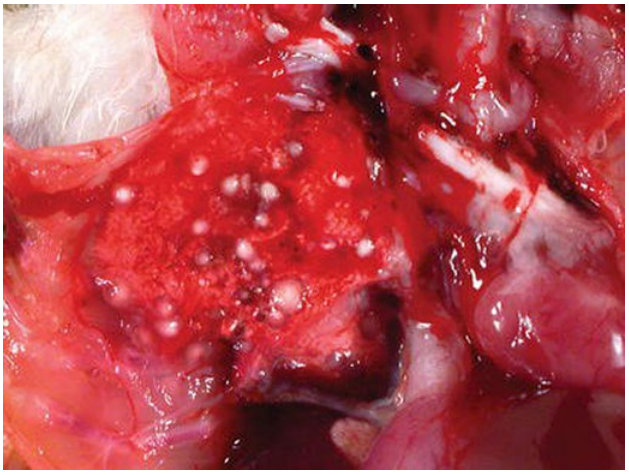


Figure 25.3 Turkey poult, 2 weeks. Numerous caseous nodules in the lung are characteristic of respiratory aspergillosis ("brooder pneumonia"). (H.J. Barnes)

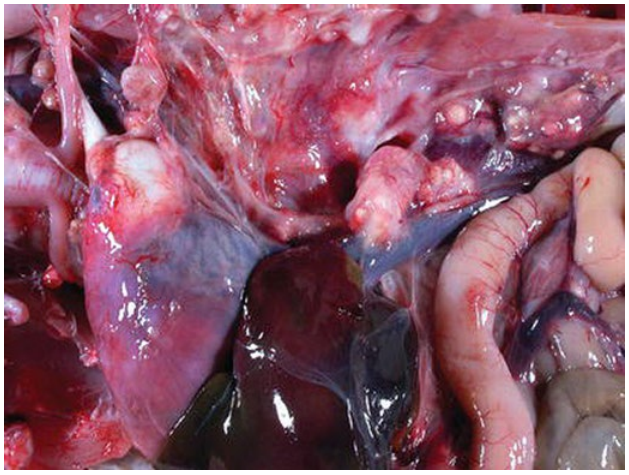


Figure 25.4 Turkey poult, 8 weeks. Air sacs containing numerous spherical nodules are granulomas caused by *Aspergillus flavus*. (H.J. Barnes)

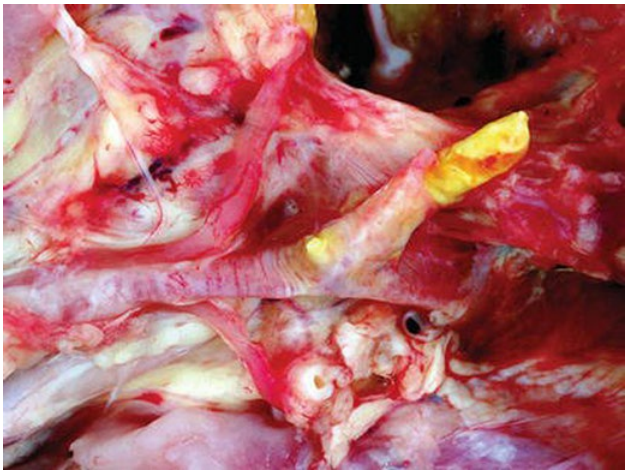


Figure 25.5 Broiler breeder, 61 weeks. Caseous exudate in the syrinx was caused by aspergillosis. (H.J. Barnes)



Figure 25.6 Broiler chicken, cerebral aspergillosis. Granuloma is present unilaterally. (M.D. Lee, Poultry Diagnostic and Research Center, The University of Georgia)

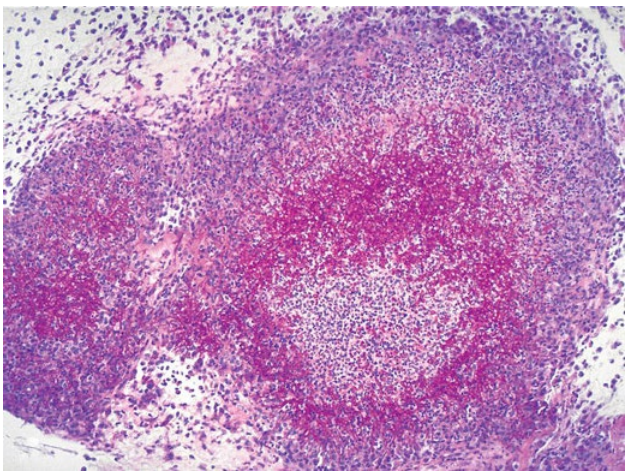


Figure 25.7 Experimentally induced granulomatous lesion in the air sac due to aspergillosis. A central caseous core is bordered by a narrow, uniform palisade of macrophages and small giant cells surrounded by a less distinct broad zone of predominantly macrophages and scattered heterophils. Periodic acid–Schiff stain, ×90. (R.A. Kunkle and H.J. Barnes)

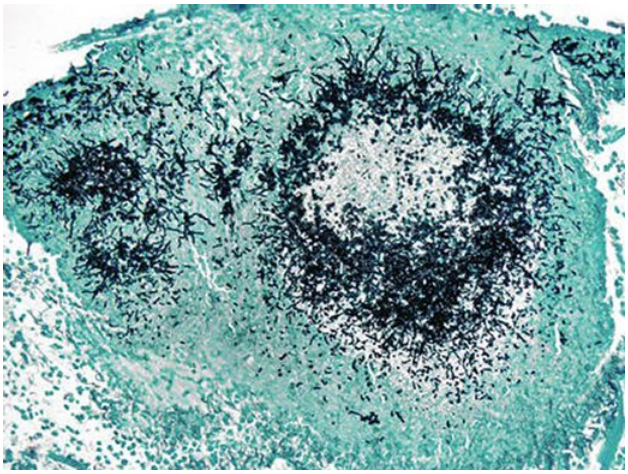


Figure 25.8 Organisms stain well with Gomori methenamine silver, ×90. (R.A. Kunkle and H.J. Barnes)

Lesions in the brain have been described as white to yellow circumscribed areas that are usually visible on the surface. They may be in the cerebellum or cerebrum or, less frequently, both (1, 88) (Figure 25.6).

Ascites in chickens has been a frequent sequela to pulmonary aspergillosis caused by *A. fumigatus*. Acute *cor pulmonale* was the suspected cause of vascular failure (41, 110).

Histopathology

Based on histopathological differences, a deep nodular form and a superficial diffuse form of aspergillosis can be distinguished. A well-organized granulomatous reaction develops in nonaerated parenchyma whereas a superficial diffuse form, containing fungal elements and a nonencapsulated pyogranulomatous reaction, predominates in serosae and lungs. Organized granulomas are clearly encapsulated by an outer thick fibrous layer whereas pyogranulomas lack clear borders (13). In a study of acute pulmonary aspergillosis in turkey poults, granulomatous airsacculitis and pleuritis were seen as early as 24 hours after intra-air sac inoculation with *A. fumigatus* (45). Air sac membranes were thickened up to 100-fold by massive infiltrates of heterophils, multinucleate giant cells, and other leukocytes. Germinating conidia were seen in the membrane interstitium, and lymphohistiocytic perivasculitis was discernible in less severely affected areas. Granulomas had centers composed of necrotic cellular debris and heterophils with a peripheral palisade of epithelioid macrophages and aggregates of lymphocytes (Figure 25.7). Examination of heterophilic granulomas stained with GMS stain revealed large numbers of germinating conidia centrally and hyphae extending peripherally through the layer of macrophages (Figure 25.8). Lung lesions consisted of heterophilic and lymphohistiocytic or granulomatous pleuritis and pneumonia with edema and hemorrhage in the initial 48 hours, but had progressed to extensive effacement of parenchymal architecture because of necrosis, hemorrhage, and massive infiltrates of leukocytes by 72 hours. In 2 alternative models of acute aspergillosis in turkeys, from 20% to 95% of viable lung parenchyma were progressively replaced by multifocal-to-coalescing necrogranulomas (56, 71). Epithelioid macrophages admixed with multinucleate giant cells were arranged in sheets. Intact and degenerate heterophils predominated in areas of necrosis. Septate hyphae were mostly localized to areas of necrosis and aggregates of multinucleate giant cells. Vascular invasion with thrombosis is often seen in severe cases (45). By 120 hours, residual fungal elements

were restricted to the cytoplasm of giant cells highlighting the ability of some birds to control the development of the infection through an effective immune response (27, 56).

Nonviable *A. fumigatus* conidia produced a transient airsacculitis and pneumonia characterized by edema and infiltrates of heterophils and macrophages (45). Multinucleate giant cells were not present in these lesions, in contrast to active infections with *Aspergillus*, in which both epithelioid macrophages and multinucleate giant cells were prominent features.

In a study of subacute and chronic phases of aspergillosis, examination of lung tissues from turkey poults revealed no differences in histopathologic lesions caused by *A. fumigatus* or *A. flavus* (86). Lesions seen in the first 2 weeks of the study were characterized by focal accumulations of lymphocytes, some macrophages, and a few giant cells. Later, lesions consisted of granulomas with a central area of necrosis containing heterophils surrounded by macrophages, giant cells, lymphocytes, and some fibrous tissue. By 8 weeks postexposure, surviving poults had mature fibrous granulomatous lesions consisting of necrotic centers surrounded by giant cells and a thick layer of fibrocytes and collagen containing a few scattered heterophils. Using Gridley's fungal stain, the organisms could be seen within the necrotic areas of the lesions. Areas of conidial production were seen in tissue sections of the well-oxygenated bronchi, bronchioles, and air sacs upon which plaques became velvety and change color to shades of green, olive, brown, or black depending on the fungal species involved (13). The presence of a fibrous capsule at the periphery of the granulomas and of multinucleated giant cells associated with focal lymphoid infiltration in the pulmonary parenchyma might be an important sign of the chronic form of the disease (10).

Brain lesions consisted of solitary abscesses with necrotic centers infiltrated with heterophils and surrounded by giant cells. Hyphae were seen in the central area of some lesions.

Eye lesions were characterized by edema of the pecten, which was infiltrated heavily with heterophils and mononuclear cells. Granulomas were found in the pecten. Fungal hyphae, heterophils, macrophages, and cellular debris were found in the chambers and retina of the eye. Edema and some heterophils were found in the sclera and surrounding tissues. In cases of ophthalmitis in turkeys, primary involvement was in the vitreous humor and adjoining tissues. In 1 turkey, hyphae were seen in the center of the lens (44).

In the trachea, occlusions consisted of fungal hyphae and heterophilic granulomatous exudate, the mucosa was necrotic and infiltrated with macrophages, and fibroplasia was evident in the subadjacent tracheal wall (5).

In a case of diffuse granulomatous bilateral arthritis of the hip joints affecting turkey poults a severe multifocal heterophilic and granulomatous osteoarthritis was associated with the presence of myriads of filamentous fungal structure invading the articular cartilage, the bone, and the synovial membrane (64).

Pathogenesis

The main route of entry of conidia is through the airways. More rarely, conidia may be introduced by puncture wounds, during surgery, or induce keratitis by contact (44). Both host and fungus characteristics explain the particular susceptibility of birds to *A. fumigatus* infection. Conidia are probably continuously inhaled and are generally rendered innocuous by innate immunity mechanisms. Upper respiratory clearance mechanisms rely on mucous-covered epithelial cells possessing cilia and lining the trachea, the primary bronchi, and the roots of the secondary bronchi. The epithelium of the upper airway presents also a highly lytic activity. *A. fumigatus* conidia are small enough, 2–3 μm in diameter, to bypass initial physical barriers and disseminate deeply in the respiratory system. The gas pathway through lungs accounts for the susceptibility of the caudal air sacs to infections, compared with the cranial air sacs. The larger diameter of *A. flavus* conidia (3.5–4.5 μm) may explain their lower pathogenicity when compared with *A. fumigatus* in experimental infections (3, 82).

Conditions that make *Aspergillus* strains able to penetrate host tissue in a shorter time interval than that necessary to mount an efficient immune response are likely to be effective in terms of colonization and subsequent infection of the host (70). The follow-up of the fungal load by quantitative polymerase chain reaction (PCR), colony-forming-unit counts and galactomannan dosage in the lung of turkeys experimentally infected showed that birds which died quickly had a consistently higher *Aspergillus* burden than other poults (56). The detection of viable *Aspergillus* was possible in a high proportion of apparently healthy turkeys which were euthanized 7 days after intratracheal aerosolization but may persist up to least 8 weeks postexposure (82).

In a study of infected chick lung tissue, conidia adhered to epithelial surfaces and smooth muscle cells lining the apices of interatrial septa of parabronchi within 1 hour after their inhalation (44). Conidia were translocated from the luminal surface to the interior of the membrane, where conidial germination was evident within 24 hours (45, 46). The avian lung-associated immune system includes bronchus-associated lymphoid tissue localized at the junctions of primary and secondary bronchi and at the ostia to the air sacs, an interstitial immune system combining lymphocytes and macrophages and a phagocyte system. The latter should provide an immediate

front line defense of the extensive gas-exchange surface area (3). The avian respiratory system responds efficiently to invasion by pathogens with a rapid influx of heterophils and macrophages from the subepithelial compartment and pulmonary vasculature. During the acute phase response, heterophils represent vital cellular components of innate immunity and function by killing the pathogens following phagocytosis. Necrosis of tissue occurs concurrently with the fulminant inflammatory response, which, by 24 hours, consists of massive numbers of heterophils admixed with cellular debris. Classic avian macrophage properties include chemotaxis, phagocytosis, pathogen elimination, and cytokine production. Many macrophages of turkeys (86) or pigeons (103) exposed to *A. fumigatus* had conidia attached to them or had ingested 1 or more conidia. Avian macrophages may prevent early establishment of the infection by killing conidia or inhibiting their germination unless *A. fumigatus* spores are in excess of the phagocytes killing capacity. Hyphae were frequently seen within the cytoplasm of giant cells with little evidence of detriment to either (44).

Potential ways of multifocal infections are by direct contamination of wounds with conidia, contiguous spread from an adjacent infected organ, or hematogenous spread from a distant site most often the lung (44). *A. fumigatus* was isolated from the blood of turkeys immediately after a 15-minute aerosol exposure of conidia. At this time, macrophages harvested by lung lavage contained numerous ingested conidia (86). This may be the route of dissemination that results in eye and brain lesions (88). The organism usually was cleared from the bloodstream by 24 hours postexposure.

Immunity

Evidence for immunity against aspergillosis in poultry is lacking; however, the majority of turkeys experimentally infected with *A. fumigatus* recover from pulmonary aspergillosis within 4–5 weeks if they are not re-exposed to the agent (46, 47). Likewise, resolution of pulmonary aspergillosis has been described in Japanese quail surviving experimental infection (14). The mechanisms involved in convalescence from aspergillosis in avian species have not been described.

Experimental vaccination of turkeys against aspergillosis has yielded only limited protection by decreasing mortality (87) or lessening early histopathologic lesions (84). Vaccination did not protect against pulmonary lesions and may have increased the likelihood of chronic *A. fumigatus* infection. Some vaccinates remained culture-positive and unvaccinated controls were culture-negative at 8 weeks postchallenge.

Natural recovery from aspergillosis does not appear to confer protection in turkeys. Convalescent turkeys remained susceptible to pulmonary aspergillosis in which recovery from unilateral *A. fumigatus*-induced airsacculitis did not protect against contralateral air sac challenge (48). Likewise, passive cellular immunization failed to protect turkeys against *A. fumigatus* challenge and splenic lymphocytes did not respond to conidial antigen preparations, regardless of the previous exposure status of the donor turkey (47).

This underlines the actual importance of animal models in therapeutic protocols and physiopathology research. At the species level, recent genetic approaches to immune modulation and disease resistance via lines of targeted selection could constitute an alternative way to complement our knowledge on avian immunity toward fungal infections and advantageously complement medical treatments and improve management in poultry farms (2).

Diagnosis

Isolation and Identification of Causative Agent

Diagnosis of aspergillosis, particularly in chronic cases, remains a challenge. It relies on a combination of clinical signs, epidemiological considerations, and eventually culture and cytology on tracheal or air sac lavage in poultry. Necropsy may reveal characteristic caseous nodules in the lungs or plaques in the air sacs of affected birds. Aerated tissues are sometimes lined by a grey-green velvet when fungal sporulation occurs. Definitive diagnosis requires the identification of *Aspergillus* isolated by culture or by the detection of the organism during histological examination on biopsy specimens (3, 25).

Because of the ubiquitous nature of *Aspergillus* in the environment, positive culture from integument or respiratory tissues without any macroscopic lesions may be frequent but should not be interpreted as a positive diagnosis of aspergillosis. Although *A. fumigatus* is the most likely agent of avian aspergillosis, other species of *Aspergillus* and other fungal genera can cause the disease especially if the environment is heavily contaminated (3, 25).

Because most agents causing the mycoses are ubiquitous saprophytes, diagnostic samples need to be carefully collected using aseptic techniques. These samples can be examined microscopically by placing a small portion of the nodule in 20% potassium hydroxide (KOH) on a microscope slide, teasing the material apart, and covering it with a glass coverslip. Following gentle heating of the slide over a flame, the specimen can be examined for hyphae within the exudate. If the preparation is too thick, the slide will need to be incubated for 12–24 hours in a moist chamber and re-examined. To aid in elucidating the fungus, KOH can be mixed with ink dye (Ink blue,

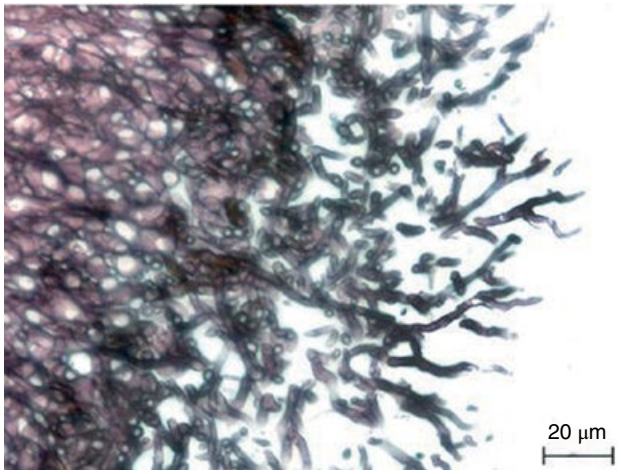


Figure 25.9 Hyphae consistent with *Aspergillus* sp. in air sac wall of a chicken. Note the dichotomous branching toward the right side of the image. Gomori methenamine silver, ×405. (M.J. Dykstra)



Figure 25.10 Hyphae consistent with Aspergillus sp. in air sac wall of a chicken. Note the numerous cross walls (septa). Gomori methenamine silver, ×800. (M.J. Dykstra)

Parker Pen Co., Janesville, WI). Hyphae of *Aspergillus* stained with the ink dye appear as blue-stained, septate, dichotomously branched structures 2–8 µm in diameter with hyphal walls that are generally parallel (Figures 25.9 and 25.10) (25).

Aseptically obtained specimens can be plated directly onto appropriate mycologic media. Alternatively, specimens can be placed into saline, minced briefly in a tissue grinder, and then streaked onto culture media. Collected fluids can be centrifuged and the sediment examined microscopically or cultured as above (25).

Satisfactory media for isolating and identifying most isolates from cases of aspergillosis include SDA and PDA. Recommended media for *Aspergillus* isolation from difficult cases are CYA and MEA consisting of 20 mL of medium per 90 mm Petri dishes which are incubated reverse side up at 25 °C with additional CYA plates incubated at 30 °C and 37 °C. Confirmation of their identity can be achieved by stimulating conidial production by culture on PDA or cornmeal agar at 37 °C (92). All cultures should be examined daily, and peripheral portions of fungal colonies transferred to fresh media to lessen the chance of bacterial overgrowth, which can suppress fungal growth (25).

Identification can be made using light microscopic examination of hyphae and conidiophores produced after 7–10 days. A small portion of the colony containing reproductive structures can be placed into a drop of suitable mounting medium (e.g., lactic acid 70%, Shear's solution or lactophenol cotton blue) on a clear glass slide, teased apart, covered with a coverslip, and examined (25, 92). Alternatively, clear adhesive tape can be used to touch the growth at the edge of the sporulating culture then placed onto a slide containing a drop of lactophenol cotton blue.

The use of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry fingerprinting for routine identification of *Aspergillus* isolates is promising but needs an improvement in spectra (92).

When the identification at the species level of an isolate exhibiting, for example, an atypical resistance pattern (acquired resistance in *A. fumigatus* or intrinsic resistance in a cryptic species) is necessary, the consensus sequence of internal transcribed spacer rDNA region is not enough for *Aspergillus* and should be completed by the sequence of other targets (multilocus gene sequence), such as β-tubulin, calmodulin, or rodlet A genes (2).

Serologic Tests

Serologic tests are of limited value because of the nonspecific nature of the fungal antigens. Agar gel precipitin has been reported in comparisons of *A. fumigatus* and *A. flavus* infections in turkey poults. Although most of the *A. fumigatus*-infected poults were positive for precipitating antibodies, poults infected with *A. flavus* were not (82). Antibody response, as measured by enzyme-linked immunosorbent assay (ELISA) and agar gel immunodiffusion, was erratic, although most poults with high antibody scores had marked lesions and low weight (71). Additionally, a direct ELISA technique has been used in turkeys with a correlation occurring between exposure level and ELISA titer (44). Perhaps use of serologic methods to identify poults with aspergillosis for culling would be advantageous because there is no legal or effective therapy for treating positive birds.

Aspergillus isolates produce a range of antigenic molecules but relevant serological biomarkers and reliable diagnostic tools allowing improved aspergillosis diagnosis or experimental infection monitoring are still lacking in birds (3).

Detection of galactomannan, a major cell wall component of *Aspergillus* spp., in sera or bronchoalveolar lavage fluid with an immunoenzymatic test (Platelia, Bio-Rad, Hercules, CA) or a latex agglutination test (LA Pastorex *Aspergillus*, Bio-Rad, Hercules, CA) has proven useful in aiding early diagnosis of invasive pulmonary aspergillosis in humans (42). Attempts to detect galactomannan in avian practice to obtain a diagnosis have been performed in various species (psittacine birds, falcons, penguins) with contrasting results because of low sensitivity or specificity but also depending on the species tested and the stage of the disease (18). However, they may constitute valuable tools to monitor the response to therapy. Within the framework of an experimental inoculation of *A. fumigatus* spores in the air sac of turkeys circulating galactomannan should be considered as an interesting biomarker because its serum concentration discriminates clearly infected turkeys from controls (3). Because it is produced by active hyphae only, galactomannan concentrations could allow the monitoring of the first steps of fungal development in tissues. However, the ubiquitous presence of galactomannan in feed may lead to false positives and reduces its efficacy in field conditions. A recent study described a cohort study using serum antibodies and galactomannan data in turkey and broiler farms. The mean antibodies and galactomannan indices of affected birds were significantly higher than the control group. However, higher antibody indices were found in broilers versus turkeys, and lower antibody indices were associated with *A. flavus* compared with *A. fumigatus* outbreaks (29). The use of serological assays could be helpful when performing health assessments of high-value breeder populations.

Use of a modified limulus assay to detect endotoxin (FungitellBeacon Diagnostics Laboratory, East Falmouth, MA) for testing human patient serum for the presence of 1-β–D-glucan residues found in the cell walls of many fungi has successfully demonstrated the presence of fungi such as *Aspergillus* spp. and *Candida* spp. (42, 58, 63). High serum levels of beta-glucans have been measured in broilers originated from either farm cases (aspergillosis outbreak) or control farms (no known episode of aspergillosis). These results may suggest sample contamination or they may be a reflection of an environmental exposure or colonization by *Aspergillus* or other fungal species (29).

Despite the fact that PCR techniques have been in use for almost 20 years by various investigators to help diagnose invasive aspergillosis in humans, the lack of standardized methodologies has hampered the interpretation and reliability of PCR results (107). PCR assays have been tested on different body fluids or for experimental purposes in turkey models in combination with other biomarkers (56) but need further research to be eventually included in the work-up of the avian practitioner.

Differential Diagnosis

Clinical signs of avian aspergillosis are nonspecific and depend on the organ systems involved. Sudden mortality with respiratory signs can be caused by carbon monoxide poisoning, acute bacterial septicemia, or mycotoxicosis (68). Aspergillosis should be ruled out in early mortality of broiler chicks.

Pulmonary aspergillosis is usually differentiated from other avian respiratory diseases by the granulomatous lesions observed at necropsy; however, *Staphylococcus aureus* pneumonia in newly placed poults can appear similar (51). Exudative fibrinous or fibrinoheterophilic airsacculitis and pneumonia are also frequently seen in cases of mycoplasmosis, colibacillosis, fowl cholera, and chlamydophilosis. Mycobacteriosis and other mycoses also need to be considered when granulomas predominate. Ocular swelling in *Aspergillus* infections resembles infectious coryza or vitamin A deficiency in chicks (44).

Intervention Strategies

Management Procedures

Aspergillus fumigatus is a contaminant of virtually every environment because of its adaptability to different growth substrates and the production of spores that remain viable under extremely harsh conditions (46). Consequently, prevention is by far the best way to control high-risk situations such as can be encountered in poultry farms particularly during the winter in closed rearing houses. One-day-old chicks should come from a hatchery with effective egg and hatchery sanitation supported by microbiological monitoring programs to help minimize the occurrence of brooder pneumonia. *A. fumigatus* infection in young chicks and poults can be reduced by hatchery sanitation. Animal facilities, transport crates, incubators, and hatchers should be cleaned and disinfected with antifungal agents before use.

Potential sources of conidia such as moldy litter materials and feed should never be used. Good litter management combined with daily assessment of its quality throughout the lifetime of the flock is the key to prevention of the disease. Bedding should be kept dry, nondusty, and clean (25). Sporadic or repeated antifungal treatment may be useful in order to control environmental contamination. Spraying of fungistatic agents like thiabendazole (26), nystatin, or copper sulfate (23) have been shown to decrease fungal contamination of bedding. Enilconazole may be sprayed, fogged, or nebulized to decontaminate surfaces or indoor volumes (3). Floor feeding should be avoided (1, 19). Other biosafety measures refer to proper storage of straw bales or loads of shavings which should be kept dry before use to prevent

fungal growth, and should be checked visually before being used for bedding. Any bales that are moldy should be discarded.

Areas around feed hoppers and watering places are fertile areas for growth of molds. Therefore, it is important to manage drinkers, avoid water spillage and if it occurs, remove the wet material from the barn. Unless a permanent yard system is used, frequent moving of feed troughs and watering places is advisable. Placing feed containers and watering fountains on screened, elevated platforms helps prevent turkeys from picking up molds that develop in such places. Drainage is advisable for areas where water is likely to stand after it rains. Daily cleaning and disinfection of feed and water utensils aids in eliminating infection. Spraying the ground around containers with chemical solutions may be advisable if it is impossible to change feeding areas frequently. In outbreaks, a 1:2,000 aqueous solution of copper sulfate for all drinking water may be used to aid in preventing the spread, although it should not be relied on as a permanent method of control (25).

Ensure that the ventilation capacity is adequate for the density of birds present in order to reduce humidity and dust levels to an acceptable concentration. Air flora density of the major fungal genera within the poultry house decreased when the windows were opened during the spring (21). Reducing dust in poultry houses and improving ventilation resulted in a 75% decrease in the incidence of fungal disease (81). Elimination of moldy feed from the diet and environment, along with proper management of sawdust litter, prevented reoccurrence of fungal ophthalmitis in flocks in poultry houses in which there had been previously affected flocks (6). Use of sprinklers may help to manage dusty environments. Some antifungal molecules may be spread on bedding or fumigated with success.

Care should be taken to minimize any stress, especially heat stress, in poultry facilities by using good animal husbandry practices. Birds suffering from aspergillosis can ward off the infection if it is not too severe and if global stress is minimized and environmental quality maximized.

Although numerous antifungal protocols have been proposed to cure birds with aspergillosis, treatment of the disease in poultry farms is virtually impossible. Exposure of chicks to enilconazole fumigation at the time of experimental infection with *A. fumigatus* reduced morbidity and mortality (100). In another experimental aspergillosis study comparing the efficacy of azole compounds, treatment of poults by crop gavage with itraconazole was the most effective in reducing lesion scores and weight loss (76). Vaccines are not a practical alternative, and none are commercially available.

Candidiasis (Crop Mycosis)

Definition and Synonyms

Candidiasis is a mycosis caused by infection with the mycelial yeasts of the genus *Candida*, principally *C. albicans*. Birds are particularly susceptible to oral and crop candidiasis, which resembles thrush in humans. Crop mycosis is the most commonly used term for infection in birds but stomatitis oidica, muguet (French), soor (German), moniliasis, oidiomycosis, and sour crop are other terms for the disease. Thrush is often used for *Candida* infections of the upper digestive tract in humans and other animals.

Significance

Candidiasis affects chickens, turkeys, geese, pigeons, guinea fowl, pheasants, quail, and other avian species (44, 59, 79, 99, 104). Occurrence of avian candidiasis is sporadic, but outbreaks can be costly. The first major reported outbreak resulted in mortality up to 20% in young turkeys, and another report the following year described the loss of 10,000 chicks because of candidiasis (44). Forty percent mortality has been reported from a *C. rugosa* outbreak in 6-week-old turkeys (59).

History

The history of avian candidiasis has been extensively reviewed in previous editions of this book (25).

Etiology

The primary agent of candidiasis is *C. albicans*, although other *Candida* species have been isolated from both healthy and diseased birds. In a mycological survey of crops from broilers, *C. albicans* comprised 95% of isolates but other species included *C. ravautii*, *C. salmonicola*, *C. guilliermondii*, *C. parapsilosis*, *C. catenulata*, and *C. brumptii* (32). Only *C. albicans, C. parapsilosis*, and *C. rugosa* have been associated with crop mycosis. An outbreak of candidiasis in turkeys yielded isolates of *C. albicans*, *C. rugosa*, *C. famata*, *C. tropicalis*, and *C. guilliermondii* from the crop suggesting that the

conditions can favor growth of a broad diversity of *Candida* species.

Candidiasis is an opportunistic endogenous mycosis that results from a disturbance of the microflora or other debilitation of the host, rather than dissemination of a pathogenic strain. Extremes of age and concurrent disease are often implicated when immunosuppression is suspected to be the underlying problem. Prolonged or otherwise inappropriate antimicrobial therapy that upsets the ecology of the microflora can initiate candidiasis, although feed additives may also contribute.

Pathogenesis and Epidemiology

Incidence and Distribution

Candida species constitute part of the normal microflora of the digestive system and can be readily isolated from the intestines and mucocutaneous surfaces of clinically normal birds, humans, and other animals.

Clinical Signs

Signs are not specific for candidiasis. Affected chicks show poor growth, stunting, listlessness, and roughness of feathers. When it is a secondary infection, signs of the primary disease may predominate.

Young birds are more susceptible than older birds to mycosis of the digestive tract. As infected birds grow older, they tend to recover from the infection. In one outbreak, losses amounted to 10,000 of 50,000 chicks younger than 60 days of age. Also, turkeys younger than 4 weeks of age succumbed rapidly to infection. In contrast, a high percentage of recoveries occurred when birds were older than 3 months of age (44).

Gross

Lesions occur most frequently in the crop and consist of thickening of the mucosa with white to off-white, raised circular or rugose lesions (Figure 25.11). Often, curd-like pseudomembranous patches that are peeled easily from the eroded mucosal surface are seen. The mouth and esophagus may be diphtheritic and eroded.

When the proventriculus is involved, it is swollen, serosa has a glossy appearance, and mucosa is hemorrhagic and often covered with catarrhal or necrotic exudate. The frequent association of candidiasis with other debilitating conditions, such as ventricular (gizzard) erosions and intestinal coccidiosis, needs to be considered. It is unlikely that gizzard erosions are directly related to candidiasis. Likewise, thickened intestines with watery contents that are frequently noted in cases of candidiasis are probably caused by coccidia or other protozoa.

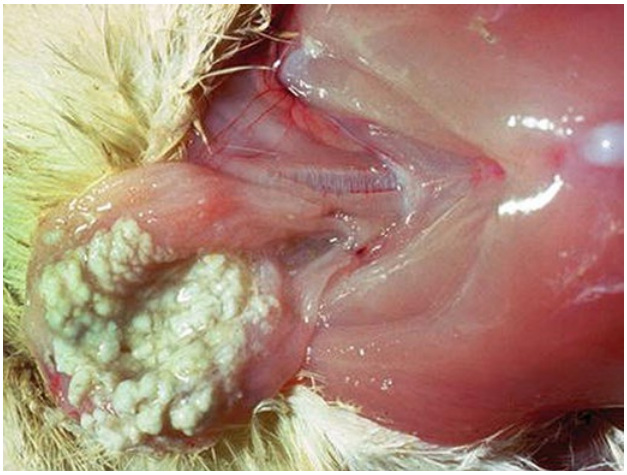


Figure 25.11 Candidiasis (crop mycosis). Crop is markedly thickened by a soft, yellow-white pseudomembrane that has a curd-like appearance. (H.J. Barnes)

Cutaneous candidiasis resulted in feather loss and superficial dermatitis in 70% of a flock of 18-month-old laying chickens. Comb candidiasis in roosters in a broiler breeder flock produced up to 10% morbidity without increased mortality or reduced fertility (65).

Microscopic

Colonization of the keratinized stratified squamous epithelium of oral, crop, and esophageal mucosa is typically limited to the stratum corneum, which may spread into the superficial stratum spinosum. The mucosal surface is covered by exudate composed of necrotic debris, sloughed epithelial cells, leukocytes, bacterial colonies, and the yeast and pseudohyphal forms of *Candida*. Epithelial edema and parakeratotic hyperkeratosis may be evident. Epithelial inflammation is characterized by mixed infiltrates of macrophages, lymphocytes, plasmacytes, and heterophils. Superficial epithelial microabscesses, submucosal edema, and interface inflammation may be present. Bacteria are often numerous in the exudate. Submucosal colonization with attendant inflammation is less frequent.

In typical *Candida* infections, there may be both mycelial and yeast forms within the lesions. Yeast cells are oval and 3–6 μm in diameter. Mycelia consist of both hyphae and pseudohyphae. Pseudohyphae are composed of elongated yeast-like cells arranged in chains that appear similar to hyphae but have prominent constrictions between adjoining cells. Hyphae have parallel sides, are septate, and measure 3–5 μm in width. Fungal morphology in tissue sections is best seen with either PAS or GMS stains (Figure 25.12).

Focal periportal necrosis in the liver of some birds suggests the action of a toxin. A soluble endotoxin, toxic for mice, has been isolated from *C. albicans*. Vascular

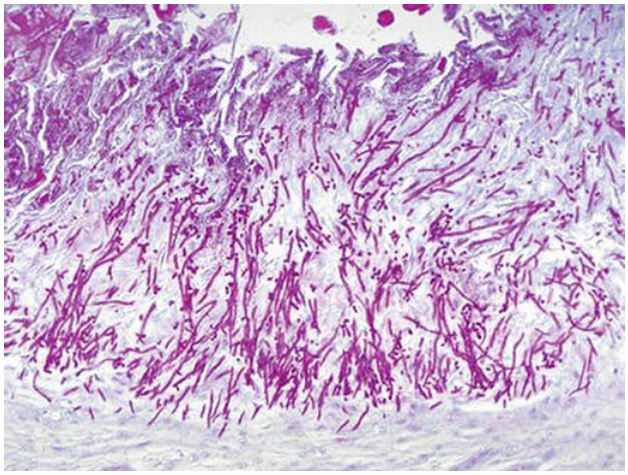


Figure 25.12 In this section of affected crop, the perpendicular arrangement of hyphae, minimal inflammation, and lack of invasion of deeper tissues are characteristic microscopic changes in crop mycosis caused by *Candida*. Periodic acid–Schiff, ×70. (H.J. Barnes)

damage in infected turkeys may be associated with *Candida* endotoxin (44). Atheromatous lesions were present on the intimal surface of the abdominal aorta in more than 50% of turkeys exposed to *C. albicans*, whereas only 12.5% of uninfected turkeys had similar lesions. Human *Candida* isolates may produce a metabolite similar to gliotoxin (*Aspergillus*). Gliotoxin, an epipolythiodioxopiperazine, possesses immunomodulating and antiphagocytic properties (94).

Diagnosis

The clinical history of turkey candidiasis often includes long-term antimicrobial therapy or certain feed additives in vegetarian diets. Observation of characteristic proliferative white curd-like lesions and heavy growth of yeast on primary culture is supportive of a diagnosis of candidiasis. Because it is possible to cultivate *C. albicans* from normal tissues, an original heavy growth of the organism is required for diagnosis. Direct microscopic examination of fresh tissue samples is useful for demonstrating abundant Gram-positive pseudohyphae and budding yeast among the bacterial flora indicating yeast overgrowth of the mucosa.

Aseptically collected scrapings of mucosal lesions can be streaked onto SDA with 50 µg/mL chloramphenicol for inhibiting bacterial growth. Some *Candida* isolates are sensitive to cycloheximide therefore its use should be avoided. Incubation of duplicate plates at both 27 °C and 37 °C is recommended. Plates should be examined daily for 5 days, but not discarded until after 1 month. On SDA, *Candida* produces a white to cream-colored, high-convex colony after incubation for 24–48 hours at 37 °C.

Microscopic morphology of young cultures consists of oval budding yeast cells approximately 5.5 × 3.5 µm. Hyphae and occasionally chlamydospores, which are spherical, swollen cells with a thickened cell wall, are seen in older cultures. Formation of chlamydospores is facilitated by growth on cornmeal-Tween 80 agar. Clusters of blastoconidia on the sides of pseudohyphae are a distinguishing feature, as is germ-tube production in appropriate media (32).

Candida species can be identified by their utilization of carbohydrates. Commercial carbohydrate assimilation panels (API20C AUX and AP Candida [bioMerieux, Inc., Hazelwood, MO] and RapID Yeast Plus System [Thermo Fisher Scientific, Remel Products, Lenexa, KS]) are available (80, 106).

Treatment and Control

For treatment, 1 level teaspoon of powdered copper sulfate ("bluestone," $CuSO_4$) can be added to 2 gallons of drinking water in nonmetal containers every other day for 1 week. Using a 1:2,000 solution of $CuSO_4$ for turkeys as the sole source of drinking water during the course of the outbreak can be effective despite poor performance in experimentally infected chicks and poults (44). An Epsom salt flush after treatment is recommended. Because candidiasis in turkeys can result from broad-spectrum antibiotic treatment, prophylactic treatment with $CuSO_4$ after the antibiotic treatment is completed can reduce the occurrence of disease.

If allowed by regulations, nystatin is useful for treating candidiasis in chickens and turkeys. A 220 mg nystatin/kg diet was effective in reducing symptoms in an outbreak in turkeys and 110 mg/kg provided significant protection against infection. In experimental candidiasis in chickens and turkeys, the severity of crop lesions was significantly reduced with 11 mg/kg nystatin. Turkeys fed 110 mg/kg (100 g/ton) nystatin had higher average weights and milder crop lesions than untreated controls. Dispersing nystatin in drinking water at 62.5–250 mg/L with sodium lauryl sulfate (7.8–25 mg/L) for 5 days successfully treated crop mycosis in chickens. Candidiasis in chickens has been successfully prevented by 142 mg/kg nystatin in feed for 4 weeks (1). However, nystatin should be administered by water because poultry with candidiasis tend to show decreased feed consumption but increased water consumption (50). In Europe, parconazole mixed in the feedstuffs is authorized in order to treat (60 ppm) or prevent (30 ppm) candidiasis of guinea fowls.

Sporadic Fungal Infections

Dermatophytosis (Favus)

Dermatophytosis, dermatomycosis, ringworm, and favus are terms applied to fungal infections of skin. Favus usually is used to denote the disease in poultry. Favus has a worldwide distribution but its occurrence is sporadic. The infection is contagious and zoonotic similar to dermatophytoses of other animals.

The primary etiologic agent of favus, *Microsporum gallinae*, first described in 1881, has had a variety of names including *Epidermophyton gallinae*, *Achorion gallinae*, and *Trichophyton gallinae*. Favus has been reported in chickens, turkeys, ducks, quail, and canaries (44) but is rarely encountered in commercial flocks (12, 22, 28).

Microsporum gallinae is a primary pathogen, although damaged skin is more susceptible to infection. It typically produces white scaly or crusty lesions on the comb and skin of the head and neck with loss of feathers. Other than skin lesions, affected birds are typically healthy and will spread gradually through a flock. It can produce ringworm lesions in people in contact with the birds.

Microscopically, fungal infection is limited to the epidermis. The skin surface is thickened by orthokeratotic hyperkeratosis and serocellular crusts with heterophils admixed with hyphae. Acanthosis and acantholysis with hydropic degeneration may be present. Lymphohistiocytic and heterophilic epidermitis and dermatitis are seen. Examination of feather follicles reveals fungal colonization of the keratinized shaft. In tissues stained with PAS or GMS stains, fungal morphology consists of branching, septate hyphae with parallel sides, 2–5 μm in diameter. Skin scrapings placed into a drop of 10% KOH on a glass slide, overlaid with a coverslip and gently heated over a flame, can be used to visualize intact and fragmented intralesional hyphae.

Scrapings can be cultured on SDA with 50 μg/mL chloramphenicol and 0.5 mg/mL cycloheximide and incubated at 27 °C or room temperature. Colonies of *M. gallinae* usually develop within 1–2 weeks at 27 °C, or about 4 weeks at 20 °C. Colonies are initially white and velvety but become tinged with pink as the culture ages. The colony reverse is initially yellow, which gradually changes to red. Microscopically, cultures are composed of slender (2–5 μm) branching, septate hyphae bearing abundant microconidia and fewer macroconidia. Microconidia are pyriform (pear-shaped) and measure 2 × 4 μm. Macroconidia (6–8 × 15–50 μm) have thin smooth or echinulate walls, contain 4–10 cells, are blunt tipped, and have a curved and tapering base (32).

Introduction of infected birds into existing flocks should be avoided. Birds with favus need to be segregated to prevent transmission of the disease. Other reservoirs, such as contaminated soil, may exist, but to date *M. gallinae* has been isolated only from infected animals. There is no label-approved treatment for poultry, but topical application of miconazole ointment on affected areas can be efficacious (22). Use of gloves with proper disposal is encouraged to prevent transmission to people.

Ochroconosis (Dactylariosis)

Ochroconosis is a sporadic fungal encephalitis of birds caused by the dematiaceous, thermophilic fungus *Ochroconis gallopava* (formerly named *Diplorhinotrichum gallopavum* and *Dactylaria gallopava*) (44). Recently, based on genome analysis, the organism has been assigned its own genus and renamed *Verruconis gallopava* (91). Young chickens, turkey poults, and quail chicks have been affected with the disease (44, 95). Infections occur in people, especially immunosuppressed transplant patients, but the organism is not considered to be zoonotic (109).

Although ochroconosis occurs infrequently, it can be serious in an affected flock causing moderate mortality approximating the morbidity rate. An outbreak in a flock of young chickens resulted in fatal encephalitis in 200 birds from a flock of 65,000. The disease has been reproduced experimentally by injecting a spore suspension into the left posterior thoracic air sac, left maxillary sinus, and cerebrum. A mortality rate of 3%–5% occurred in an outbreak of 60,000 broilers. Ochroconosis in turkey poults caused 20% mortality (44) and mortality ranged from 15% to 20% in an outbreak involving Japanese quail chicks (95).

Clinical signs are those of central nervous system disease and include incoordination, loss of equilibrium, tremors, torticollis, paralysis, and death. Gross lesions are frequently confined to the brain with involvement of both cerebellum and cerebrum, but pulmonary granulomas may also be seen. Lesions have been described as focally extensive, circumscribed, firm, and gray or red (44, 95).

Histologically, lesions are characterized by multifocal to coalescing areas of necrosis infiltrated with numerous heterophils, macrophages, and multinucleated giant cells. Dematiaceous (pigmented) hyphae of *Ochroconis* are readily apparent in H&E-stained tissue sections. Hyphae typically are scattered throughout the lesion in a random arrangement and are yellow to light brown, septate, irregularly branched, and 1.2–2.4 μm in diameter. Fungal elements are frequently located within multinucleated giant cells.

For culture, pieces of lesions can be ground and inoculated onto SDA and incubated at both 24 °C and 37 °C. *O. gallopava* grows well at both room temperature and 37 °C; however, maximum growth of this thermophilic fungus is achieved at 45 °C. Chloramphenicol (0.05 g/L) can be included in SDA to retard bacterial growth but growth of *Ochroconis* is inhibited by cycloheximide (44). After 2–5 days on SDA agar at 24 °C or 37 °C, colonies are velvety, gray-brown with a flat or wrinkled surface. The reverse side of the colony is a deep purple-red and may diffuse into the surrounding medium. Microscopic examination reveals light tan to brown septate hyphae with numerous oval, 2-celled, brown conidia (3.2 × 9.0 µm) borne on short unbranched conidiophores.

Ochroconis prefers acidic environments with moderately high temperatures and has been isolated from decaying vegetation, effluents of acid hot springs, thermal soils, and coal waste piles (1, 3). Ochroconosis in poultry has been associated with contaminated litter and egg incubators (44). Removal of contaminated litter and decontamination of incubators by fumigation is recommended when the disease occurs.

Zygomycosis (Mucormycosis)

Zygomycosis is caused primarily by fungi belonging to the genera *Mucor*, *Rhizopus*, *Absidia*, *Rhizomucor*, and *Mortierella*. Mucormycosis is a commonly used term for these infections because most zygomycosis cases are caused by members of the Order Mucorales, but this is a problematic usage because *Mortierella* is in the order Mortierellales.

Clinical syndromes associated with zygomycosis depend on the organ or system infected. Zygomycoses are acquired from environmental sources, are not contagious, and occur in birds, mammals, and humans. Infection of mammals is associated with immunosuppression or diabetes.

Zygomycoses are not uncommon in avian species if the environment is heavily contaminated with fungal spores (44, 67). Both localized and systemic infections in birds have been reported (39) and commonly concurrent with aspergillosis in a flock. Multifocal white nodules in the lungs have been reported in a chicken with pulmonary zygomycosis. Diagnosis of zygomycosis was confirmed by histopathology (57). Disseminated zygomycosis with concurrent pulmonary aspergillosis affected a flock of layer cockerels, but not pullets, although they were housed together. Mortality was increased and granulomas were found in lung, air sac, peritoneum, liver, spleen, and kidney. *Rhizopus* was isolated from granulomas in various tissues, but only *Aspergillus* was isolated from lung lesions (60). *Absidia corymbifera* caused zygomycosis in broiler chicks. Zygomycotic airsacculitis in a duck, with involvement of intercostal muscles, yielded growth of *Mucor* (55). Zygomycotic ventriculitis and proventriculitis resulting from *Rhizopus* (75) or *Mucor* infection (39) have been described in ostriches. Impaction predisposes to the disease (34).

Zygomycoses can be diagnosed with relative confidence by histopathology. Lesions are characterized by heterophilic granulomatous or granulomatous inflammation, usually with numerous multinucleated giant cells. Necrosis and angioinvasion occur frequently. Granulomas typically have a necrotic center. Zygomycetes are more easily visualized with PAS or GMS stains. Hyphae are relatively wide (7–20 µm) with nonparallel sides and irregular distensions, and have few or no septa and infrequent random branching.

Specific etiologic diagnosis is based on growth and colony characteristics and microscopic morphology. Samples can be streaked on SDA with chloramphenicol; cycloheximide inhibits growth. Growth is relatively rapid at 27 °C and in most cases mature colonies with conidia are obtained within 4 days. Methods to differentiate genera can be found in medical mycology texts.

Macrorhabdosis (Megabacteriosis)

Macrorhabdosis results from heavy infections with *Macrorhabdus ornithogaster*, an opportunistic Ascomycota yeast that colonizes the isthmus between the proventriculus and ventriculus. Previously the organisms were incorrectly thought to be large bacteria and called megabacteria (98). Macrorhabdosis is widespread and affects a variety of birds including poultry. Infection in pet and aviary birds ranges from serious disease with high mortality to asymptomatic carriers (8, 36, 54). Among domesticated birds, *Macrorhabdus* infection has been identified in chickens, turkeys, guinea fowls, quails, partridges, ducks, geese, pigeons, and ostriches. Affected chickens have been in noncommercial flocks and often had other concurrent diseases although the organism can also be found in clinically normal birds (8, 38, 54, 73). Occurrence of the organism in commercial poultry has not been studied. Day-old chicks are readily infected experimentally, the organism multiplies in the proventriculus and isthmus, and food utilization is impaired (36, 77). Japanese quail have been infected with proventricular mucosal scrapings from an infected chicken (54).

Macrorhabdosis is a chronic progressively debilitating, gastrointestinal disease characterized by emaciation, prostration, anorexia, cachexia, and death. Proventriculi are enlarged because of thickening of the walls. Moderate to marked lymphoplasmacytic and heterophilic inflammation of the proventriculus and ventriculus are seen microscopically. Numerous organisms, especially in areas of heterophilic inflammation, are present in the

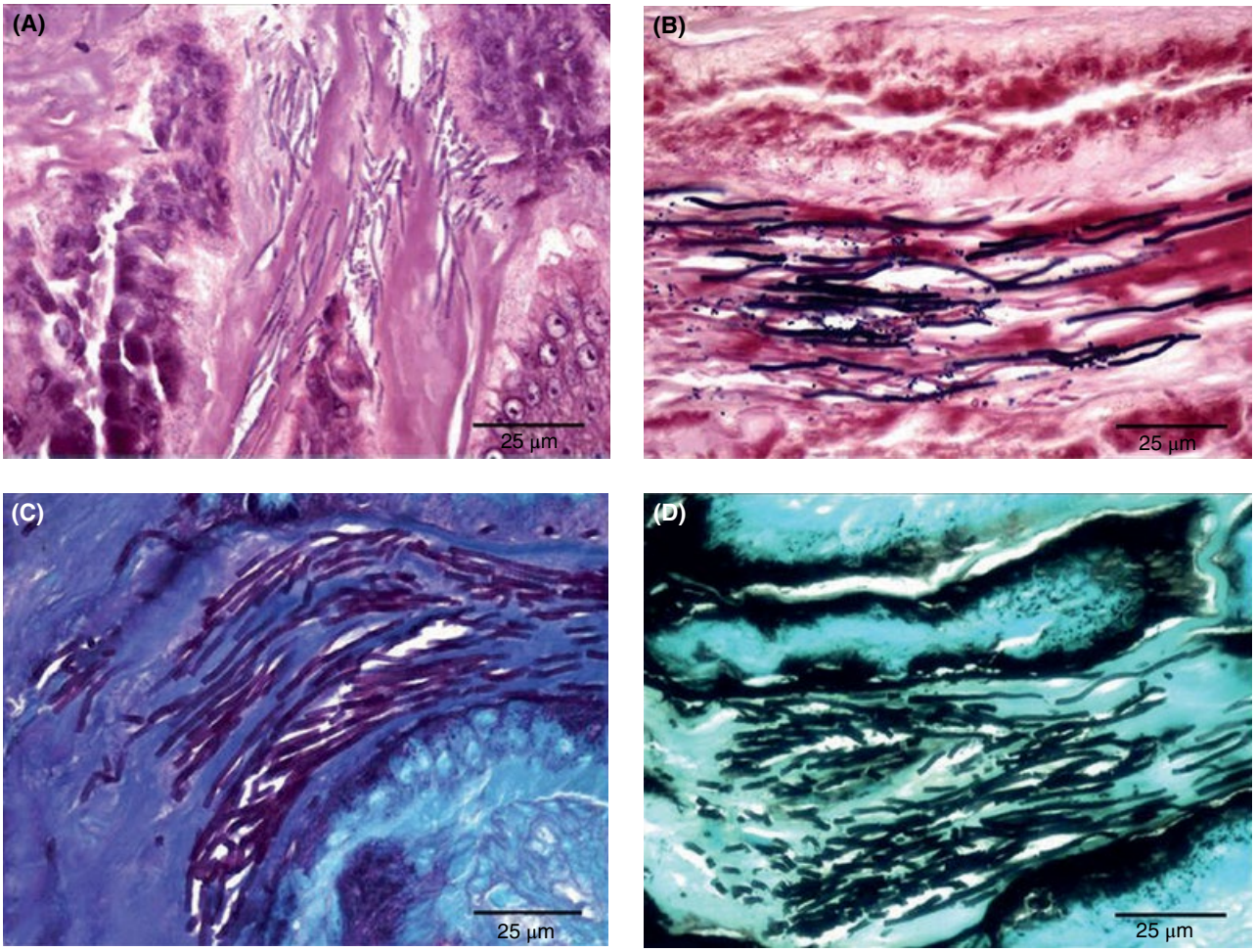


Figure 25.13 Hobby chicken, adult, gastric isthmus, *Macrorhabdus ornithogaster*. (A) Organisms are large, elongated, and arranged in parallel bundles. H&E stain. They stain variably with Gram stain (B), but are strongly periodic acid–Schiff (C) and Gomori methanine silver (D) positive. Bar = 25 µm. (O.J. Fletcher)

mucus, proventricular crypts, koilin, and, less frequently, the epithelium. Diagnosis is confirmed by finding characteristic large, Gram-variable, PAS + organisms in fecal smears, gastric mucus, or in the isthmus and adjacent proventriculus and ventriculus (8, 43, 54). Often they are arranged in parallel bundles (Figure 25.13). *Macrorhabdus* needs to be differentiated from *Candida*, which is similar in size and morphology. Treatment with a combination of antifungal and gastric acidifiers has reduced losses. Depopulation, thorough cleaning and disinfection, and leaving the premises vacant for at least 6 weeks are necessary to control the disease (44).

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Section V

Parasitic Diseases

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26

External Parasites and Poultry Pests

Nancy C. Hinkle and Robert M. Corrigan

Summary for External Parasites

Agent, Infestation, and Disease. Arthropods include insects and their multilegged relatives such as ticks and mites. Some arthropods live on chicken bodies and are known as ectoparasites or external parasites; typically, these feed on the bird's blood, skin, or dermal structures. Other arthropods thrive in the poultry environment, particularly in the manure or litter, and may affect bird health and comfort by impacting their habitat. Both ectoparasites and environmental pests, such as lesser mealworms and flies, can transmit human and bird pathogens. Ectoparasites typically cause discomfort, irritation, and itching, so birds may exhibit restlessness, scratching, and excessive grooming.

Diagnosis. Although mites and lice are small, most species are visible to the naked eye. Microscopic examination is necessary for species identification. Procuring specimens requires locating the arthropods on the host and collecting them into alcohol for preservation and identification. Environmental pests may be similarly difficult to locate and identify because many of them are highly mobile and nocturnal. Others, such as flies and beetles, may be readily evident.

Interventions. Prevention is the preferred method of forestalling arthropod infestations, both on the animals and in their environment. New animals being introduced to the facility should be examined for ectoparasites and quarantined until confirmed free of ectoparasites. Both ectoparasites (e.g., mites, lice) and environmental pests (e.g., darkling beetles, bed bugs) can invade the premises surreptitiously on personnel, vehicles, and other materials moving onto the property, so biosecurity plays a significant role in protection against arthropod pests. Some ectoparasites (e.g., mites, lice, ticks) can be introduced to the facility on wildlife, especially wild birds and rodents transiting the property. On-host ectoparasite stages must typically be addressed using acaricides or insecticides, because few alternative options exist. A broader array of suppression techniques is available for environmental pests, including management strategies, cultural, mechanical, and biological control.

Introduction

Poultry producers must contend with pest arthropods on their flocks as well as environmental pests that affect the animals, workers, facilities, and the neighborhood. Effective pest management requires an understanding of the organism's biology and behavior as well as strategies adapted for specific housing and production systems. This chapter addresses external parasites found on birds (mites, lice, fleas, ticks, etc.), insects and their multilegged relatives found in the birds' environment (flies, beetles, mites, bugs, etc.), and rodents found in and around poultry facilities.

Ectoparasites and Arthropod Pests

The dermal environment of birds provides an ideal habitat for numerous species of fleas, ticks, lice, and mites. Ectoparasites are external parasites, those that live on the skin or feathers, as opposed to endoparasites, which are internal parasites like tapeworms and flukes. Some of these groups are very closely tied to their hosts, having all life stages occurring on the bird. Typically, these are spread by bird-to-bird contact. Others, for example fleas, feed on the host and spend some life stages on the host, while other stages develop in the off-host environment.

Poultry production methods and animal housing type influence degree and type of infestation (25). For instance, in modern high-density layer units, the northern fowl mite (*Ornithonyssus sylviarum*) thrives and rapidly spreads through the flocks. By comparison, the red mite (*Dermanyssus gallinae*) fares poorly because suspended wire caging provides little off-host habitat suitable for these pests. Because breeder facilities provide a long-term stable environment, insects like bed bugs that live

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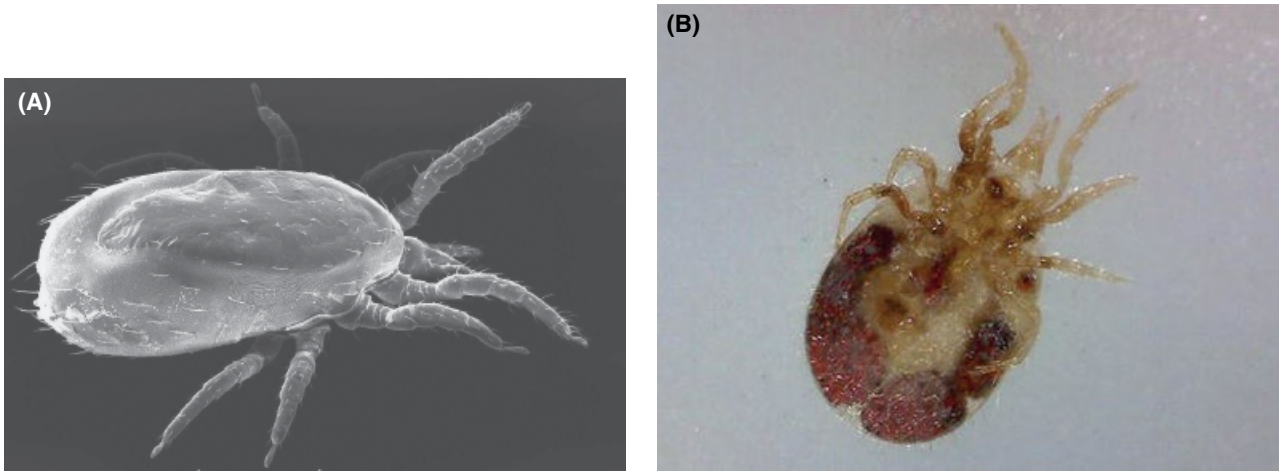


Figure 26.1 Northern fowl mite (*Ornithonyssus sylviarum*) is the most common ectoparasite found on chickens. (A) Scanning electron micrograph. (J.P. Owen) (B) Photograph. (J. Nixon)

off-host but move to the host to feed can establish and flourish. Detection is important and easier for parasites that live on the bird (lice, northern fowl mites, hard ticks, sticktight fleas) than for those that move onto the bird only to feed (bedbugs, chicken mites, soft ticks).

Although ectoparasites do not differentiate between bird breeds, they do demonstrate environmental predilections. Modern production practices, and especially modern caging, have significantly reduced external parasite exposure. However, populations of ectoparasites have been maintained in backyard flocks (28). Reversion to production practices that restore environmental conditions conducive to ectoparasites, as has been demonstrated in European practices since 2000, has allowed a resurgence of poultry pests that had not been seem as problematic in commercial production for over 50 years. A dramatic example is the poultry red mite, which had rarely been seen in commercial flocks since the 1940s. As Europe moved to cage-free or "enriched cage" environments, providing ectoparasites with off-host hiding opportunities, they saw a sharp increase in poultry red mite infestations (24).

Economically, the most prevalent and significant pests of the various segments of the poultry industry are northern fowl mites for caged layers and breeder flocks, lesser mealworms for broilers, and flies for caged layers (18). Backyard flocks and other poultry are particularly susceptible to these and other pests (28), but alterations in flock housing and husbandry practices can increase bird vulnerability to both internal and external parasites (29).



Figure 26.2 Northern fowl mite (*Ornithonyssus sylviarum*) feeding produces inflammation and scabbing around the vent. (B. Mullens)

Mites

Northern Fowl Mites

Ornithonyssus sylviarum, the northern fowl mite, is the most significant poultry ectoparasite, prevalent particularly in caged layer and breeder flocks (Figure 26.1). These hematophagous mites tend to congregate around the vent where their feeding results in skin inflammation, irritation, scabbing, and anemia (Figure 26.2). Mite numbers may reach tens of thousands per animal, resulting in

feather discoloration around the vent caused by concentrations of living and dead mites, mite eggs, and mite feces. All life stages occur on the host, allowing this mite to thrive even in suspended cage production situations. The mites quickly disperse through poultry houses, often reaching almost 100% prevalence in untreated flocks. Northern fowl mites are also common on wild birds, having been found on over 72 bird species, providing a natural reservoir and source of reinfestation. In warmer regions of the world the tropical fowl mite (*O. bursa*) replaces the northern fowl mite, producing the same severe pathology.

Although they cannot survive on mammalian hosts, dislodged mites will move to workers in egg-processing lines and attempt to feed, producing pruritus and irritation. Personnel issues may arise with egg handlers refusing to work until mites are eliminated.

Because these mites develop rapidly, from egg to adult in 4 or 5 days, they can complete their life cycle in a week, allowing mite populations to explode dramatically. The protonymph feeds once or twice, then molts to the nonfeeding deutonymph stage, and then to the adult, which feeds several times during its 3–7 day adulthood (29). Northern fowl mites can develop to extremely high numbers (tens of thousands per bird), especially upon initial infestation of caged laying hens just coming into production. In pullets the immature immune system does not suppress mites, so numbers rapidly increase and the flock becomes heavily infested. At this stage, mites are numerous on birds, moving onto eggs and producing maximum damage to birds. Mite feeding causes blood loss, scabbing, and pruritus. As the bird's immune system matures, the mite population declines, but without treatment mites never completely disappear from the house. Although only a few birds may be infested, they continue to serve as inapparent foci for reinfestation of the rest of the flock.

Because of their small size (<0.5 mm length), northern fowl mites can hide in cracks and crevices. Even on depopulated farms, mites can survive without hosts for several weeks, depending on weather conditions, and infest subsequent flocks. Mite control is based on exclusion and acaricides. Biosecurity is critical, because mites are capable of moving within and among facilities on equipment and personnel.

Bird mites are not host specific in that they can survive and reproduce on any avian species (though typically they are more successful on their preferred host), but they are limited to birds and cannot perpetuate their populations on mammals or other vertebrates. They have been recorded to move among poultry houses by hitchhiking on house mice and other peridomestic rodents.

Mite infestation affects birds adversely, requiring redirection of energy and nutrients from body growth and egg production to enhancing the immune system. Feed conversion efficiency and weight gain are reduced as the immune system expends energy to suppress mites, diverting resources from body maintenance and egg production to stimulating the immune system. In breeder flocks, pruritus and irritation to the vent region reduce rooster libido, interfering with mating and significantly lowering egg fertility.

Economic impacts of northern fowl mite infestations in caged layer operations were documented and showed that at periods of peak mite infestation, profits can be reduced by 50 cents per hen annually (26). This justifies investments in mite monitoring and timely acaricide treatments to suppress infestations before they spread throughout the house.

Acaricide treatments are not 100% effective because of several factors. Birds are missed in applications. Sprayer pressure does not allow complete coverage and product penetration to the skin where mites are located. Mite populations are resistant to most available acaricides, so are not eliminated by chemical treatments.

Because northern fowl mites are found primarily around the vent region, it is critical to deliver an acaricide to the site of infestation, penetrating thick plumage on the bird's underside. This requires considerable pressure in spray applications, ensuring the material reaches the skin beneath the feathers, producing sufficient pressure that birds are lifted off the cage bottom. In treating breeders, which are floor birds and running around loose, the challenges include getting sufficient spray delivered to each bird, ensuring it penetrates the feathers and adequately covers the vent region, and confirming that each bird is treated (20).

Nontraditional treatments have been tested for mite suppression, but results show only temporary alleviation of mite numbers. Sulfur at high rates eliminated mites from birds for 3 months, but neem, kaolin clay, diatomaceous earth, and *Beauveria bassiana* reduced mites for less than 2 weeks (27). Thorough application continues to be the challenge because even the most potent acaricides are effective only if they reach sites where mites are found. Future research will include investigation of vaccines for mite control because it has been demonstrated that birds are capable of modulating mite numbers through immune responses (26). Selecting for host animal resistance may also be possible, developing bird strains with characteristics that permit them to tolerate or avoid mite infestation (26).

Poultry Red Mite

The poultry red mite (chicken mite or roost mite), *D. gallinae*, is another haematophagous mite, but it is not a permanent ectoparasite (Figure 26.3). Bloodfeeding stages (adult and nymphal mites) move to the host to feed at night while the host is roosting, but otherwise hide themselves in cracks and crevices nearby in the environment,

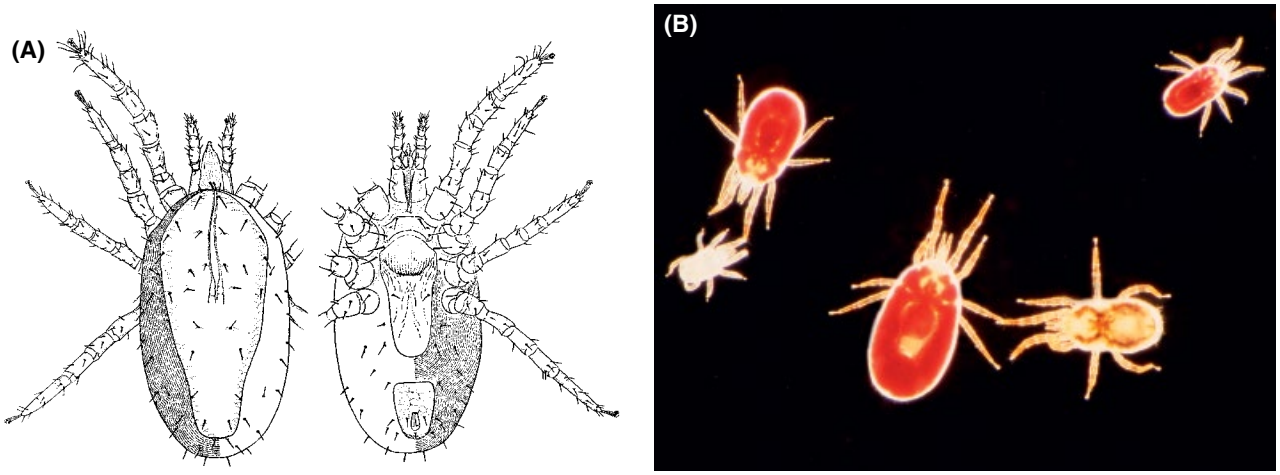


Figure 26.3 Chicken or red mite (*Dermanyssus gallinae*) hides in the roost, moving onto birds at night to feed. (A) Illustration. (E.W. Baker) (B) Photograph. (O. Kilpinen)

where the mites also deposit their eggs. Within 2 months, a female chicken mite and her descendants can amount to over 2,000 mites, causing mite populations to escalate rapidly (34). Adult chicken mites can live for several weeks in protected environmental areas even in the absence of hosts.

Dermanyssus mites are rare in US caged layer operations, but are occasionally found on breeder farms, where mite irritation may force setting hens to leave their nests. They are very common in many European caged layer operations, constituting the primary pest of laying hens in Europe, and may increase in prevalence in North America as production practices are altered (e.g., enriched cages, free range and cage-free) (29). Loss of product registrations and development of acaricide resistance in mite populations are also making mite suppression more challenging.

Not only are egg production and fertility reduced by mites, but feed conversion efficiency can be significantly impacted. In severe infestations, mite feeding can produce anemia and result in increased mortality, especially in younger birds.

Scaly Leg Mites

Scaly leg mites (*Knemidocoptes mutans*) (Figure 26.4) are small mites that burrow into the skin of the birds' shanks and feet, producing tissue swelling, enlargement of scales causing them to protrude, and lymphatic fluid leakage (Figure 26.5). This mite infests wild birds as well as domesticated fowl. All stages of this mite live on the bird and transmission occurs through direct contact. If left untreated, scaly leg mite infestation can produce leg and claw deformity. Typical treatments for scaly leg mites include spraying with pesticides or dipping the affected limbs in mixtures of linseed oil and kerosene weekly until the condition resolves.

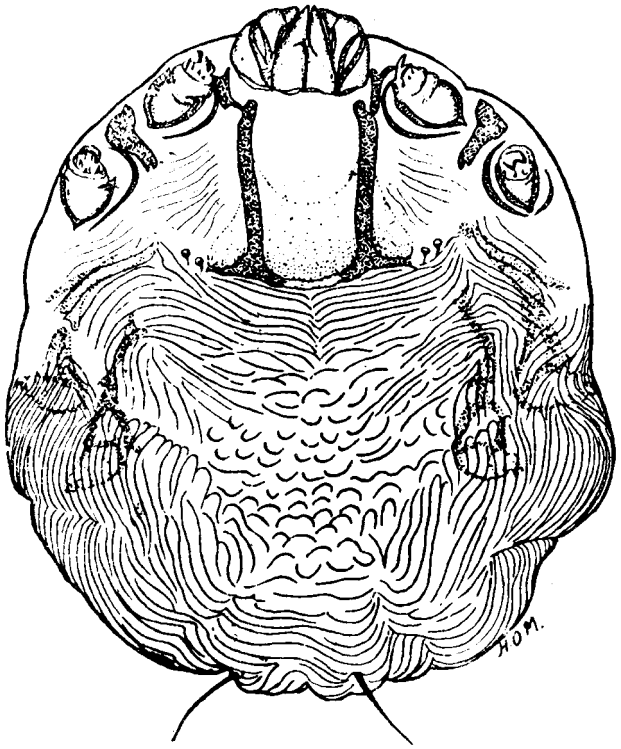


Figure 26.4 Scaly leg mite *(Knemidocoptes mutans)*. (E.J.L. Soulsby)

Veterinarians sometimes prescribe off-label ivermectin use to treat scaly leg mites.

Depluming Mites

The depluming mite (*Knemidocoptes laevis*) burrows into the skin at the base of feathers, producing severe irritation and resulting in feather-pulling. These mites are very small and may be difficult to see without magnification. When they crawl around on the bird, they may be dislodged and spread to other members of the flock.



Figure 26.5 Scaly leg mites (*Knemidocoptes mutans*) burrow under the skin, distorting the legs and feet and causing the scales to protrude. (University of California)



Figure 26.6 Larva of turkey chigger (*Neoschoengastia americana*). (N. Hinkle)

Chiggers

Larval chiggers, *Neoschoengastia americana* (Figure 26.6), are problems on turkeys allowed to range outdoors in the United States. These gregarious mites tend to attach to a bird in large numbers (ca. 100 per animal), and this clustered feeding activity produces skin inflammation and lesion formation. Chigger-damaged areas must be removed at slaughter, resulting in carcass downgrading and financial loss. After the larvae have fed (typically for 4–6 days), they drop off the turkey, and subsequent life stages are nonparasitic. In order to prevent bird infestation, these free-living stages must be eliminated from the environment, or birds must be kept off chigger-infested ranges during chigger season.

Other Pest Mites

The species described above are the most significant mite pests of poultry, but there are other mite species found on or in the quills, in the respiratory system, in air sacs, and within subcutaneous tissues. Most of these mites are microscopic so generally are discovered only upon necropsy. Poultry mites can cause retarded growth, reduced egg production, lowered vitality, damaged plumage, and even death. Typically, the main effects, which are constant irritation and blood loss, are not apparent without careful examination.

Mite Control

The most challenging aspect of treating birds for mites, lice, and other ectoparasites is getting the product through the feathers to the target pest. Because feathers are designed to repel water, aqueous pesticide sprays have difficulty penetrating plumage, resulting in inadequate treatment and wastage as the material drips off the animal. Similarly, dusting is time-consuming and laborious, because each animal must be handled individually and the acaricidal dust directed to the site of infestation. This also stresses the birds and may cause injury. The most critical component of poultry mite integrated pest management (IPM) is prevention; all animals should be quarantined and inspected before they are allowed on the property. When infestations occur, they should be addressed quickly and attacked using all appropriate IPM options.

Fleas

The main flea pest of poultry is the sticktight flea (*Echidnophaga gallinacea*) (Figure 26.7). Sticktight fleas are occasionally pests on backyard flocks, but not on commercial poultry. Mammals having contact with sticktight flea-infested backyard flocks (such as dogs,

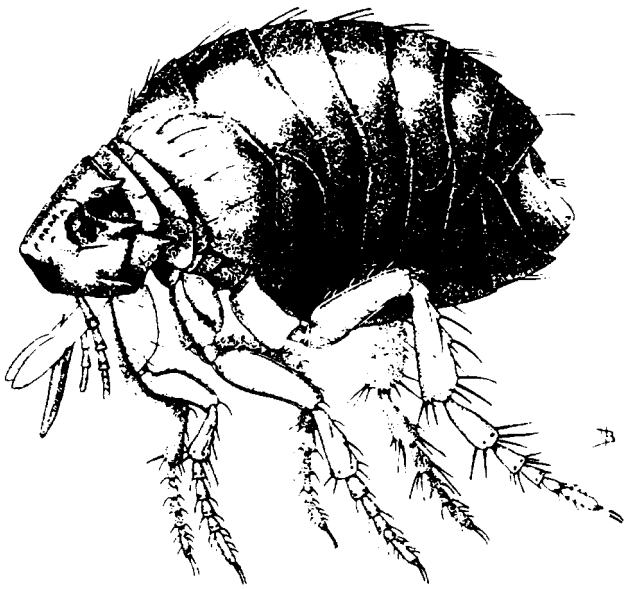


Figure 26.7 Sticktight flea *(Echidnophaga gallinacea)*. (USDA)



Figure 26.8 Sticktight fleas (*Echidnophaga gallinacea*) attach on fleshy areas. (P. Kaufman)

cats, swine, etc.) occasionally acquire sticktight fleas. These fleas are small (less than 2 mm long before feeding).

The sticktight flea can be found on a wide variety of birds. Adult females are usually attached to the skin on the host's head in clusters of dozens or hundreds (Figure 26.8). This flea is unique among poultry fleas because the females embed their mouthparts deep in the skin, making the adult females sessile; the male remains mobile on the host's body and may mate with several females. Other adult fleas of birds and mammals are intermittent feeders on poultry. Numerous sticktight fleas embedded around the eyes can produce swelling and interfere with vision.

Eggs are laid by the immobile females and fall off the host's body into the environment. Larvae develop in the litter, feeding on organic debris, and pupate in the same area. Adults may emerge within a week or, if hosts are not present, remain quiescent within the cocoon for weeks or months. This emphasizes the need for bedding removal to eliminate subsequent population resurgence.

Small numbers of sticktight fleas can be removed by hand using tweezers to pull them off individually. To prevent reinfestation, birds should be treated with a pyrethroid insecticide registered for on-bird application and the flea larval habitat should be modified to interrupt the life cycle. Because flea larvae thrive in shavings, litter, straw, and other floor covering material, the coop should be cleaned out, all contaminated materials removed (off the property to prevent fleas returning), or burned and replaced with fresh shavings. The new shavings should be sprayed with an insect growth regulator (such as methoprene or pyriproxyfen). Birds should be monitored to catch any subsequent infestation so that they can be treated prior to establishment of the flea population.

Lice

Only chewing lice (order Mallophaga) infest birds. These lice feed on feathers, feather debris, skin scales, and other dermal scurf (28). Because they can chew through feather shafts, fresh blood may be found in their gastrointestinal tracts, but they are not bloodsuckers. More than 40 louse species have been reported from domestic birds, although most of these are rare on commercial flocks. Chickens have 7 typical louse species, and 3 are commonly found on turkeys. Although the economic impact has not been determined, louse infestation can be very damaging to young birds, resulting in stunted growth, reduced feed conversion efficiency, and other adverse effects.

Each louse species is relatively host-specific, although they may transfer to atypical hosts when in close association. Poultry louse species have similar life cycles and habits. They live continuously on their feathered hosts and are strongly host-dependent, quickly dying if dislodged. Louse eggs are attached to the feathers, and a single female may produce from 50 to 300 eggs during her lifespan. Young lice go through 3 instars which are similar in appearance to adults, varying in color and size. Typically, the louse egg incubation period is about 4–7 days, and nymphs take about 3 weeks to mature. Lice mate on the host and egg laying begins a few days after they reach adulthood. Lice differ in their predilection sites and these preferences have given rise to the common names applied to louse species.

Chicken Body Louse

The chicken body louse (*Menacanthus stramineus*) is the most common species on adult birds (Figure 26.9). It tends to stay on the skin rather than on the feathers and frequently locates on less densely feathered areas such as around the vent, although in heavy infestations it may be distributed over the entire body (Figure 26.10A,B). Parting the feathers reveals straw-colored body lice

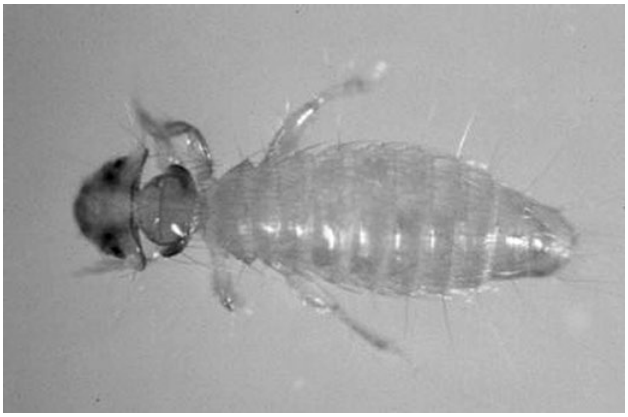


Figure 26.9 Chicken body louse (*Menacanthus stramineus*). (N. Hinkle)

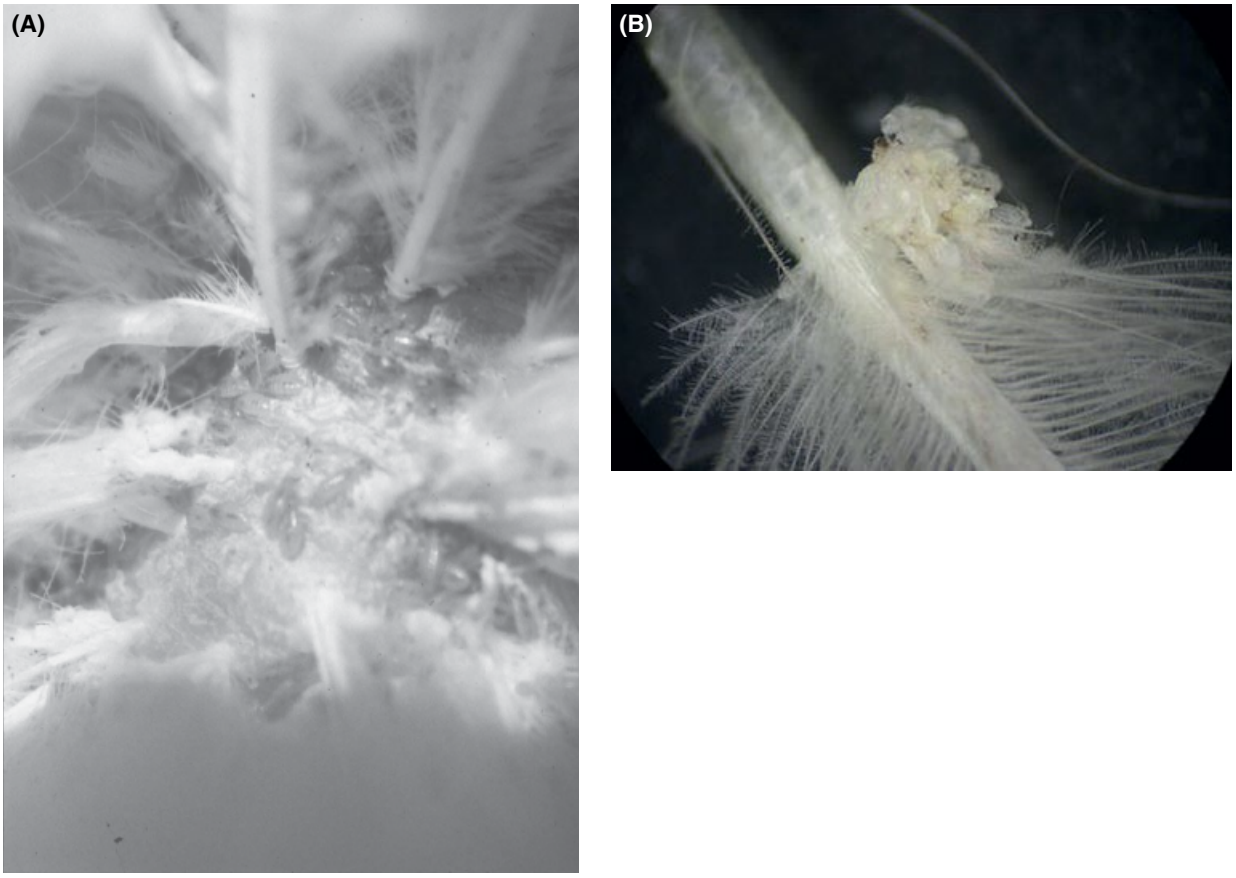


Figure 26.10 (A) Chicken body louse (*Menacanthus stramineus*). (N. Hinkle) (B) Clusters of lice eggs are glued to feathers. (N. Hinkle)

rapidly running on the skin, searching for cover. Clusters of eggs are glued to feather quills; the eggs hatch in about a week, and lice reach maturity in 3 weeks. These lice feed on skin debris and at the base of feathers, producing skin irritation and injury that may result in scabbing. Weight gain may be reduced as a result of the constant irritation.

Shaft Louse

The shaft louse or small body louse (*Menopon gallinae*) is similar in appearance to the body louse, but smaller (Figure 26.11). They are found primarily on the feathers of the breast and thighs. This louse rests on the feather shaft and rapidly descends the shaft, scurrying for cover, when feathers are parted. It feeds on feathers and typically does not infest young birds until they are fully feathered. Heavily infested birds will display restlessness.

Chicken Head Louse

The chicken head louse (*Cuclotogaster heterographa*) is an oblong (ca. 1 mm long), greyish louse found mainly around the head and neck areas of birds. It orients itself near the host's skin in the down or at the base of feathers, particularly on the top and back of the head and under

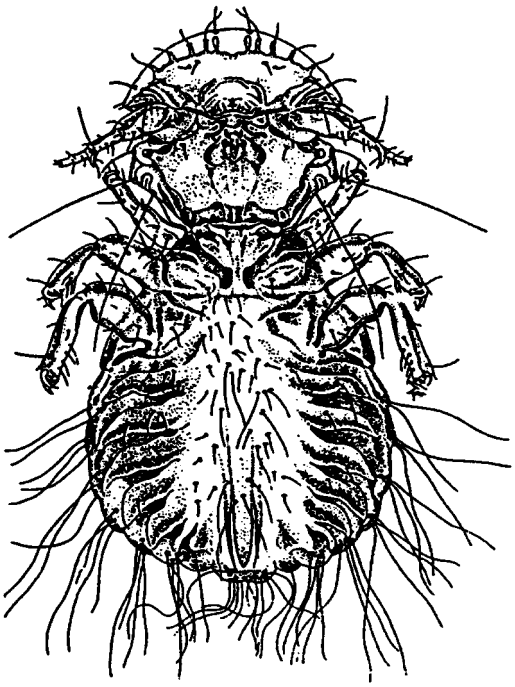


Figure 26.11 Shaft louse (*Menopon gallinae*). (Kriner)

the beak. Female lice attach individual pearly white eggs to the base of small head feathers and the eggs hatch within 5 days. Head lice do not feed on blood but can be very irritating to young chickens and turkeys. Heavily infested individuals may decline and die before they are a month old.

Ticks

Ticks are not insects but are 8-legged bloodsucking arthropods related to insects. The 2 major groups of ticks are hard ticks (Ixodidae) and soft ticks (Argasidae). The most significant avian ticks are argasids, which are long-lived ticks that feed repeatedly as adults. Because these ticks live off the host and only move onto the birds at night to feed, they may be easily overlooked and large numbers can be present, exsanguinating birds before they are noticed.

Fowl Tick

The most important tick in poultry is the soft-bodied tick *Argas persicus*, known as the fowl tick (sometimes called the "blue bug"), although many species of hard ticks will feed intermittently on poultry (Figure 26.12). Soft ticks spend most of their lives in cracks and other hiding places off the bird, moving to fowl at night to take a blood meal. Mating takes place off the host in these hiding places. A few days after each feeding, the female tick lays a batch of eggs (up to 500) in these hidden crevices. Depending on temperature, the eggs may hatch in a couple of weeks or several months. Larvae emerging from the eggs crawl to a host fowl and take their first blood meal, then detach and hide off the host while molting to the nymphal stage. Soft ticks have many nymphal instars, each of which must blood feed, so repeated feeding by large soft tick populations may result in blood loss, emaciation, and fatal anemia. In addition, the fowl tick has been shown to transmit a number of significant spirochete, piroplasmosis, rickettsial, and bacterial diseases of importance in many parts of the world. Because these ticks are nocturnally active, they may not be noticed unless the flock is checked at night. Whereas soft ticks may be common in backyard flocks, typical commercial production facilities have rarely provided environmental conditions conducive to their populations, but this will change with modifications in caging and husbandry (29). Adult argasids are highly resistant to starvation, allowing them to survive without feeding for over a year in the absence of a host, confounding eradication from infested premises. All cracks and crevices that may harbor ticks must be thoroughly treated with an appropriate acaricide to successfully eliminate a fowl tick infestation, and treatment may need to be repeated to suppress tick larvae that hatch from the remaining eggs.

Lesser Mealworms (Adults Known as Darkling Beetles)

Alphitobius diaperinus, commonly known as the darkling beetle, is the most significant pest in broiler houses worldwide (Figures 26.13). Both larval and adult stages of these omnivorous beetles feed on dead and dying birds, spilled feed, and excreta, picking up and perpetuating pathogens (Figure 26.14). Of particular concern are various avian disease agents including the viruses that cause infectious bursal disease and leucosis virus, as well as tapeworm eggs and *Eimeria* (14). Bacterial pathogens involved in human foodborne disease outbreaks that are maintained and transmitted by darkling beetles include *Campylobacter* and *Salmonella* (12). Of particular concern is the darkling beetle's capability to maintain *Salmonella* internally during pupation, so that newly emerged adult beetles coming out of the clay floor or

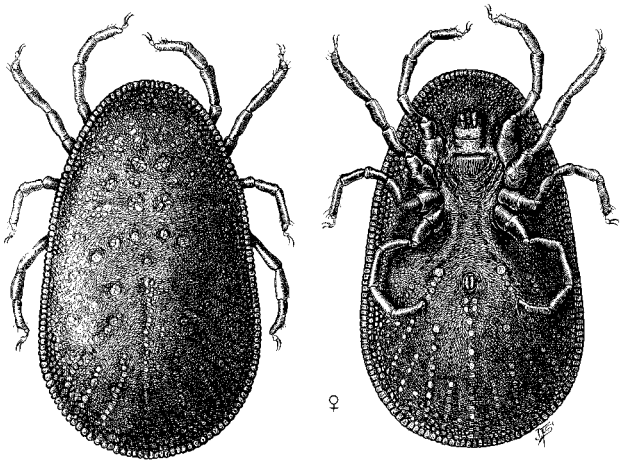


Figure 26.12 Fowl tick (*Argas persicus*). Dorsal view on left, ventral on right. (USDA)



Figure 26.13 Darkling beetle (*Alphitobius diaperinus*). (A. Roche)

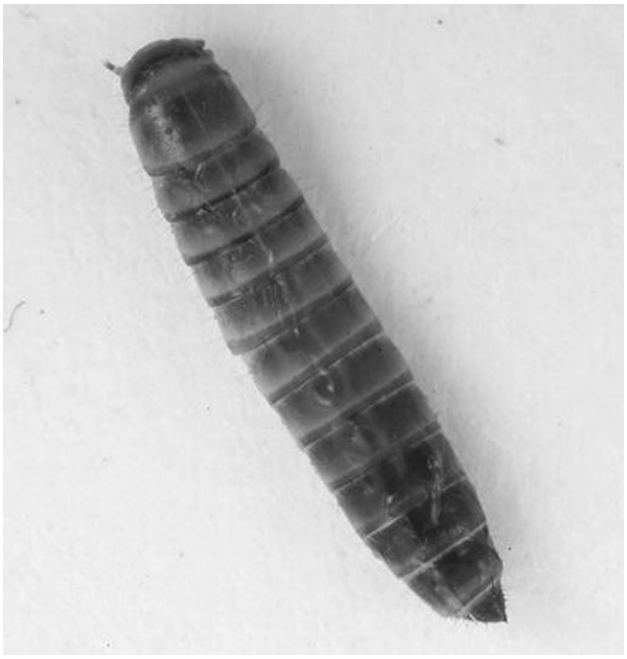


Figure 26.14 Lesser mealworm *(Alphitobius diaperinus)*. (A. Roche)

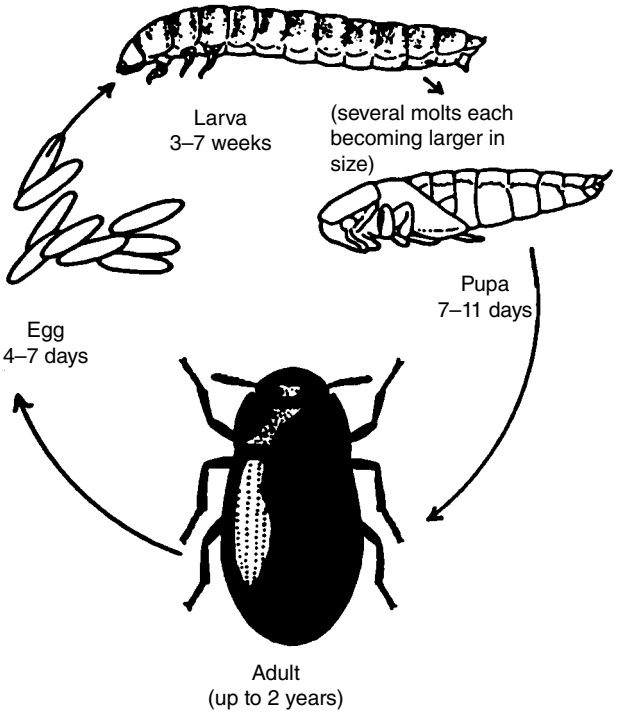


Figure 26.15 Life cycle of the darkling beetle.

foam insulation can recontaminate a broiler house and subsequent flocks (31). Without successful darkling beetle control, efforts to eliminate *Salmonella* from the premises will be thwarted.

Darkling beetles reduce feed conversion efficiency, damage housing structures, and can adversely affect community relations when adult beetles migrate from field-applied litter to nearby neighborhoods. The broiler house environment is ideal for darkling beetles, resulting in high populations. Birds consume both adult and larval beetles, resulting in reduced feed consumption and interfering with weight gain (Figure 26.15). Larval beetles seek protected niches in which to pupate, often burrowing into insulation to create pupal chambers and thus destroying the insulative capacity of the polyurethane (39). Pupae are highly desirable food to mice, which rip into the insulation to extract them, further damaging the material. This increases heating and cooling costs, while decreasing flock productivity. When litter is removed between flocks and applied to pastures or agricultural lands as a soil amendment, adult beetles in the litter frequently fly to nearby structures (attracted to lights at night) and may enter homes through spaces around doors and windows or plumbing, electrical, or cable penetrations.

Darkling beetle control is complicated by the broiler house environment, especially the litter-covered floors, food availability, and stable habitat. Pesticides applied to shavings or litter are rapidly degraded by high pH and moisture conditions, as well as litter microbes. Very few pesticides are registered for application in the presence of birds, so most products are limited to being applied prior to flock placement, leaving weeks for the chemical to lose efficacy and for beetle populations to increase without effective intervention.

Several studies are investigating control alternatives, such as nematodes, fungi, and other entomopathogens, but they are not available commercially. Diatomaceous earth, an inert powder made from siliceous remains of prehistoric freshwater unicellular organisms, has been touted for use against lesser mealworms, but research has demonstrated that it is ineffective.

Updated lists of pesticides available for darkling beetle suppression in poultry houses can be found on Cooperative Extension websites. Effective use of these products involves taking advantage of the pest's behavior such as the fact that larvae and adult beetles tend to concentrate under feeders and around the periphery, so insecticide applications should focus on these locations. Application should closely follow label instructions for best results.

Flies

Biting Flies

Although many Diptera will feed on poultry (mosquitoes, midges, gnats, stable flies), only a few are of veterinary importance, generally because they serve as intermediate hosts for other parasites. Haemosporidians are vector-born parasites (phylum Apicomplexa) that are commonly

found in birds. Avian haemosporidian parasites have a cosmopolitan distribution and are divided into 4 genera: *Plasmodium*, *Haemoproteus*, *Fallisia* and *Leucocytozoon*.

Black flies (family Simuliidae) are important as vectors for transmission of *Leucocytozoon* spp. (an apicomplexan blood parasite) to poultry such as turkeys and ducks. Infection with this protozoan causes intravascular hemolytic anemia in birds, frequently resulting in significant mortality. Black flies seldom enter buildings, so restriction of birds indoors during seasons and times of day when black flies are host-seeking (generally around dawn and dusk) offers some protection. Larval black flies develop only in flowing water, but adults may fly several miles to find hosts. If larval development sites can be located, successful control can be obtained by treating the streams with formulations of *Bacillus thuringiensis* var. *israelensis*.

Biting midges, *Culicoides* spp., serve as intermediate hosts for *H. nettionis* which can infect domestic ducks, exacting reproductive costs and affecting survival. Similarly, *H. meleagridis* infection produces muscle inflammation and significantly interferes with weight gain and growth in turkeys. Biting midge larvae can develop in virtually any standing water (puddles, ponds, treeholes, etc.) so vector suppression is unlikely to be achieved. However, biting midges are disinclined to enter structures, so keeping birds indoors during midge activity periods (typically around dawn and dusk) can reduce exposure.

Several genera of mosquitoes can transmit avian *Plasmodium* spp. (avian malaria). Mosquitoes also transmit the viruses causing eastern equine encephalitis (EEE), western equine encephalitis (WEE), and other encephalitides. Epiornitics of EEE occasionally occur in pheasants and partridges, whereas other wild birds serve as amplifying hosts for the virus. In contrast, WEE rarely produces clinical disease in avian species, although it has been reported to cause encephalitis and paralysis in turkeys. Mosquito control should focus on larval suppression to prevent emergence of bloodsucking adults. There are many reliable mosquito larvicides, including *B. thuringiensis* var. *israelensis*.

The pigeonfly (hippoboscid fly, lousefly), *Pseudolynchia canariensis*, is especially harmful to nestling pigeons and also transmits *H. columbae*, a malaria-like disease of pigeons. This fly is unusual in that the larva matures inside the female and pupates immediately following deposition.

Nonbiting Flies

Around poultry facilities, especially layer houses, house flies (*Musca domestica*) are the most common pest (Figures 26.16 and 26.17). House flies serve as intermediate hosts for some poultry parasites (e.g., tapeworms) and as mechanical vectors of several significant pathogens.



Figure 26.16 Mating house flies (*Musca domestica*). (N. Hinkle)



Figure 26.17 House fly (*Musca domestica*) puparium and larva. (N. Hinkle)

Large numbers of flies can be distracting and annoying to workers, and fly specks on eggshells render the product less attractive to consumers. House fly larvae (maggots) thrive in moist hen feces, so the continually renewed habitat under battery cages is ideal for producing abundant larvae. Similarly, the area under the slats in breeder houses provides moist undisturbed bird feces for house fly oviposition and larval development. Late-instar larvae migrate to drier portions of the house (such as along walls) to pupate, and emerging adult flies move upstairs, or sometimes exit the house and disperse through the community. For this reason, they can be serious problems in neighbor relations, with potential conflict resulting in litigation.

In some portions of the country, especially with open-sided California-style laying houses, other filth-breeding flies in the genus *Fannia* can become a severe problem in cooler months. *F. canicularis* (the little house fly) is commonly found during winter months in southern California layer houses (Figure 26.18). Adult males have

a lekking behavior in which they circle continuously about 5 feet off the ground, putting them in front of workers' faces and producing annoyance. *Fannia* tend not to enter homes, but they frequently hover on walkways and patios. Because they seldom land, they are less likely than house flies to transmit pathogens, but they are inclined to move away from the poultry facility to nearby homes and aggravate neighbors, resulting in poor community relations.

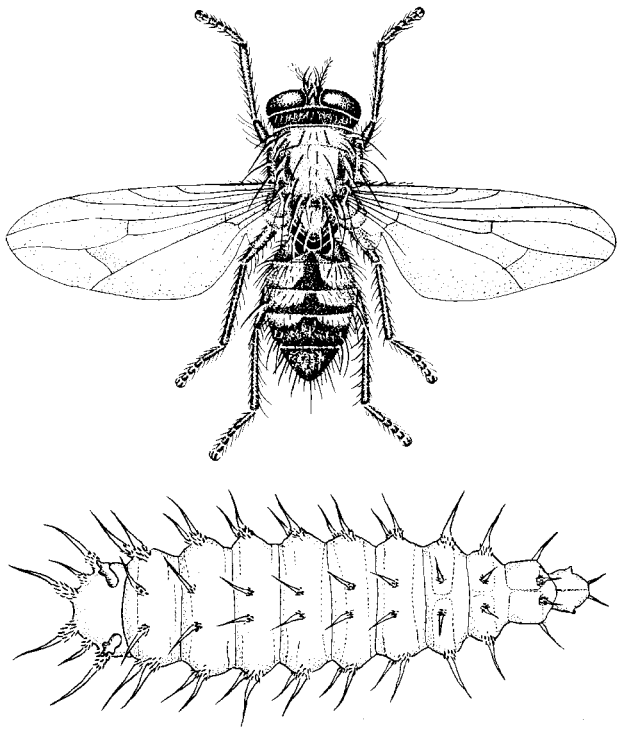


Figure 26.18 *Fannia* spp. adult and spinose larva. (Coop. Extension, University of California)

Blow flies and flesh flies (Calliphoridae and Sarcophagidae, respectively) oviposit and feed on decaying carcasses, illustrating the necessity of promptly and properly disposing of dead birds. If dead birds are to be composted, the carrion must be adequately covered with material to prevent fly access, otherwise adult flies will crawl through the overlaying litter and oviposit, resulting in maggot production from the compost.

Because flies are highly mobile and frequent materials containing potential human pathogens (*Escherichia coli*, *Salmonella*, etc.), they play a significant role in maintenance and transmission of these threats to human health (15). Fly suppression is important both from a nuisance perspective and because they serve as mechanical vectors of potential public health concern (1).

Bed Bugs

The poultry bug (*Haematosiphon inodorus*) and the common human bed bug (*Cimex lectularius*) are associated with chickens and other poultry (Figure 26.19A,B). These hematophagous bugs hide during the day in cracks and crevices around the poultry roost, moving to the birds to feed at night. Their nocturnal habit means they are seldom observed until populations have reached tremendous numbers, at which point they can have severe effects

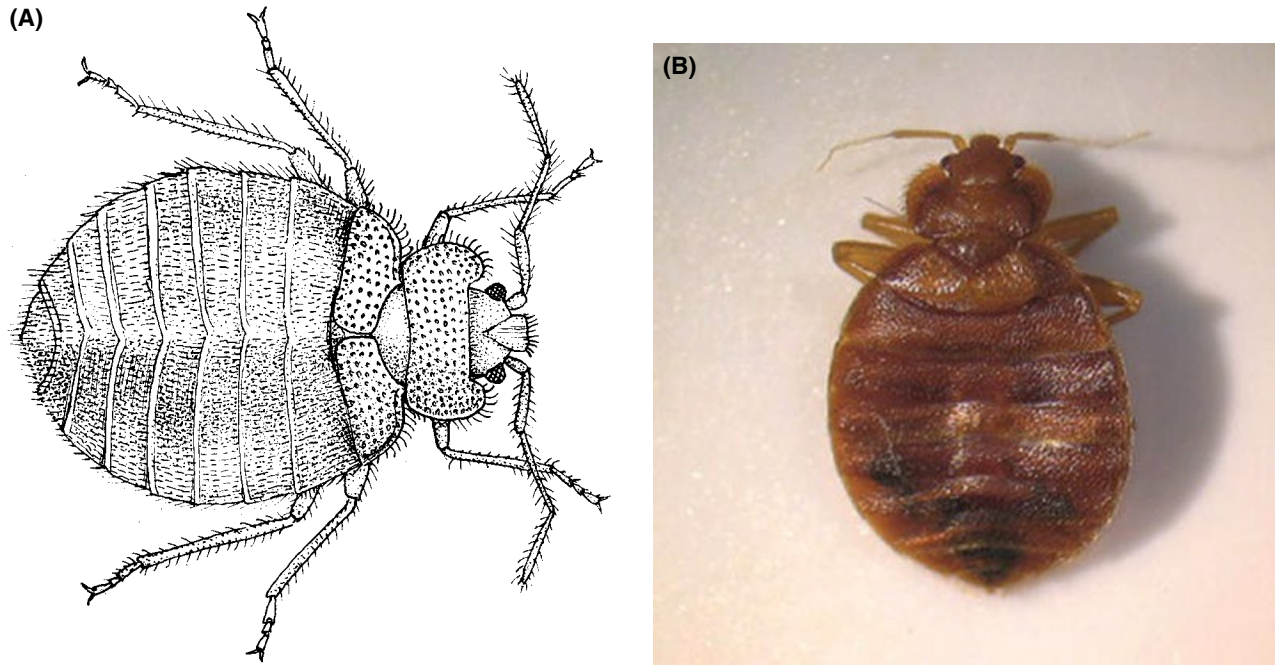


Figure 26.19 Common bed bugs (*Cimex lectularius*) hide in cracks and crevices during the day, moving onto birds at night to feed. (A) Illustration. (USDA) (B) Photograph. (Centers for Disease Control).

on production. Both the poultry bug and the bed bug are widespread around the world, and the bed bug, in particular, is currently experiencing a reemergence as a public health pest. Concerns are that bed bugs may migrate from poultry facilities, or be carried on clothing or belongings of personnel, to infest homes. Some bed bug populations demonstrate high levels of resistance to many of the insecticides available for their suppression, frustrating control efforts (35).

Control Recommendations

Pest suppression strategies include pesticides as well as mechanical, cultural, and biological control options (2, 20). Water and substrate (manure and litter) management are crucial in fly suppression. Litter management is the foundation for darkling beetle suppression. Prevention through use of mite-free stock and restricted movement of personnel and equipment on mite-infested farms is the only reliable method for northern fowl mite control.

Restrictions on equipment and personnel movement not only limit distribution of ectoparasites, but also help to reduce pathogen transmission. Before equipment is moved between farms it should be thoroughly cleaned and disinfected. When darkling beetle-containing litter is removed from houses, it should be rapidly hauled away from the premises, to prevent beetles migrating back into the facilities. Likewise, when manure is cleaned out of layer houses, it should be immediately composted or dried to kill maggots already developing in it and prevent subsequent fly oviposition.

Different aged flocks should not be raised in close proximity as older birds can serve as a reservoir for infestation of young birds. All in/all out practices, maintaining uniform flock age, helps to minimize contamination of incoming flocks.

Advances in ventilation and nipple watering systems have improved litter conditions to where they are no longer favorable for fly larval development in broiler houses. Inattention to equipment service and maintenance, however, can result in leakage and soaked litter, producing maggots and fly outbreaks. Identifying problems and instituting timely corrective actions can prevent an incipient problem from turning into a disaster.

Integrated pest management incorporates a range of options for arthropod suppression based on pest biology and behavior, production conditions, and host attributes. In order to develop successful control strategies, pest identification is the first step (2). As discussed previously, changes in facilities management practices likely will allow resurgence of pests rarely seen in over 50 years, so suppression efforts will have to adjust to these situations (25). In poultry pest management, chemical insecticides play a critical role, protecting bird health and profitability (17). Wise pesticide use requires understanding modes of action so that insecticide classes can be appropriately rotated to forestall resistance. All label requirements and warnings must be followed to ensure human, animal, and environmental safety. Excellent information on poultry pests and their suppression is available (2, 20). Current registrations and recommendations are available through Cooperative Extension (or at sites such as the online *Georgia Pest Management Handbook*), along with assistance identifying pest arthropods.

Rodent Integrated Pest Management for Poultry Operations

Summary for Poultry Pests

Commensal rodents find commercial poultry facilities near-perfect habitats for reproducing and expanding their populations. Infestations can become severe and, as a result, the threat of economic damage to an operation and the potential for disease transmission can escalate quickly. Consequently, rodent IPM programs for poultry operations require *tailored* programs employing *site-specific* inspections, sanitation, exclusion, traps, new asphyxiant burrow treatments, rodenticide baits, and skilled installment of bait stations. Rodent monitoring technology and first alert systems offer great potential in disease prevention strategies. Poultry rodent IPM programs are unlike almost any other urban rodent control operations that even professionals in the exterminating industry encounter. Both the poultry producer and the pest professional must recognize this prior to initiating control efforts. Do-it-yourself rodent control programs are strongly discouraged for multiple reasons including cost-effectiveness, but also for safety to nontarget wildlife and companion animals.

Introduction

Commensal (or domestic) rodents are important economic and health-related pests in poultry facilities. On a global scale, 3 species are of greatest significance: the house mouse *(Mus musculus/domesticus*), the Norway (brown) rat *(Rattus norvegicus*), and the roof (black) rat *(Rattus rattus).* The roof rat is mostly restricted to tropical and subtropical regions. Depending on the country or specific locale, other rodent pest species may occur and be important, but the 3 commensal species comprise the majority of rodent pest issues to poultry and other livestock. For additional information on other rodent pest species, consult the World Health Organization document (40).

Maintaining rodent-free poultry facilities can be challenging, and for many commercial-level operations it may be nearly impossible on a practical level. All 3 rodent species require relatively small openings to gain entry to animal husbandry buildings. Further, rodents (mice in particular) are among nature's best-equipped stowaways and can be delivered directly into a poultry facility hidden in delivery boxes, trucks, trailers, on pallets, or within the voids of processing equipment.

Once inside and left unchecked for even relatively short periods of time, rodents can multiply to serious pest levels quickly because of the uniquely abundant and easily accessible resources of poultry structures. Nutritious food (grains, eggs, birds, insects) is essentially available *ad lib*. Rodent harborages may be limited only by the size of the structure itself.

For example, in layer facilities that contain shallow and deep pits collecting poultry manure, the conical mounds of dried manure provide excellent rodent harborages. The manure is an additional harborage resource to what is available in the soil below slab walkways and within the structural voids (walls, ceilings) of the house. Because poultry facilities are typically uniform in their construction, and well supplied with feed and water, rodents can utilize an entire layer house, which might measure up to 15,000 m^2 or more. Finally, by utilizing the ubiquitous feathers found in poultry facilities, rodents can build protected, dry, and well-insulated nests, all of which contribute toward unusually high survival rates of rodent litters.

Consequently, poultry facilities have produced some of the highest densities of mouse populations ever recorded (3, 30). Selander (32) estimated mouse densities of 3,000 mice/414 m^2 (70,000 mice per hectare) in Texas chicken barns.

Economic Impact

Rodents pose significant economic threats to poultry and other livestock operations along several fronts (23). On a daily basis, rodents consume and contaminate feed meant for the poultry. They constantly gnaw on structural, mechanical, electrical, and other utility components of buildings. Rodents undermine concrete slabs and walkways via their burrowing activities, causing collapses and walkway deterioration, which in turn can affect operations and production. Because of their tunneling and burrowing activities, rodents are particularly destructive to livestock style building insulation, which also jeopardizes poultry production (38). Finally, the costs associated with the customized IPM programs necessary to control and prevent rodents in poultry operations can be substantial.

Table 26.1 A selection of poultry diseases in which rodents have been implicated or directly involved in transmission.

Disease	Agent	Rodents Implicated
Bordetellosis	Bacteria	Rats
Campylobacteriosis	Bacteria	Rats, mice
Erysipelas	Bacteria	Rats
Fowl cholera	Virus	Rats, mice
Fowl pox	Virus	Rats
Leptospirosis	Bacteria	Rats, mice
Salmonellosis	Bacteria	Rats, mice

Public Health Significance

When rodents exist in and around any type of poultry facility, biosecurity of the operation is compromised. Similar to the threat of other wild animals being active around livestock facilities, rats and mice can serve as reservoirs and potential vectors for numerous vertebrate disease agents (40) as well as several poultry pathogens (23) (Table 26.1).

Concern exists for food safety on a global scale regarding salmonellosis, campylobacteriosis, and other microbial pathogens potentially associated with rodent pests and foodborne illnesses (22). Several *Salmonella* serotypes have been associated with rodents (16) including *S. enteriditis*, *S. typhimurium*, and *S. dublin*. However, because rodents are capable of both mechanical and biological transmission of pathogenic microbes, there may be others identified in the future.

Rodents can spread or accelerate the spread of established disease agents from contaminated areas to uncontaminated areas via their droppings, feet, fur, urine, saliva, or blood. They can introduce a disease to nearby uninfected poultry buildings on the same or on different farms within a company operation. With rats, the contamination threat can be measured in farms and properties separated by a few miles (36). More research is needed on the precise role wild rodents play as disease threats in poultry production from the field to the market.

Rodent Biology and Behavior in Poultry Environments

Commensal rodents adapt well to specific habitats whether they are city streets, residential homes, strip malls, livestock operations, warehouses, crop fields, and so on. In fact, differences can exist in the biology (e.g., reproductive rates and success) (4) and behavior (territories, foraging, nesting, etc.) among rodents within different habitats.

The following discussion addresses each of the 3 commensal rodent species in poultry environments. Table 26.2 provides their generalized comparative life histories.

Table 26.2 Reproductive capabilities of the 3 commensal rodents, assuming 1 year of life (13).

	Sexual Maturity in Females (Days)	Gestation Period (Days)	Number of Litters: range (Avg.)	Pups per Litter: range (Avg.)	Total Production Per Female	Age at Weaning: Begin to Forage Outside of Nest (Days)	Cycle of Days in Heat
Norway rat	75–90	21–25	2–7 (4)	6–14	36–39	28	4
Roof rat	68–90	20–23	3–9 (5)	5–8	31–34	28	4
House mouse	35–60	18–21	6–8	2–13 (5)	43–45	21–28	4

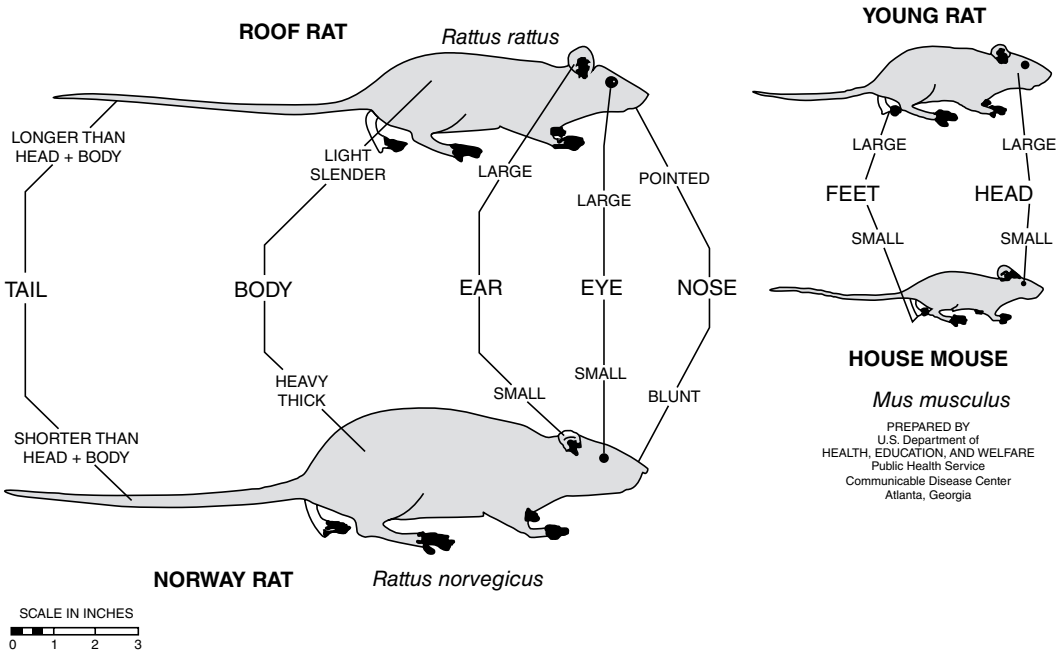


Figure 26.20 Field identification of domestic rodents.

House Mouse

The house mouse is the poultry industry's number one pest in most parts of the world and is probably familiar to most people. It has been described as a mammalian weed (3) and the second most successful mammal on Earth (5).

The house mouse is typically a brown to grayish rodent with relatively large ears, small eyes, and weighing from 0.5 to 1 oz (15–30 g). Most adults measure from 5.5 to 7.5 inches (14–19 cm) in length including the 3–4 inches (7–10 cm) tail (Figure 26.20).

Poultry operations are ideal rodent breeding environments for the reasons listed earlier, and this is particularly true of the small house mouse that can find harborages in virtually any component of a poultry operation. A house mouse litter typically contains 4–7 pups; the dam can produce upwards of 8 litters in a year's lifetime (Table 26.2). Inside warm protected livestock facilities with abundant food resources, mice can produce litters at 24–28 day intervals and the young can achieve sexual maturity in as little as 35–40 days. This is one of the fastest rates for house mouse reproduction in any environment (4). Most wild mice live for less than 1 year, but some individuals may live as long as 2 years.

In and around poultry operations, mice feed on the most abundant food which is various grain feeds. Their grain diet is supplemented with broken poultry eggs, opportunistic killing and eating of chicks, dead poultry carcasses, insect larvae, and any other natural foods that exist near the facility (weed seeds, berries, acorns, soil invertebrates, etc.).

Mouse territories in poultry houses are among the smallest recorded, with mice traveling only 3–30 ft (1–10 m) from their nests (19). However, mice will disperse several hundred feet or farther should disruptions occur (e.g., poultry house cleanouts, construction, etc.). In some locales, mice may migrate from fields to structures and back again with changing seasons.

Norway Rats

Norway (brown) rats are large, robust rodents with most adults weighing from 12 to 16 oz (340–453 g). Larger rats approaching 27 oz (770 g) or more occur, but are uncommon. The Norway rat's fur ranges from reddish to grayish brown on the back and sides, with grey to yellow-white bellies underneath. Norway rats are about 13–18 inches (33–46 cm) long including the 6–8.5 inches (15–22 cm) tail (Figure 26.20).

The average Norway rat litter contains 8–12 pups, and the dam, depending on her longevity, can produce from 4 to 7 litters over the course of 1 year. Rat pups attain sexual maturity as early as 8 weeks of age in good environments (i.e., poultry houses), but more typically about 12 weeks are necessary. Females can enter estrus every 4–5 days and may mate within 24–48 hours following the birthing of a litter (Table 26.2). Wild rats typically live 5–12 months, but some may survive for over 2 years.

The Norway rat is mostly a burrowing animal. Rat burrows are usually 6 ft (2 m) long and 6–12 inches (15–30 cm) deep, containing a main entrance and 2 escape holes. Burrows tend to be established along wall foundations, beneath heavy objects or within dense vegetation, but may occur anywhere if food is nearby. The Norway rat will also occupy wall spaces and elevated areas of enclosed ceilings in a similar manner to the roof rat.

Like mice, rats too are highly opportunistic around poultry facilities. The rat consumes from 0.5 to 2.5 oz (14–60 g) of food daily. Thus, a large infestation of rats can consume considerable quantities of poultry feed in a short period of time. Rats, each of which requires at least 1 oz (28 mL) of water daily, constantly gnaw into water lines and cause leaks.

Rats forage at distances of 50–450 ft (15–137 m) from their nests, depending on various factors. For severe infestations in poultry operations where there are abundant food and shelter, the territories are at the lower end of the range. When disruptions occur to the rat's nesting areas (house cleanouts, construction, etc.), dispersing rats may travel more than 5 miles (8 km) over a few days and establish new colonies at neighboring farms.

Roof Rats

The roof (black) rat is a medium-sized rat with a sleeker appearance than the Norway rat. Adults weigh from 5 to 9 oz (150–250 g) and are grayish black to solid black in color with a buff-white to gray belly.

Roof rats measure about 16 inches (41 cm) in total length from the nose to the end of the tail (Figure 26.20). This is about the same total length as the Norway rat, but the roof rat's tail is longer than the body and is one of its key identifying characteristics. The tail reaches the snout when pulled back over the top of the body. The snout is pointed; the ears are large and cover the eyes when pulled down.

As the name implies, the roof rat tends to occupy elevated areas such as ceilings, soffits, trees and bush canopies, the head spaces of grain elevators, and so forth. The reproductive rates of Norway and roof rats are similar, although the roof rat is slightly less prolific (4–8 pups per litter) (Table 26.2).

The roof rat is also an opportunistic forager and will eat nearly anything that is nutritional and available around the poultry operation as well as berries, nuts, seeds, fruits, slugs, snails, birds, and a wide range of insects found in the natural environs. Roof rats have foraging ranges similar in length to or longer than Norway rats.

Rodent IPM for Poultry Operations

As discussed earlier, poultry houses intrinsically provide a "perfect storm" for commensal rodent infestations once rodents achieve a foothold. Unless rodent management programs are proactive, well-designed, and comprehensive (i.e., integrated pest management), rodent populations in poultry operations rebound explosively following inadequate control efforts (9). Furthermore, designing, implementing and sustaining effective and efficient IPM programs for poultry operations is challenging, even for highly skilled pest management professionals (6). Rodents may infest the entire length of a facility from the manure pit and ground floors to the attic, as well as along the immediate exterior walls of each building. Secondary and tertiary rodent populations may exist around the peripheral areas of poultry farms causing "waves" of new rodent immigrations as the established infestations are eliminated or reduced. Moreover, when rodent baiting programs are employed, the baits must compete with the copious amounts of food already available to the rodents.

Therefore, effective poultry rodent IPM programs must be comprised of 4 elements: (1) on-going inspections and monitoring, (2) rodent exclusion, (3) sanitation and cultural practices, and (4) population reduction strategies. Unfortunately, rodent control efforts around livestock environments are often underestimated in their complexity, scope, and costs necessary to attain control and to sustain the program over the long term.

Conducting Rodent Inspections

The most important step in poultry rodent IPM programs is to establish routine and on-going inspections of the premises and the immediate surrounding areas. Proactive inspections to prevent infestations from ever occurring (or from accelerating to a serious level) can save poultry producers headaches and, in some cases, the survival of the business itself. Should an infestation occur, inspections will provide insight as to both the severity and the scope of the infestation. Moreover, once

a control program is initiated, ongoing inspections will track progress and identify areas of persistent infestation.

Rodent sightings, droppings, tracks, burrows, pathways, fresh gnaw marks, and dead rodents can indicate where rodents are active as well as pinpointing suspected nesting zones. The most revealing rodent inspections are performed using bright flashlights within the first 2 hours after sunset or the hour before sunrise. If rodents are present, such inspections will reveal the location, distribution, and severity of the infestation. This knowledge is essential in determining control procedures (e.g., areas to eliminate harborages, necessary repairs to eliminate feed and water spillage, where to concentrate baits and/or traps, and so on).

Because mice produce 40–100 droppings in a single evening and rats about 20–50, droppings are common signs seen by poultry personnel when the rodents themselves are not noticed.

During inspections, rodent burrows are another common and easily spotted sign. The insulated walls and ceilings are common nesting locations for rodents, and their burrow entrances are easily spotted. Rodents (especially mice) will readily burrow into dry poultry manure of just a few inches in diameter and height. The earth below slab walkways will be tunneled out by all 3 species. Rat burrows around poultry buildings are conspicuous because of their large openings of 1–2 inches (2.5–5 cm diameter). When rodents are seen repeatedly during the day, it indicates an established and probably severe infestation.

Technology using remote electronic sensors can provide pre-emptive notifications to smart phones, computers, tablets, etc. of rodents moving onto a property. Similarly, they can provide alerts about rodents moving into highly sensitive rooms or difficult-to-reach structural spaces. For rodent-free poultry operations, this technology offers important disease prevention advantages. A search of the internet term "remote rodent sensors" will provide product listings, equipment, price ranges, and so on. Remote sensor programs, however, are not a good fit for poultry operations battling ongoing rodent infestations (unless only a few sensitive buildings or rooms need to be monitored) because alerts would be constant. Heavy rodent infestations would be obvious without the sensor technology.

Pest Exclusion

Although rendering a commercial poultry facility 100% rodent-proof can be cost prohibitive, rodents can be excluded from many areas to reduce threats to poultry as well as to help maximize a producer's comprehensive food safety program. Sometimes livestock producers dismiss conducting *any* rodent-proofing, assuming that because rodents cannot be completely eliminated from farm structures, it is not worth doing at all – an unfortunate assumption. Stuffing steel wool into rodent holes is not effective rodent-proofing, nor is attaching "weather strips" to door bases.

Performing effective elementary pest exclusion can make the difference between poultry rodent infestations being minor and easily corrected, or being severe and requiring significant time and effort, while the operation remains at serious economic and disease-related risks.

For example, all grain storage bins and containers can be made rodent proof or kept in rodent proof rooms. Delivery doors, building foundations, roof areas, and cooler boxes can all be modified to deny rodents entry and harborage without major expense or time commitments. Gaps around augers, pipes, and wires where they enter structures can be readily sealed using Portland cement mortar, masonry, or metal collars.

Technology and methodology options exist for structural rodent exclusion for doors and other entry points. These are reviewed in Corrigan (8) and via the internet on rodent Xcluder technologies. Additional publications exist for consultation (7, 13, 33, 37).

Sanitation and Cultural Practices

Once rodents become established *inside* poultry houses, eliminating their food source is obviously not feasible. And, because the rodents utilize the walls, ceilings, ground, and poultry manure for nest sites, it is also impossible to eliminate all indoor rodent harborages.

An important exception to this, however, is the management of dry manure in deep pit and shallow pit egg layer facilities for controlling mice. In these facilities, mouse populations can reach high densities because mice commonly construct their nests within the accumulating manure mounds in the pit and on the various shields and support trusses. The conical mounds of manure below the cages can essentially serve as thousands of high-rise condominiums for mouse families from one end of an egg layer house to the other. Mice also nest within the smaller manure piles that accumulate on manure shields directly below the cages in stacked cage arrangements.

Many egg layer facilities conduct their pit manure cleanouts on a yearly schedule. But if mice get a foothold in these facilities, the manure may need to be removed several times in the course of the year until the mouse population is brought under control. At a minimum, any manure build-up on the manure shields or the support trusses must be removed as frequently as is feasible (every 14–30 days). One family of mice (8–15 mice) can nest within a pile of dried manure measuring only a few cubic inches.

Even loose feathers will be gathered by mice and used to construct feather nests along the floors of houses in

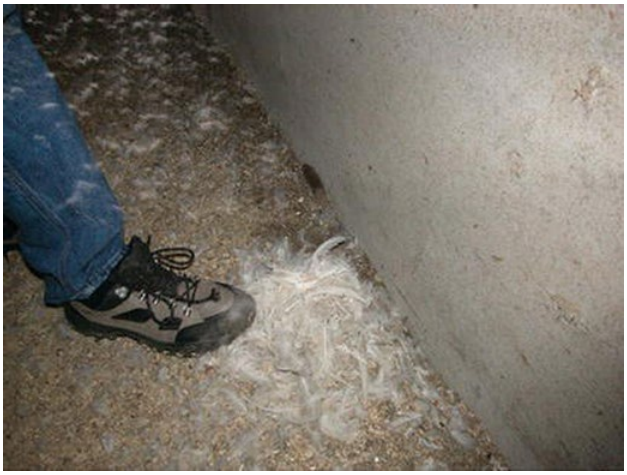


Figure 26.21 A mouse nest built from a collection of feathers. These simple nests can house mouse families and should be disrupted via weekly sanitation programs. Note the scurrying mouse once the nest was touched with the inspector's shoe.

which the manure has been removed (Figure 26.21). These nests are easy to spot, so the mice can be dispatched and the nests destroyed.

Conscientious maintenance and repair of all interior feed and watering systems are essential for effective rodent IPM. Quick repairs of any leaking lines are important because rodent numbers will rapidly increase near malfunctioning feed and water systems and spread to the rest of the house.

Sanitation efforts, especially of surrounding exterior areas, also can make the difference between minor and severe infestations, as well as the frequency with which new rodents colonize a poultry facility following successful extermination campaigns. Weeds and rodents, for example, have always gone hand-in-hand. Weeds provide rodents with food, water, nesting material, and cover from predators. Thus, exterior weed control is a critical element of controlling rodents indoors. Maintaining an uncluttered 3 ft (1 m) weed-free perimeter around all buildings is the standard practice for rodent prevention.

Rats are discouraged from burrowing near building foundations when a perimeter strip of heavy gravel is present. Gravel should be at least 1 inch (2.54 cm) in diameter and laid in a band at least 2–3 ft (0.75–1 m) wide and 6 inches (15 cm) deep.

Exterior feed bins must be kept in good repair and all spillage should be cleaned daily (preferably before dusk). All exterior debris (e.g., old equipment, boards, pallets, etc.) that rodents can utilize for cover must be eliminated.

Population Reduction

When rodent infestations develop in and around poultry operations, initial population reduction to supplement the other steps of IPM is usually necessary. This is achieved by using traps and poison (rodenticide) baits (where allowed), as well as approved burrow treatments for exterior burrowing Norway rats.

Traps

Rodent traps can help check incoming rodents and supplement baiting programs for existing infestations. In areas or cases where poison baits are not allowed, trapping programs may comprise the largest part of the population reduction program beyond sanitation and pest exclusion efforts. Skillful trap installment in strategic locations can capture substantial numbers of rodents and prevent infestations from escalating.

Multiple-capture live mouse traps (also called "curiosity traps") are commonly used inside poultry operations (Figure 26.22). As their name suggests, these traps can capture multiple mice. Several models and trade names are available to the poultry producer (e.g., EZ Force, Ketch-All, Repeater, Tin Cat, Kwik-Katch, Pro-Ketch, etc.) and are effective and useful as a supplemental method for controlling mice.

The ordinary rat snap trap can be effective for rat infestations and capturing elusive rats. But it must be stressed that beyond merely capturing a rodent here and there (which even the homeowner can do), skill and experience are necessary for any meaningful impact on a rodent infestation using traps.

An important drawback of trapping programs is that they require daily servicing by personnel, and this can amount to several hours every day. Discussion on curiosity traps, snap traps, glue traps, and the various models and techniques for using traps on the scale necessary for poultry IPM efforts are provided by Corrigan and Timm (10) and Corrigan (7).



Figure 26.22 One brand of a multiple catch trap used for capturing mice inside commercial livestock facilities. Several different models exist on the market and information is easily gathered by searching the internet.

Rodenticide Baits and Bait Containers

If used carefully and correctly, rodenticide baiting programs can provide high levels of control in relatively short periods of time (i.e., a cost-effective approach). But for some cases and for some locales, poison baits are either not appropriate because of pesticide contamination risks, or they are not permitted because of company or governmental restrictions on the use of pesticidal baits. In these cases, traps, sanitation, cultural practices, and exclusion comprise the program profile.

When rodenticide baiting programs are allowed, their use is not a job for the do-it-yourselfer unless the infestation is at a minimal level. Although many livestock producers and operation managers may be certified to apply various chemicals and pesticides, rarely, if ever, are producers adequately trained to address the complexity of rodent baiting and trapping efforts required in poultry facilities.

Poultry rodent IPM programs require cost-effective and sustainable results, but they also demand high levels of safety to both the poultry product and the various nontargets that may live on or visit the facility grounds. Nontarget animals such as dogs, cats, hawks, owls, coyotes, and foxes can be harmed if rodenticide baits are not competently applied and if caution is not exercised at all times. Safety issues associated with use of poison baits around poultry environments goes beyond simply reading the label and following product directions.

Implementation of rodenticide campaigns in poultry facilities should be conducted by a trained experienced pest professional with expertise specifically in poultry rodent IPM. Installing baits and traps at prescribed spacing around the building perimeter and then simply servicing them on a weekly or monthly basis thereafter results in a never-ending "harvesting" program and rarely achieves population elimination.

A variety of rodenticide baits are available that are labeled for use around poultry operations. Rodenticides are of 2 broad chemical groups: anticoagulants and nonanticoagulants (Table 26.3).

For several safety reasons, rodenticide baits are often installed inside protective bait containers (also called bait boxes or bait stations). Norway rats, however, can be safely baited without bait stations via

Table 26.3 Examples of rodenticide baits used for poultry rodent integrated pest management programs.

Active Ingredient	Examples of Trade Name[1]	Formulations Available	Dose Required to Kill/Comments
Anticoagulants			
Brodifacoum	Jaguar, D-Con, Final, Ropax, Havoc	Pellets, blocks, packs, soft sachets	Single dose; kills warfarin resistant rodents; relatively higher secondary threats to raptors and companion animals
Bromadiolone	Contrac, BootHill, Hawk, Trax-One, Just One Bite, Maki, Brigand, Resolv	Pellets, blocks, packs, soft sachets	Single dose; kills warfarin resistant rodents in USA; resistance has developed in some countries
Diphacinone	Ramik Green, Contrax-D, Ditrac	Pellets, blocks, packs	Death may occur in rats after 1 feeding, but multiple feedings usually necessary
Difenacoum	Multi-kill	Pellets, blocks, packs	Single dose, genetic resistance has developed in some countries
Difethialone	Generation, First Strike	Pellets, bait packs, sachets	Single dose; kills warfarin resistant rodents
Flocoumafen	Storm	Blocks, pellets	
Warfarin	Ferret, Contrax-W	Bulk pails, place packs, 50 lb bulk	Best results are achieved when warfarin is ingested in repeated doses over 4–10 days for rats, sometimes longer for mice. Genetic resistance in some locations
Nonanticoagulants			
Bromethalin	Assault, Fastrac, Trounce, TakeDown	Pellets, blocks, packs	Single dose; stop-feed action; kills warfarin resistant rodents
Cholecalciferol	Selontra, Rampage	Place packs (meal, pellets and canaryseed)	1–3 feedings are lethal. Low toxicity threats to birds of prey; kills warfarin resistant rodents
Zinc phosphide	Many brands	Pellets and mixed grains	Single dose (acute) bait; quick kill results; some rodents may become bait shy to zinc phosphide

[1] Many bait products exist under several trade names depending on the country. No endorsement of named products is intended, nor is criticism implied of similar products not mentioned. Check product labels for livestock applications clearances. In most cases, tamper-resistant bait stations must be used with all baits.

direct burrow treatment, although specific techniques must be complied with to avoid poisonous bait exposure of nontarget organisms (7).

As with the baits themselves, there are also different types of bait stations. Some are more effective or appropriate for use in one type of poultry operation, or for specific locations within particular poultry houses, than others (7, 10). Contemporary bait stations include models designed as PVC T-tube stations (and variations thereof) to facilitate bait installments onto narrow ledges or for baiting rats in elevated areas. Information on all of the rodenticide baits and bait stations are readily accessible via the internet.

Baiting Strategies

There is no one-size-fits-all baiting strategy for commercial poultry facilities. Pest control workers not experienced in poultry rodent IPM programs sometimes try to implement the same conventional baiting schemes as are employed for other buildings they service (e.g., warehouses, factories, etc.). However, the different types of poultry facilities and the specific operation (layer, broiler, turkey, hatchery, etc.) require that each operation and its particular infestation be analyzed prior to proceeding with any type of baiting program. Site-specific baiting strategies are available (7, 10).

Rat Burrow Treatments Using Carbon Dioxide and Carbon Monoxide

In 2017 carbon dioxide (via the use of dry ice pellets) and carbon monoxide (via the use of smoke generating machines) were approved by the US Environmental Protection Agency for use against burrowing Norway rats on exterior yard areas. These treatments are highly effective, offer no secondary threats to nontargets (e.g., raptors, companion animals, wildlife, or people), and are highly humane in the lethal treatment of the rats themselves (i.e., slumber occurs prior to asphyxiation within the rat's burrow chambers).

Property owners can purchase dry ice from ice stores and download directions for its use against rats from the internet (e.g., a Bell Laboratories product produces a label "Rat Ice" that can be followed to achieve quick and safe control of burrowing rats). The carbon monoxide machines for rodent control can also be researched online.

The mode of action of both carbon dioxide and carbon monoxide is asphyxiation (not fumigation). The materials and the techniques are considered by pest management experts (and most veterinarians and animal welfare enthusiasts) to be effective, humane, environmentally responsible, and cost-effective. These materials and approaches are a novel case within vertebrate pest management of 'some things old are smart again' (11).

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27

Internal Parasites

Larry R. McDougald

Introduction

The worm parasites of poultry (trematodes, acanthocephalans, cestodes, and nematodes) are represented by numerous species and often seriously limit productivity of poultry, the well-being of pet or zoo birds, and threaten the survival of wildlife. In highly developed poultry industries, the threat of damage from these parasites has been limited by technology; the practice of keeping poultry indoors may effectively eliminate access to arthropods and lower animals known to serve as essential intermediate hosts for the parasites. Today, only a handful of parasites are important in commercial poultry, although many are found in small flocks reared in natural environments. A rich fauna of internal parasites can be found by examination of birds from backyard flocks and feral or free-range poultry. Wild birds of nonpoultry species have an abundant fauna of worms of all types. Many are important in the commercial production of game birds. The important parasites of poultry normally have a short, direct life cycle, and are fecund enough to prosper in the poultry house environment. This is particularly true in cases in which management does not require frequent cleanout between flocks. Even though all cestodes have intermediate hosts, they may be important because the intermediate host is well suited to the poultry house environment.

The difficulty in control of internal parasites is underscored by the fact that there have been no new products registered specifically for control of worms in chickens in many years. Excellent products are approved for use in other animals which can be used off-label by veterinary prescription. Even though mild infections of most worms cause little damage, some bring in other diseases, such as the well-known relationship between *Heterakis gallinarum* and blackhead disease (*Histomonas meleagridis*). Recent widespread clinical outbreaks of blackhead disease underscore the need for advances in this area. Among anthelminthics, few have been approved for use in poultry, and there is currently no product approved for treatment of cestode infections. Recent changes in the FDA's regulation of off-label use of products by veterinarians has provided some relief by allowing the use of some modern products, but there are still major parasites in many bird species for which there is no treatment.

Nematodes and Acanthocephalans

Introduction

Birds in general are hosts to a great number of worm parasites, particularly the nematodes (roundworms). However, most commercial poultry are reared indoors, restricting the opportunity for exposure to worms requiring intermediate hosts mainly to those with direct life cycles. The shortened growing period for broilers further reduces the exposure to worms, because many of the nematodes require 4 weeks or more to reach sexual maturity and produce infective ova. Worm burdens are more likely to reach damaging populations in warm weather and in birds that are kept for 7 weeks or more (such as broiler-breeders or layers, turkeys, and heavy broiler/roasters).

The nematodes reported from chickens in North America are listed in Table 27.1. One can see that host specificity is not as strict with nematodes as with some other classes of parasites. Most of the nematodes infect the turkey and a variety of game birds, although a sharing of these parasites is not important except where more than 1 species of bird is mixed with others in common

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Table 27.1 Nematodes reported from chickens in the United States.

Nematode	Location	Intermediate Host	Other Definitive Host
Baylisascaris procyonis	Brain		Raccoons (accidental parasite in chicken, turkey, partridge, quail)
Oxyspirura mansoni	Eye	Cockroach	Turkey, duck, grouse, guinea fowl, peafowl, pigeon, quail
Syngamus trachea	Trachea	None	Turkey, goose, guinea fowl, pheasant, peafowl, quail
Capillaria contorta	Mouth, esophagus, crop	None or earthworm	Turkey, duck, guinea fowl, partridge, pheasant, quail
C. annulata	Esophagus, crop	Earthworm	Turkey, goose, grouse, guinea fowl, partridge, pheasant, quail
Gongylonema ingluvicola	Crop, esophagus, proventriculus	Beetle, cockroach	Turkey, partridge, pheasant, quail
Dispharynx nasuta	Proventriculus	Sowbug	Turkey, grouse, guinea fowl, partridge, pheasant, pigeon, quail
Tetrameres americana	Proventriculus	Grasshopper, cockroach	Turkey, duck, grouse, pigeon, quail
T. fissispina	Proventriculus	Amphipod, grasshopper, cockroach, earthworm	Turkey, duck, goose, guinea fowl, pigeon, quail
Cheilospirua hamulosa	Gizzard	Grasshopper, beetle	Turkey, grouse, guinea fowl, pheasant, quail
Ascaridia galli	Small intestine	None	Turkey, duck, goose, quail
Capillaria anatis	Small intestine, cecum, cloaca	None	Turkey, duck, goose, partridge, pheasant
C. bursata	Small intestine	Earthworm	Turkey, goose, pheasant
C. caudinflata	Small intestine	Earthworm	Turkey, duck, goose, grouse, guinea fowl, partridge, pheasant, pigeon, quail
Capillaria obsignata	Small intestine	None	Turkey, goose, guinea fowl, cecum pigeon, quail
Heterakis gallinarum	Cecum	None	Turkey, duck, goose, grouse, guinea fowl, partridge, pheasant, quail
Subulura brumpti	Cecum	Earwig, grasshopper, beetle, cockroach	Turkey, dove, duck, grouse, guinea fowl, partridge, pheasant, quail
S. strongylina	Cecum	Beetle, cockroach, grasshopper	Guinea fowl, quail
Strongyloides avium	Cecum	None	Turkey, goose, grouse, quail
Trichostrongylus tenuis	Cecum	None	Turkey, duck, goose, guinea fowl, pigeon, quail

rearing pens. Also, some of the parasites are accidental, such as *Baylisascaris procyonis*, a parasite of the raccoon. Nematodes not found in chickens but reported from game birds or other poultry are listed in Table 27.2. In many cases, experiments have not been performed to determine whether the chicken is a suitable host for these worms. Wild birds are host to numerous nematode species, but most are not known to infect commercial poultry. Some of these are highly pathogenic to their hosts and even cause mortality. Table 27.3 lists nematodes from wild birds that are also known to infect poultry or game birds. It cannot be expected that commercial chickens or turkeys could become infected with these worms because many require intermediate hosts not found indoors. However, birds reared outdoors in pens, or free-range birds, could become exposed, because many of these parasites exist in reservoirs of backyard poultry and game birds. The life cycles of these parasites are largely unknown.

Worm parasites of poultry have received little research attention for many years, so that much of the literature involves case reports or superficial surveys. The scientific names are those of Yamaguti (49), except where modified by modern authorities on nomenclature. The classification of families follows that used in a series on nematode parasites of vertebrates edited by Anderson and Bain (2). This edition of *Diseases of Poultry* has been streamlined by the elimination of detailed morphological descriptions, the basis of classical parasite taxonomy. The detailed information is of course available in most previous editions of this reference, especially the 12th edition. The figures illustrating the key taxonomic

Table 27.2 Nematodes reported from poultry or commercially raised game birds other than chickens.

Nematode	Location	Intermediate	Other Definitive Host
Cyathostoma bronchialis	Trachea	None or earthworm	Turkey, duck, goose, (chicken)
Cymea colini	Proventriculus	Cockroach	Turkey, grouse, prairie chicken, quail, (chicken)[1]
Tetrameres crami	Proventriculus	Amphipod	Duck
Microtetrameres helix	Proventriculus	Grasshopper	Pigeon
Amidostomum anseris	Gizzard	None	Duck, goose, pigeon
A. skrjabini	Gizzard	None	Duck, pigeon, (chicken)
Ascaridia columbae	Small intestine	None	Pigeon, dove
A. dissimilis	Small intestine	None	Turkey
A. numidae	Small intestine	None	Guinea fowl
Omithostrongylus quadriradiatus	Small intestine	None	Pigeon, dove
Heterakis dispar	Cecum	None	Duck, goose
H. isolonche	Cecum	None	Duck, grouse, pheasant, prairie chicken, quail
Capillaria columbae	Large intestine	None	Pigeon, dove

[1] Experimental.

Table 27.3 Nematodes reported from wild birds in the United States that pose a potential problem for poultry or commercially raised game birds.

Nematode	Location	Intermediate Host	Definitive Host
Oxyspirura petrowi	Eye	Unknown	Grouse, quail, pheasant, prairie chicken
Splendidofilaria californiensis	Heart	Unknown	Quail
Singhfilaria hayesi	Subcutaneous	Unknown	Turkey, quail
Splendidofilaria pectoralis	Subcutaneous	Unknown	Grouse
Chandlerella chitwoodae	Connective tissues	Unknown	Grouse
Aproctella stoddardi	Body cavity	Unknown	Turkey, dove, quail
Cardiofilaria nilesi	Body cavity	Mosquito	Chicken
Echinura uncinata	Esophagus, gizzard, proventriculus, small intestine	Water flea	Duck, goose
E. parva	Proventriculus, gizzard	Unknown	Duck, goose
Tetrameres pattersoni	Proventriculus	Grasshopper, cockroach	Quail
T. ryjikovi	Proventriculus	Unknown	Duck
Cymea neeli	Proventriculus, gizzard	Unknown	Turkey
C. pileata	Proventriculus	Unknown	Quail
Physaloptera acuticauda	Proventriculus	Unknown	Chicken, pheasant
Amidostomum acutum	Gizzard	None	Duck
A. raillieti	Gizzard	None	Duck, dove
Cheilospirura spinosa	Gizzard	Grasshopper	Grouse, partridge, pheasant, quail, turkey
Cymea eurycerea	Gizzard	Unknown	Pheasant, quail, turkey
Epomidiostomum uncinatum	Gizzard	None	Chicken, duck, goose, pigeon

(*Continued*)

Table 27.3 (Continued)

Nematode	Location	Intermediate Host	Definitive Host
Streptocara crassicauda	Gizzard	Amphipod	Chicken, duck
Ascaridia bonasae	Small intestine	None	Grouse
A. compar	Small intestine	None	Grouse, partridge, pheasant, quail
Porrocaecum ensicaudatum	Small intestine	Earthworm	Chicken, duck
Capillaria phasianina	Small intestine, cecum	Unknown	Partridge, pheasant, guinea fowl
C. tridens	Small intestine	Unknown	Turkey
Aulonocephalus lindquisti	Cecum, large intestine	Unknown	Quail
A. pennula	Cecum	Unknown	Turkey
A. quaricensis	Cecum	Unknown	Quail

Note: Some of these have been reported from domestic poultry outside of the United States.

characters are retained for use by those wishing to make detailed examination of worms.

Nematodes

Morphology of Nematodes Used in Identification

Nematodes are usually spindle-shaped with the anterior and posterior ends attenuated. The body covering or cuticle is often marked by microscopic transverse grooves. Longitudinal folds (alae) may be present at the anterior or posterior ends. The posterior alae are sometimes modified to form a bursa (see Figure 27.21B), which functions in copulation. Cuticular ornamentations occasionally found on the anterior extremities are spines, cordons, or shields (see Figure 27.19A).

The mouth opening on the anterior end of the worm is usually surrounded by lips bearing sensory organs (see Figure 27.16A). The mouth may lead directly into a cavity immediately anterior to the esophagus (see Figure 27.18), or this may be reduced or absent. The esophagus may be simple (consisting of 1 undivided part) or more complex (consisting of a short, anterior, muscular part and a long, posterior, glandular part). A bulb may or may not be present at the posterior end (see Figure 27.8). The intestine follows the esophagus and leads to a short rectum connecting to the anal or cloacal opening near the posterior end.

Nematodes usually have separate sexes. These may be fairly similar in appearance, such as in *Ascaridia galli*, or may be remarkably different, as in *Tetrameres americana* (see Figure 27.28), in which the elongate male is much smaller than the globular female. The male worm usually can be identified by the presence of 2 (rarely 1) chitinous structures known as spicules, located near the posterior end of the body (see Figure 27.8). Spicules are considered as organs for use during copulation, keeping the vulva and vagina open, and possibly guiding the amoeboid sperm into the female reproductive tract. Eggs (ova) or larvae are discharged through the vulva, the location of which varies between genera of nematodes.

Development and Life Cycles

About half of the species of nematodes in poultry have a direct life cycle (depending only on the bird host), whereas others depend on such intermediate hosts as insects, crustaceans, snails, and slugs. Several use paratenic hosts, in which no development takes place, but which facilitate parasite survival and dispersal between hosts.

Nematodes normally pass through 4 developmental stages and 4 successive molting events (shedding of the cuticle) before maturing and producing eggs. Eggs laid by the female are excreted in the droppings, regardless of the site of infection of the adult worm. Some eggs are embryonating before leaving the host, but most require suitable conditions outside the host for the development of infective larvae. Most hatch only when consumed by a new host, but a few hatch in the environment and release larvae. Eggs require several days to weeks to embryonate (develop larvae inside). Nematodes with direct life cycles infect birds when the egg or larva is consumed. Worms with indirect life cycles infect the bird when the intermediate host or vector is consumed. Blood-feeding arthropods serving as intermediate hosts may inject larvae in saliva during feeding. For reasons discussed above, nematodes with direct life cycles predominate in commercial poultry.

After infection takes place, the worm requires several weeks to mature and produce eggs (the prepatent period). Some larval development takes place, followed by a final molt, allowing the worm to become an adult.

Nematodes Important to Commercial Poultry Production

Genus *Ascaridia*

The worms of the genus *Ascaridia* invade the small intestine where they cause light-to-moderate damage.

These all have a direct life cycle and resemble each other except for size, contributing to possible confusion. Microscopically, the tails of male worms are used for identification (Figure 27.1). It is doubtful that the reported host range for these worms is accurate. Eggs embryonate in 14–30 days in the environment. Eggs hatch after ingestion by a bird host, releasing second-stage larvae. These invade the mucous layer of the small intestine for a few days before molting to the third stage. It is common to find numerous third-stage larvae in a bird, with low numbers of adults. After 18–30 days, there is a final molt, and the adults begin producing eggs.

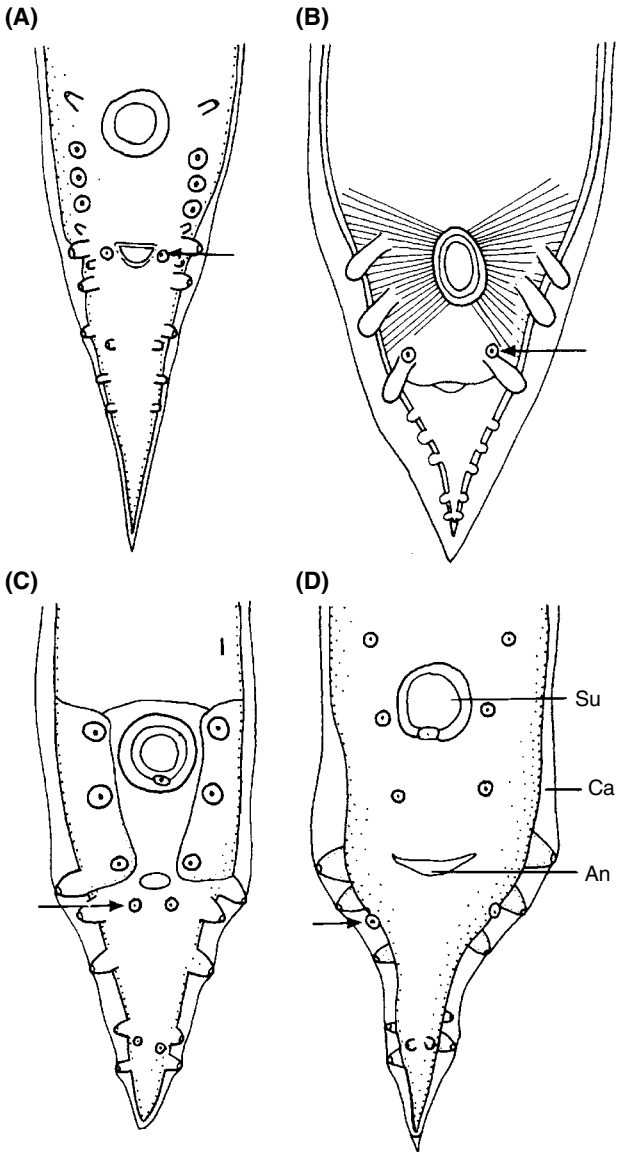


Figure 27.1 Male tails. (A) *Ascaridia columbae*. (After Wehr and Hwang) (B) *Ascaridia compar*. (After Linstow) (C) *Ascaridia dissimilis*. (D) *Ascaridia galli*. (After Wehr)

Ascaridia bonasae. The host of *A. bonasae* is the grouse and Bobwhite quail. The worm is normally much smaller than *A. galli*.

Ascaridia columbae. The hosts of *A. columbae* are pigeons and doves, usually found in the lumen of the small intestine, but also in other parts of the digestive tract. They are of low pathogenicity.

Ascaridia compar. The hosts of *A. compar* are grouse, partridge, pheasant, and Bobwhite quail.

Ascaridia dissimilis. The host of *A. dissimilis* is the turkey. *A. dissimilis* is very common in commercial turkey flocks with numbers of worms in natural infections reaching 2,000/bird (30). Mortality and low productivity are associated with this worm (18, 29). Aberrant migration of larvae may cause hepatic foci and granulomas (31). Infections cause anorexia, intestinal inflammation, diarrhea, and possible depression of immune competence.

Ascaridia galli. Chickens are the host of *A. galli*, but this worm also has been reported in turkeys, doves, ducks, and geese; it is not known whether all these reports are accurate. These are large worms, sometimes reaching 116 mm in length (approximately 4.5 inches). The eggs are very similar in appearance and size to those of *H. gallinarum* (Figure 27.2), and may survive outdoor conditions for more than a year (14). These worms are common in poultry, even cage layers, where worm eggs are mechanically carried by flies. Pathogenicity is generally low, although in heavy infections there is decreased weight gain (35). It is not uncommon to find worm burdens so high that the intestine is blocked. Birds commonly develop age resistance after 3 months, resulting in significantly lower worm burdens (27, 45).

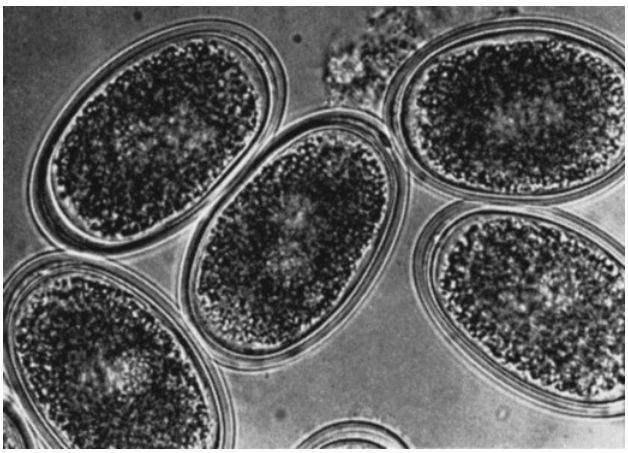


Figure 27.2 *Ascaridia galli* eggs freshly voided from a chicken. ×400. (Benbrook)

An important nuisance factor with this worm is the occasional finding of adult worms in table eggs (36). This is apparently associated with the use of older dewormers such as piperazine, which narcotize the worm rather than killing it. As the worm passes out through the cloaca, it may recover and begin migrating up the oviduct to the shell gland where it may be included in the egg. Worms inside eggs can be detected by candling, although this is more difficult with brown-shelled eggs. This nuisance has been greatly reduced by the use of modern anthelminthics to effectively kill both larvae and adult worms.

Ascaridia numidae. *A. numidae* is a parasite of the guinea fowl. It is found in the small intestine or occasionally the ceca.

Genus *Capillaria*

Several species in the genus *Capillaria* may infect the crop or upper digestive system, whereas others are intestinal. They are usually thin and threadlike. The eggs are easily recognized by the prominent opercula at both ends. Some use intermediate hosts, whereas others have a direct life cycle. The structures of the male bursa and female vulva are used to identify species of this genus (Figure 27.3 and Table 27.4).

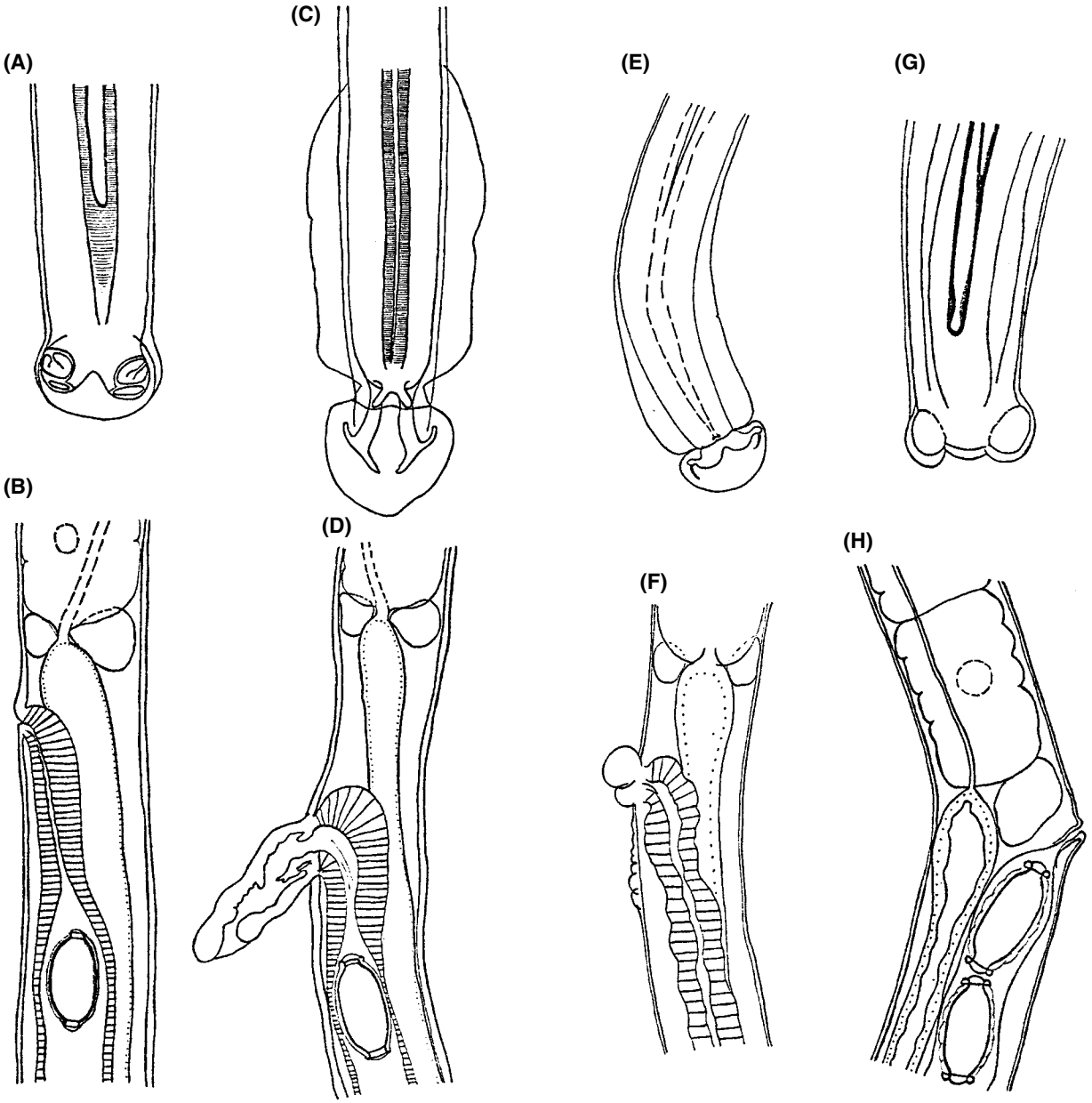


Figure 27.3 Male bursa (A,C,E,G) and female vulva (B,D,F,H) of *Capillaria obsignata* (A,B), *Capillaria caudinflata* (C,D), *Capillaria bursata* (E,F), and *Capillaria anatis* (G,H). (After Wakelin)

Table 27.4 Characteristics of *Capillaria* from chickens in the United States.

Characteristic	*C. anatis*	*C. bursata*	*C. caudinflata*	*C. obsignata*
Male				
Lateral caudalae	–	+	+	–
Spicule sheath	Spines	No spines	Minute spines	No spine
Female				
Vulvar appendage	None	Semicircular	Pronounced	None

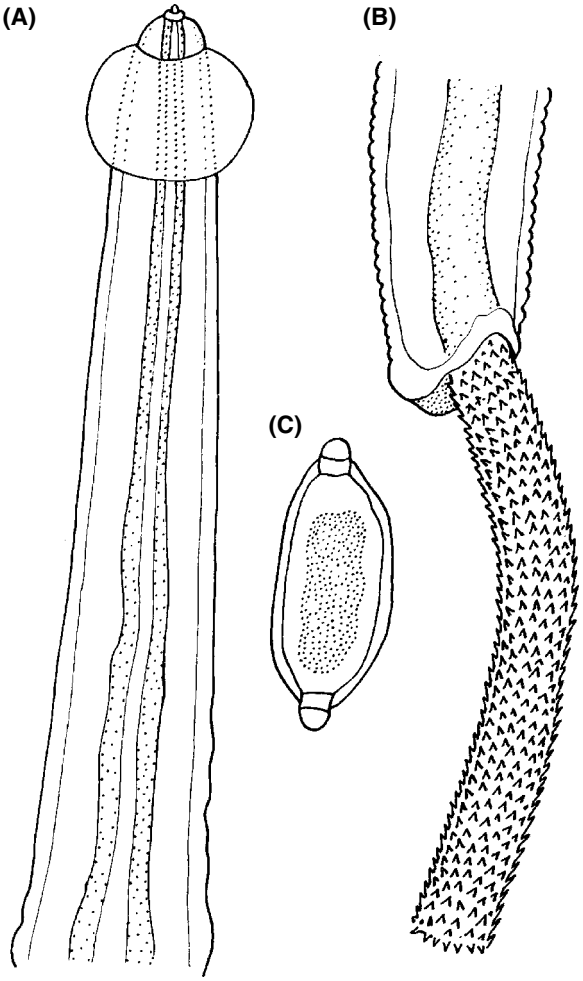


Figure 27.4 *Capillaria annulata*. (A) Head end. (B) Male tail. (After Ciurea) (C) Egg.

Capillaria annulata. *C. annulata* (Figure 27.4) is a parasite that has been reported in turkeys, pheasants, quail, chickens, and other game birds. It invades the mucosa of the crop. It may result in morbidity and mortality, and uses earthworms (*Eisenia foetidus* and *Allobophora caliginosus*) as intermediate hosts.

Capillaria contorta. *C. contorta* (Figure 27.5) infects all gallinaceous birds and invades the mucosa of the crop and esophagus. It may result in morbidity and mortality. The life cycle is direct.

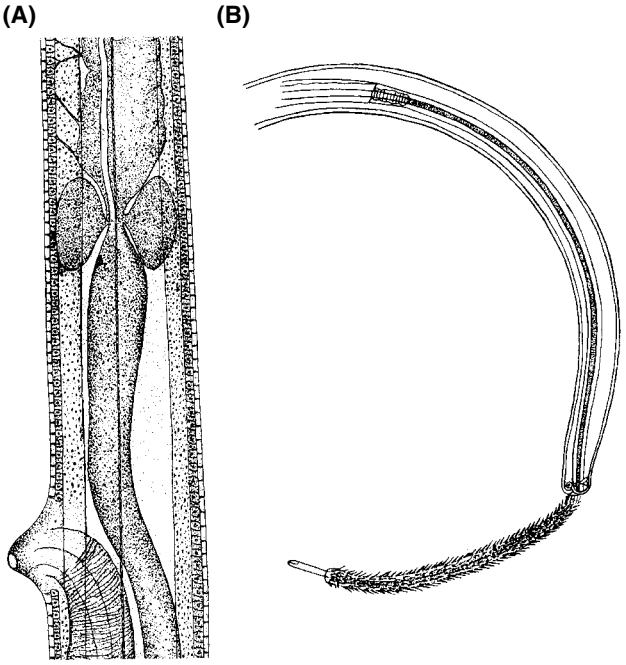


Figure 27.5 *Capillaria contorta*. (A) Region of vulva. (After Eberth) (B) Male tail. (After Travassos)

Capillaria obsignata. *C. obsignata* (Figure 27.6), one of the most important worms in commercial chickens, is also reported in the turkey, guinea fowl, pigeon, and Bobwhite quail. It is found in the small intestinal mucosa where it induces a catarrhal exudate and thickening of the wall. Morbidity and mortality are possible in heavy infections.

Capillaria caudinflata. *C. caudinflata* infects the small intestine of all gallinaceous birds. It uses earthworms (*Eisenia foetidus* and *Allobophora caliginosus*) as intermediate hosts.

Capillaria bursata. *C. bursata* infects the small intestine of gallinaceous birds and uses earthworms as the intermediate host.

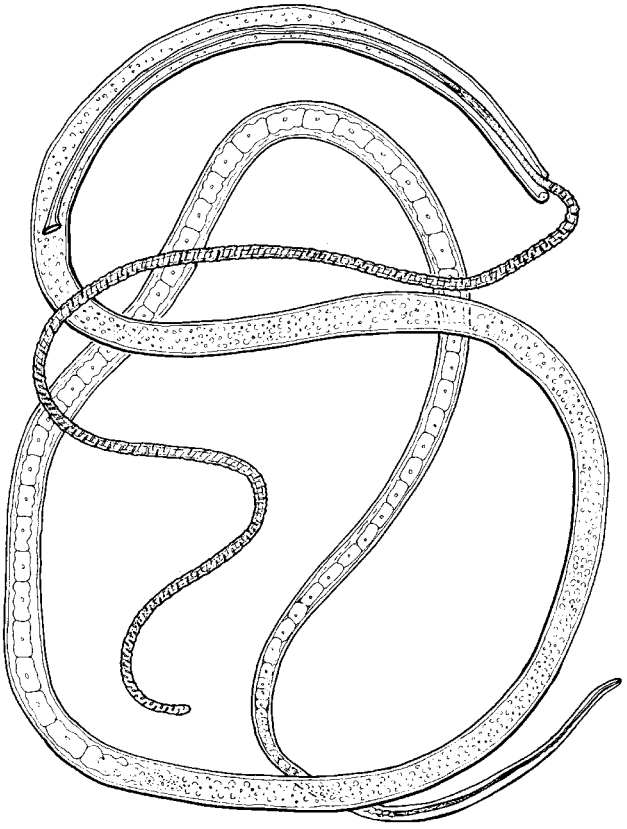


Figure 27.6 *Capillaria obsignata*. (After Gagarin)

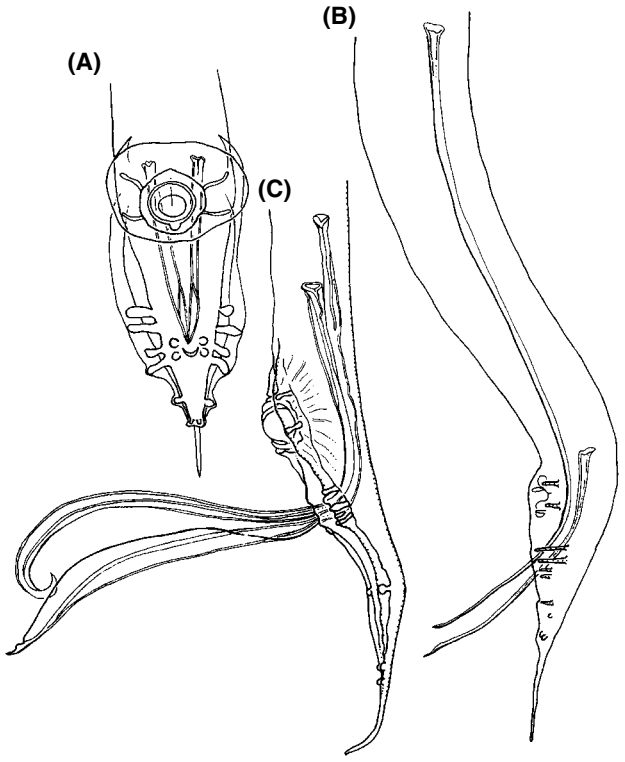


Figure 27.7 Male tails. (A) *Heterakis dispar*. (After Madsen) (B) *Heterakis gallinarum*. (After Lane) (C) *Heterakis isolonche*. (After Cram et al.)

Capillaria anatis. *C. anatis* is reported from the chicken, turkey, duck, goose, partridge, and pheasant. This worm is found in the cecum or sometimes in the small intestine. The life cycle is not known.

Genus *Heterakis*

Worms of the genus *Heterakis* are small and thin, and are found primarily in the ceca of birds. Several species of these worms are common in commercial poultry and game birds.

Heterakis bonasae. *H. bonasae* infects the ceca of the ruffed grouse and Bobwhite quail. Prevalence and worm burdens are sometimes high (17, 23). In contrast to results with *H. gallinarum*, *H. bonasae* could not serve as a vector for blackhead disease (histomoniasis) in Bobwhite quail (10).

Heterakis dispar. *H. dispar* infect the ceca of ducks and geese but are relatively nonpathogenic (Figure 27.7A).

Heterakis gallinarum. The hosts of *H. gallinarum* are the chicken, turkey, duck, goose, grouse, guinea fowl, partridge, pheasant, and quail. This nematode is an abundant parasite of replacement layers and all chickens that are maintained on soil or litter. Worms are small and white, and generally thin. Microscopic identification is based on the head and esophagus (Figure 27.8A), but eggs are similar to those of *A. galli*. Without doubt, this is the most important nematode in poultry because it serves as a transport host for *Histomonas meleagridis*, the causative agent of blackhead disease (47). Although there are other species of cecal worms, *H. gallinarum* is the only one known to carry blackhead disease. Blackhead disease can be produced by dosing birds orally with embryonating eggs, larvae, or male worms (38, 43). The life cycle of the worm is direct, although worm eggs may be ingested by earthworms, where they hatch and live for months before being ingested by a bird. The ring-necked pheasant is the best host for *H. gallinarum*, followed by guinea fowl and chickens (27). Turkeys rarely develop patent infections, but larval parasitism is sufficient to allow initiation of histomoniasis. The worm itself is of low pathogenicity, although inflammation and thickening of the cecal wall and nodule formation on the mucosa and submucosa are seen in heavy infections (24). Larvae may migrate to the liver, causing hepatic granulomas (37).

Heterakis isolonche. *H. isolonche* is reported from the duck, grouse, pheasant, prairie chicken, and Bobwhite quail. It is very similar to *H. gallinarum*, but differentiated by the size and appearance of the spicules (Figure 27.7C).

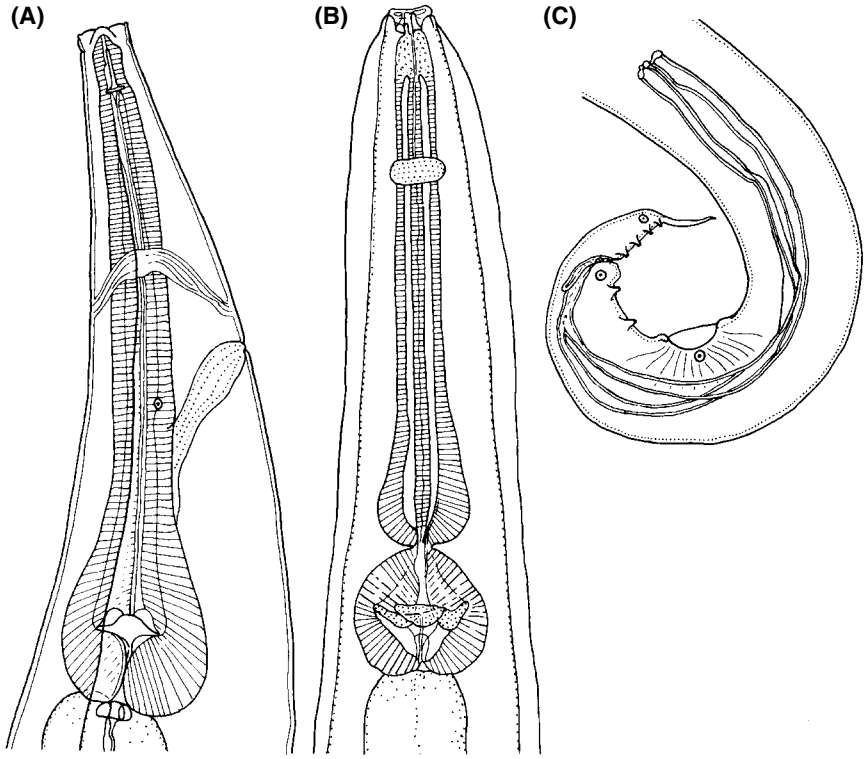


Figure 27.8 (A) *Heterakis gallinarum*, head. (B) *Subulura suctoria*, head. (After Skrjabin and Shikhobalova) (C) *Subulura strongylina*, male tail. (After Barreto)

It is somewhat more pathogenic than other species, reportedly causing high mortality in pen-reared pheasants, but is less pathogenic in quail and grouse.

Nematodes Important in Game Birds, Waterfowl, or Poultry Reared Outdoors

Aproctella stoddardi

Aproctella stoddardi is found in Bobwhite quail, doves, grouse, and turkeys, in the body cavity. Morphology is illustrated in Figure 27.9. A biting arthropod is thought to be the intermediate host. Heavy infections may result in mortality in doves.

The eggs apparently hatch *in utero*, as unsheathed larvae are present in the uterus.

Aulonocephalus lindquisti

The hosts of *A. lindquisti* are Bobwhite quail and blue or scaled quail, mostly in western Texas. These are bright pink worms, mostly in the ceca, but sometimes in the large intestine (Figure 27.10). The pathogenicity and life cycle are unknown.

Baylisascaris procynois and *Baylisascaris columnaris*

Baylisascaris procynois and Baylisascaris columnaris are parasites of raccoons and skunks, respectively, occasionally infecting birds through consumption of infected feces. Birds are not a suitable host for the worm, but the larvae migrate to the brain, causing severe neurological signs.

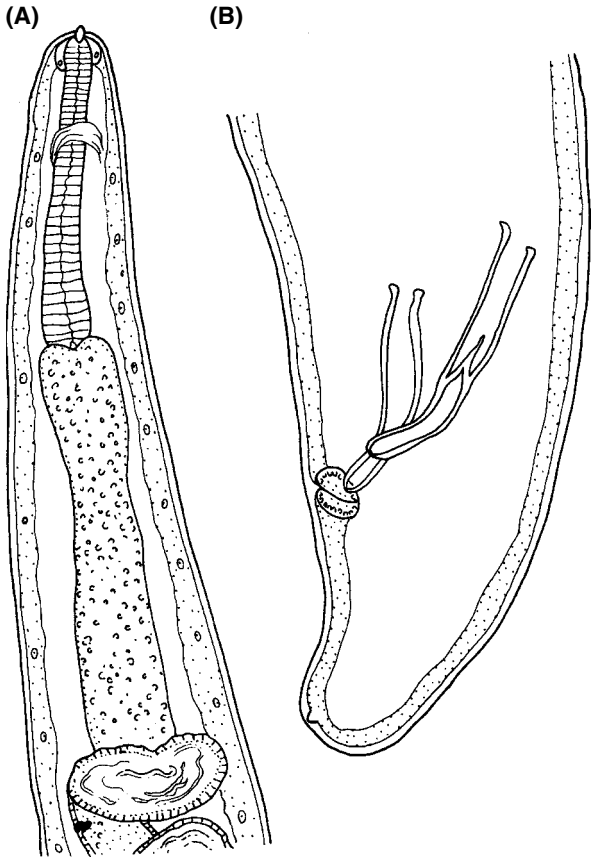


Figure 27.9 *Aproctella stoddardi*. (A) Head. (B) Male tail. (After Anderson)

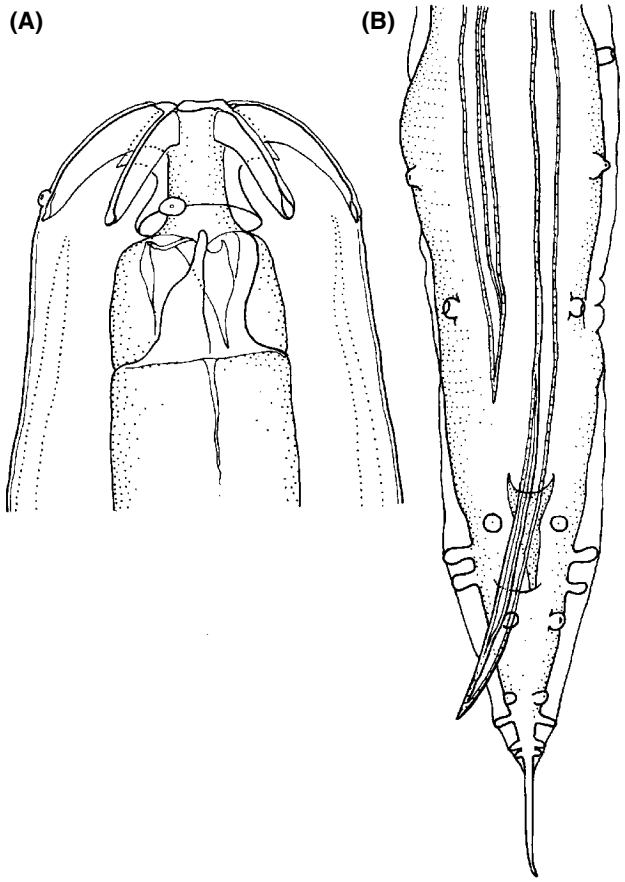


Figure 27.10 *Aulonocephalus lindquisti*. (A) Head. (B) Male tail. (After Chandler)

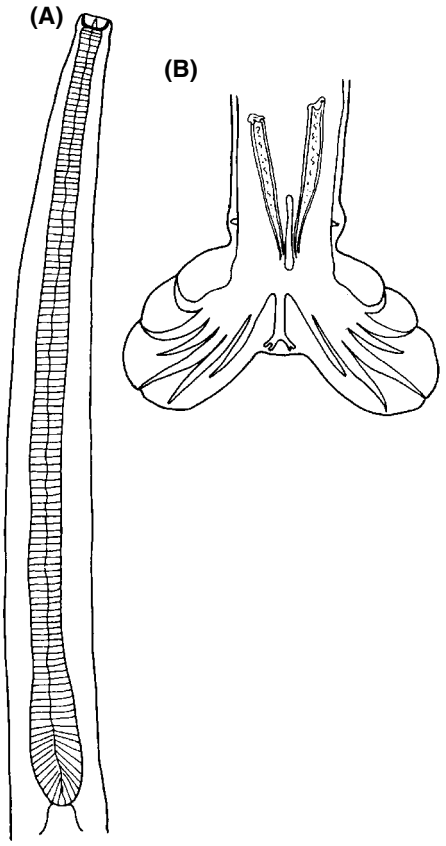


Figure 27.11 *Amidostomum anseris*. (A) Anterior. (After Boulenger) (B) Male bursa. (After Railliet)

This condition has been reported from partridges, emu, quail, and other birds, and has been produced experimentally in chickens (25).

Genus *Cheilospirura* and Relatives

Genus *Cheilospirura* and their relatives are worms that are found under the horny lining of the gizzard (ventriculus).

Amidostomum anseris. The *A. anseris* (Figure 27.11) infects the gizzard of ducks, geese, and pigeons. A slender and reddish worm, 10–24 mm long, is found under the horny lining of the gizzard and occasionally in the proventriculus. Eggs are thin-shelled, 85–110 × 50–82 µm. Heavy losses among geese have been attributed to this nematode. The lining of the gizzard may be necrotic and detached, with loss of blood. The life cycle is direct, and eggs are passed in the partly developed stage. Eggs may hatch and invade the birds through the skin or orally.

Amidostomum skrjabini. *A. skrjabini* infects ducks, geese, and pigeons. It is smaller and distinguished microscopically from *A. anseris* and *A. raillieta* (Figure 27.12). Eggs are laid partly developed.

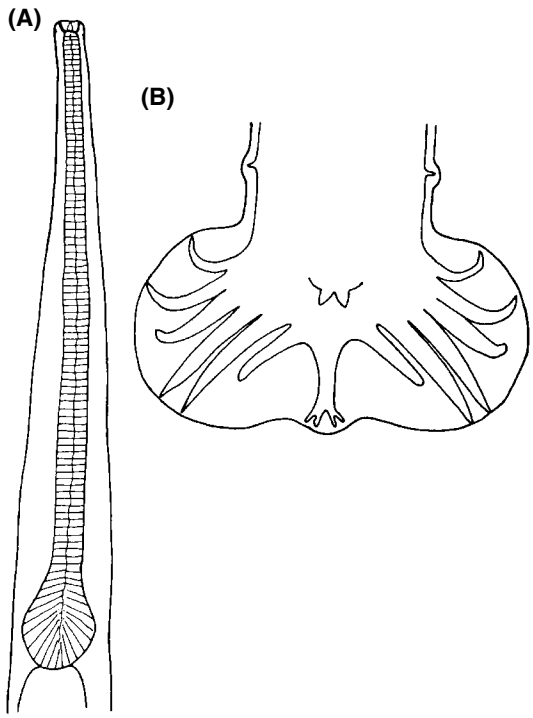


Figure 27.12 (A) Head, *Amidostomum raillieti*. (B) Male bursa, *Amidostomum skrjabini*. (After Boulenger)

Cheilospirura hamulosa. *C. hamulosa* (Figure 27.13) infects most gallinaceous birds. It uses grasshoppers, beetles, weevils, and sandhoppers as intermediate hosts. In heavy infections the wall of the gizzard may show small local lesions in the lining and muscular tissue, with soft nodules enclosing parasites in the muscular portion and sometimes causing severe damage.

Cheilospirura spinosa. The hosts of *C. spinosa* (Figure 27.14) are grouse, partridge, pheasant, quail, and wild turkey. Grasshoppers serve as the intermediate host. Heavy infections in quail are known to cause the gizzard lining to be hemorrhagic and necrotic, with marked proliferative changes in the gizzard wall.

Cyrnea colini. *C. colini* (Figure 27.15) is a proventricular worm of the turkey, grouse, prairie chicken, and quail in the United States which is similar in appearance to *C. hamulosa* but smaller and lacking cuticular adornments on the anterior of the body. The cockroach (*Blattella germanica*) serves as an intermediate host. No pathology has been reported.

Epomidiostomum uncinatum. *E. uncinatum* (Figure 27.16) is reported in ducks, geese, and pigeons (42).

Hadjelia truncate. *H. truncate* is similar in appearance to other gizzard worms; this parasite was recently reported as a clinical problem in meat pigeons in California (41). Infections were characterized by severe ventriculitis and emaciation.

Codiostomum struthonis

Codiostomum struthonis parasitizes the ceca and colon of the ostrich. The life cycle is unknown but is likely direct. Pathogenesis is not determined.

Cyathostoma bronchialis

Cyathostoma bronchialis is reported in ducks, geese, and turkeys in the United States. Chickens have been experimentally infected. A closely related species (*C. variegatum*) has been reported from the emu in the United States. The worm is found in the larynx, trachea, bronchi, and sometimes the abdominal air sacs, causing "gaping." This species is very similar to *Syngamus* but is larger and less firmly united *in copula* (Figure 27.17). The life cycle may be direct, or the worm may use an earthworm as a paratenic host. Infective larvae migrate to the lungs through the peritoneal cavity and air sacs (15). Mortality as high as 20% with high morbidity has been reported in domestic geese. The disease may last several months, with birds showing signs of respiratory distress. Morbidity and mortality also have been reported from Mandarin ducks (53). Recovering birds may be stunted.

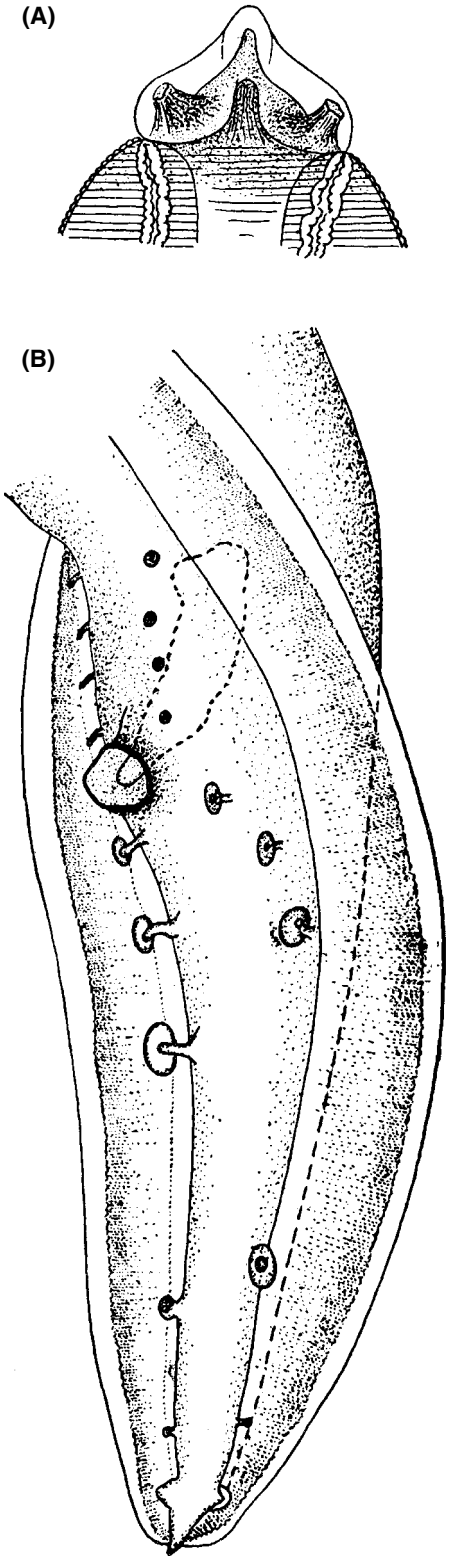


Figure 27.13 *Cheilospirura hamulosa*. (A) Head. (After Drasche) (B) Male tail. (After Cram)

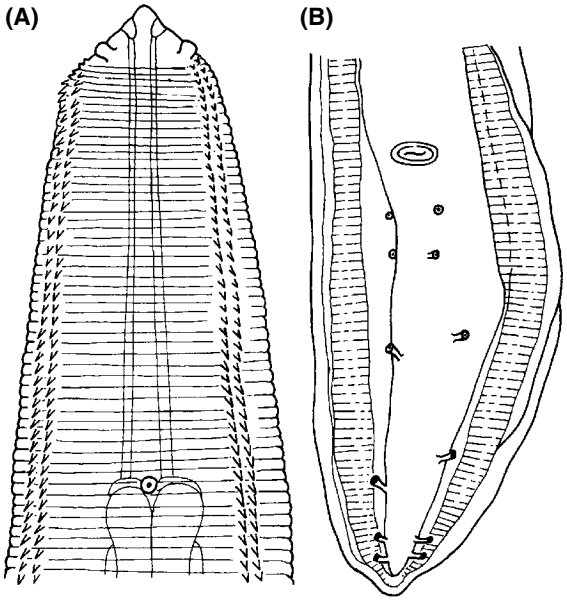


Figure 27.14 *Cheilospirura spinosa*. (A) Head. (B) Male tail. (After Cram)

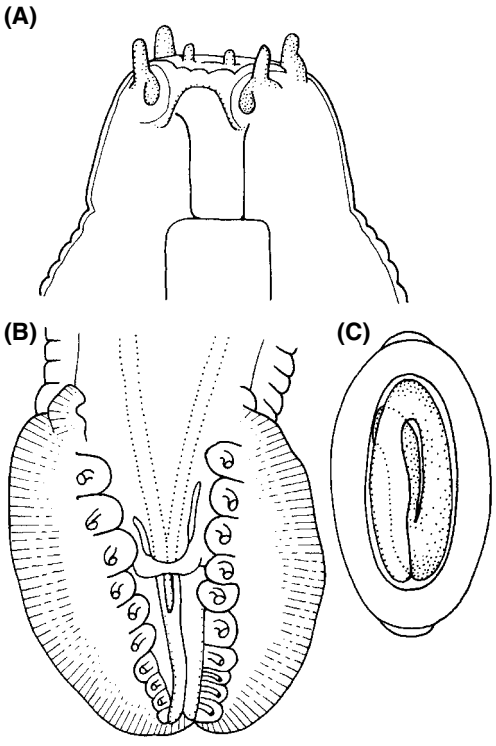


Figure 27.15 *Cyrnea colini*. (A) Head. (B) Male tail. (After Cram) (C) Egg.

Deletrocephalus dimidiatus

Deletrocephalus dimidiatus parasitizes the small and large intestines of the rhea. The life cycle is probably direct and causes anemia from consuming blood.

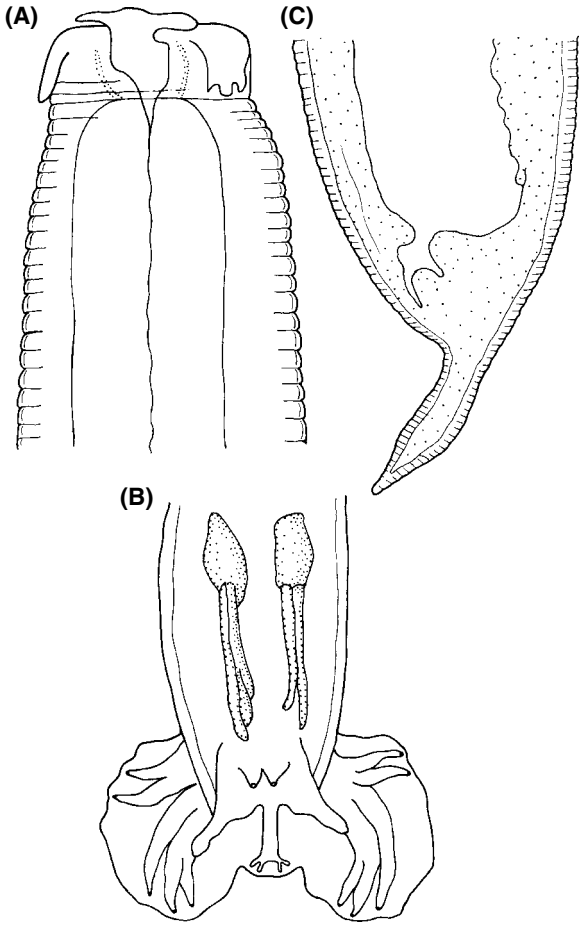


Figure 27.16 *Epomidiostomum uncinatum*. (A) Head. (B) Male tail. (C) Female tail. (After Skrjabin)

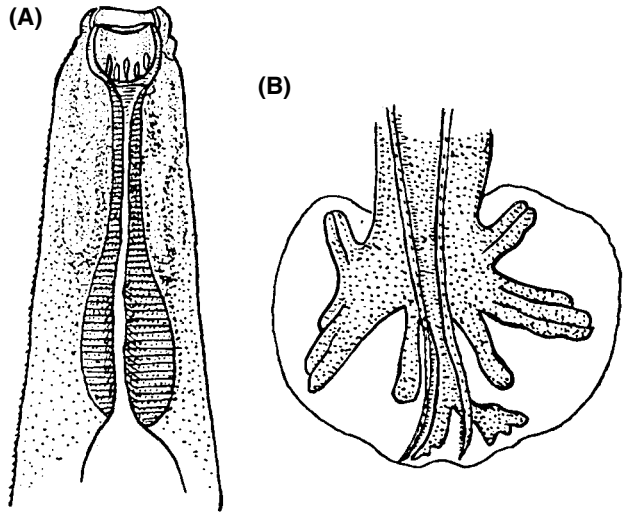


Figure 27.17 *Cyathostoma bronchialis*. (A) Head. (B) Male tail.

Dicheilonema rhea

Dicheilonema rhea is a filariid worm infecting the rhea. It is found in the abdominal and appendage fascia but causes no remarkable pathology. The life cycle is unknown.

Dispharynx nasuta

The nematode *D. nasuta* (Figure 27.18) parasitizes most galliform and passerine birds in the United States. It is found in the wall of the proventriculus, sometimes the esophagus, and occasionally the small intestine. It uses the pillbug (*Armadillidium vulgare*) and the sowbug (*Porcellio scaber*) as intermediate hosts. Heavy infections are known as grouse disease in the northeastern United States and may kill pigeons. Ulcers in the proventriculus and destruction of tissues are common in heavy infections.

Echinura uncinata

Echinura uncinata is reported from wild and domestic ducks and geese in Canada. This worm (Figure 27.19) invades the mucosa of the esophagus, proventriculus, gizzard, small intestine, and possibly the air sacs and can cause morbidity and mortality. It uses water fleas of the genus *Daphnia* as intermediate hosts.

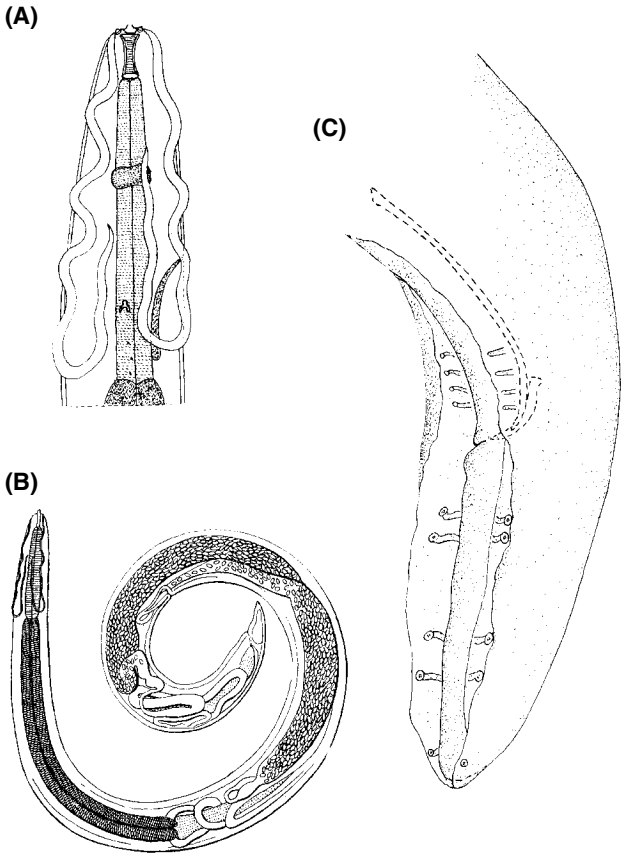


Figure 27.18 *Dispharynx nasuta*. (A) Head. (After Seurat) (B) Female. (After Piana) (C) Male tail. (After Cram)

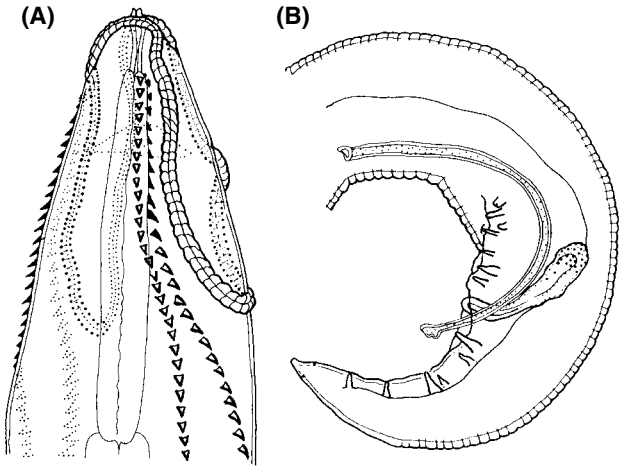


Figure 27.19 *Echinura uncinata*. (A) Head. (B) Male tail. (After Romanova)

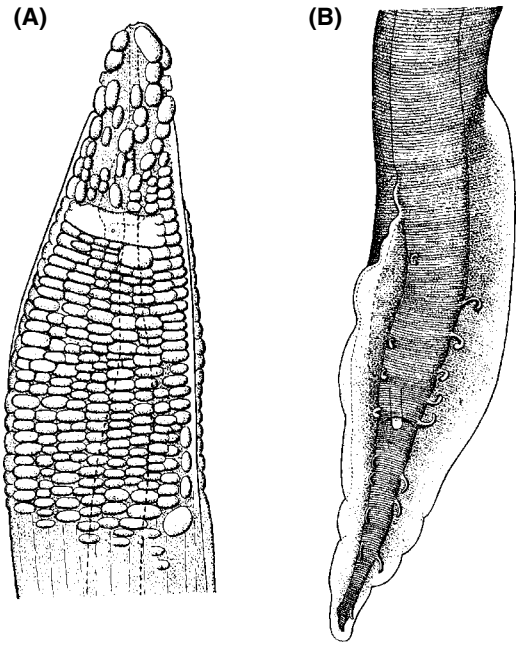


Figure 27.20 *Gongylonema ingluvicola*. (A) Head. (B) Male tail. (After Ransom)

Gongylonema ingluvicola

Gongylonema ingluvicola is reported in the chicken, turkey, partridge, pheasant, and Bobwhite quail. Adults (Figure 27.20A) are found in the mucosa of the crop, and sometimes in the esophagus and proventriculus. The beetle *Copris minutus* and cockroaches serve as intermediate hosts. This worm is of low pathogenicity, but burrows into the crop mucosa, causing white convoluted tracks.

Libyostrongylus douglassii

Libyostrongylus douglassii is found in ostriches, where it parasitizes the wall of the proventriculus and causes up

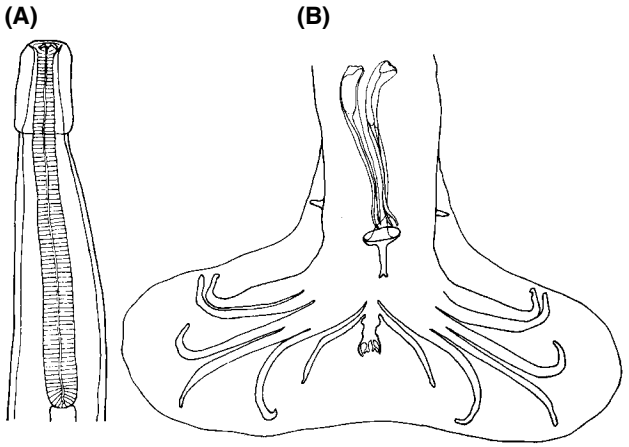


Figure 27.21 *Ornithostrongylus quadriradiatus*. (A) Head. (B) Bursa of male. (After Stevenson)

to 50% mortality. The life cycle is direct, and the eggs hatch on the ground where they remain infective for as long as 30 months.

Ornithostrongylus quadriadiatus

Ornithostrongylus quadriadiatus is found in the small intestine of pigeons and doves (Figure 27.21). These worms are delicate, slender worms, and red when freshly collected, apparently from ingested blood. The life cycle is direct; eggs hatch within 25 hours after voiding. Infective larvae are swallowed by a host and mature in the small intestine. The worm matures in 5–6 days. Pigeons may suffer catarrhal enteritis and blood loss. Birds may become moribund and anorexic and have pronounced greenish diarrhea. Intestines are markedly hemorrhagic and have a greenish mucoid content with masses of sloughed epithelium.

Oxyspirura mansoni

Oxyspirura mansoni, the eye worm, is reported from the chicken, turkey, duck, grouse, guinea fowl, peafowl, pigeon, quail, and a wide variety of passeriform birds (1) (Figure 27.22). It is located beneath the nictitating membrane and in the conjunctival sacs and nasolacrimal ducts. Eggs are swallowed and passed in the droppings where they are eaten by a cockroach (*Pycnoscelus surinamensis*). Upon ingestion of cockroaches, the infective larva migrates from the crop, up the esophagus to the mouth, and through the nasolacrimal duct to the eye. Severe opthalmia may develop, with white cheesy material collecting beneath the eyelids (40). Many wild birds also are host to this parasite.

Oxyspirura petrowi

Oxyspirura petrowi is reported from the grouse, pheasant, and prairie chicken. The life cycle and pathogenicity are similar to that of *O. mansoni*.

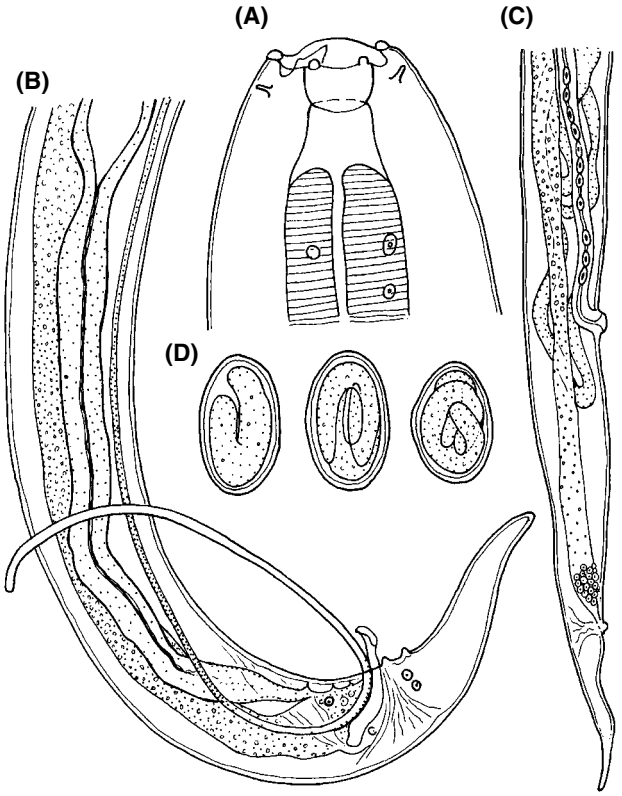


Figure 27.22 *Oxyspirura mansoni*. (A) Head. (B) Male tail. (C) Female tail. (D) Eggs. (B–D after Ransom)

Sicarius uncinipenis

Sicarius uncinipenis parasitizes the gizzard of the rhea.

Sicarius waltoni

Sicarius waltoni parasitizes the gizzard of the rhea.

Singhfilaria hayesi

The hosts of *S. hayesi* are turkeys and quail, where it infects the subcutaneous tissues around the esophagus, crop, and trachea (Figure 27.23). It is of low pathogenicity and the life cycle is unknown.

Strongloides avium

Strongloides avium infects quail, grouse, turkeys, chickens, and geese (Figure 27.24). It also has been reported from the junco and the coot and in chickens in Puerto Rico (6, 29). It is found in the ceca or sometimes in the intestine. In the intestine of the avian host, the population consists of only parthenogenetic females. Eggs hatch in the soil soon after being passed. Larvae may develop into free-living males and females or into infective larvae. Infection takes place when larvae are swallowed by a host or if the larvae penetrate the skin of the host. Larvae of this genus are known to penetrate the skin of humans, resulting in cutaneous eruptions, which is of some medical concern. In heavy infections the walls of the ceca are thickened and bloody. It may cause mortality.

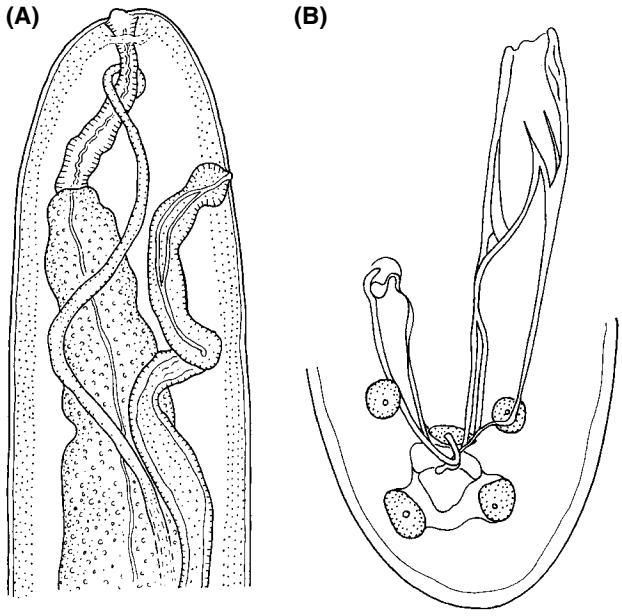


Figure 27.23 *Singhfilaria hayesi*. (A) Head. (B) Male tail. (After Anderson and Prestwood)

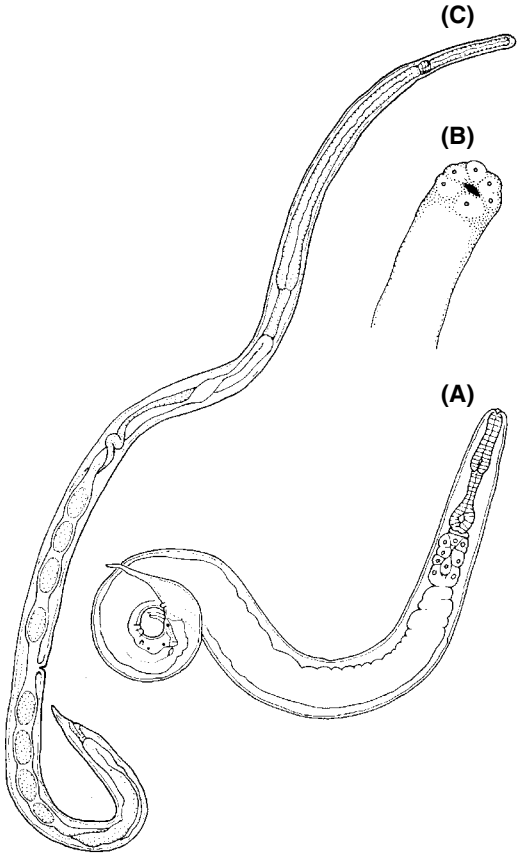


Figure 27.24 *Strongyloides avium*. (A) Free-living male. (After Cram) (B) Head, parasitic female. (C) Parasitic (parthenogenetic) female. (B and C after Sakamoto and Sarashina. 1968. *Jpn J Vet Res*.16:44–47)

Genus *Subulura*

The genus *Subulura* is known to parasitize a wide variety of galliform birds and even ducks. These are largely cecal worms

Subulura brumpti. *S. brumpti* parasitizes most gallinaceous birds and ducks. They are small worms which can be separated from *H. gallinarum* by microscopy. The eggs are almost spherical (82–86 × 66–76 µm), and fully embryonated when laid.

Subulura strongylina. *S. strongylina* is reported from chickens, guinea fowl and quail, and is identified microscopically by tail morphology (Figure 27.8C).

Subulura suctoria

S. suctoria is reported from several gallinaceous birds (Figure 27.8B). They are larger than *S. brumpti, with* eggs measuring 51–70 × 45–64 µm.

Syngamus trachea

Syngamus trachea is reported from the chicken, turkey, goose, guinea fowl, pheasant, peafowl, emu, and quail. Known commonly as gapeworms, these worms are found in the trachea and cause labored breathing (Figure 27.25). They are also called redworms because of their prominent color and forked worms because the male and female are always locked in copulation to form a "Y" (Figure 27.26). Lesions or nodules result

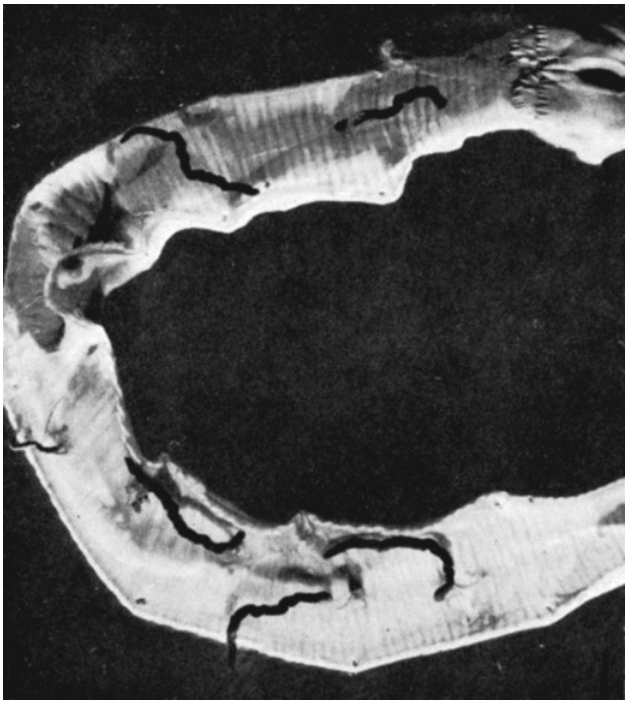


Figure 27.25 *Syngamus trachea*. Trachea showing attached gapeworms. (After Wehr)

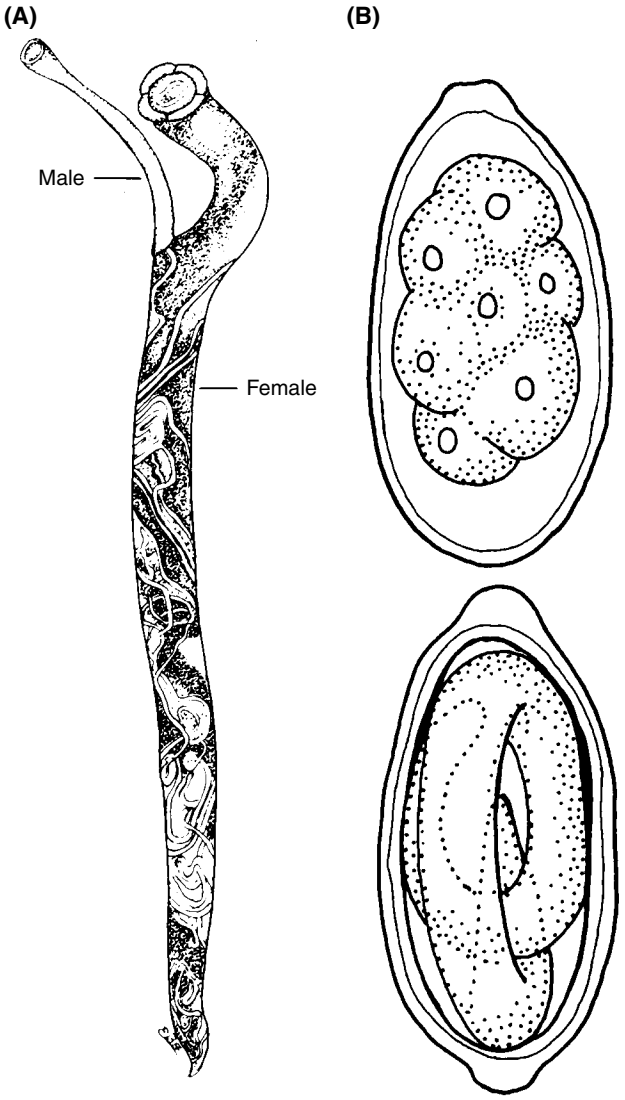


Figure 27.26 *Syngamus trachea*. (A) Male and female worms. (After Wehr) (B) Egg.

from inflammatory reactions at the site of attachment of male worms. This parasite is common in poultry or game birds reared in outdoor pens where birds have access to the intermediate hosts. Females apparently attach and reattach. The life cycle may be direct, by ingestion of embryonating eggs or infective larvae, or may involve an intermediate host. The earthworms *Eisenia foetidus* and *Allolobophora caliginosus* are known to become infected, with the larvae encysting in the body musculature. Encysted larvae are known to remain infective for up to 4 years. Slugs and snails also may serve as a transfer host. When ingested, infective larvae penetrate the wall of the crop and esophagus and migrate to the lungs, or penetrate the duodenum to be carried to the lungs by the portal bloodstream. Young turkey poults, chicks, and pheasant chicks are most susceptible and may suffocate from the inflammation and obstruction by worms. Some birds also may suffer marked changes in the blood (22).

Genus *Tetrameres*

Worms of the genus *Tetrameres* are parasites of the upper digestive tract, mostly the proventriculus. These relatively pathogenic nematodes use an intermediate host and are not seen in poultry reared indoors. They are easily recognized by the bright red color and distinctive globular shape of the female. The host range includes gallinaceous birds and waterfowl.

Tetrameres americana. *T. americana* (Figure 27.27) is reported from the chicken, turkey, duck, grouse, pigeon, and Bobwhite quail. At necropsy these bright red worms can be seen through the wall of the unopened proventriculus. Grasshoppers (*Melanoplus femurrubrum* and *M. differentialis*) or cockroaches (*Blatella germanica*) serve as intermediate hosts. These worms are easily recognized because of the extreme sexual dimorphism and the red color (Figure 27.28); the female is globular (Figure 27.28B), whereas the male (Figure 27.28A) is elongate like other nematodes. Eggs are 42–50 × 24 μm, and embryonating when laid. Chickens become emaciated and anemic, although quail are affected less (8). In chickens, the wall of the proventriculus may be thickened to the point of blocking the lumen. *T. Americana* has been reported from wild (13) and laboratory-raised pigeons (16).

Tetrameres crami. The hosts of *T. crami* are wild and domestic ducks, where they are found in the proventriculus. Intermediate hosts include the amphipods *Gammarus*

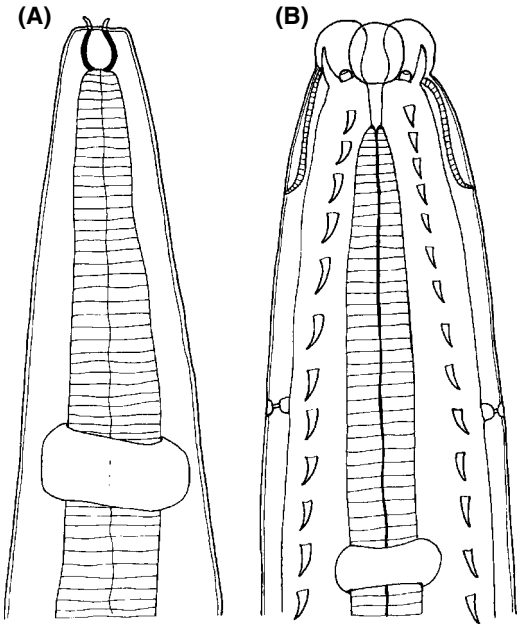


Figure 27.27 (A) *Tetrameres americana*, head. (Courtesy Graybill) (B) *Tetrameres fissispina*, head. (Travassos)

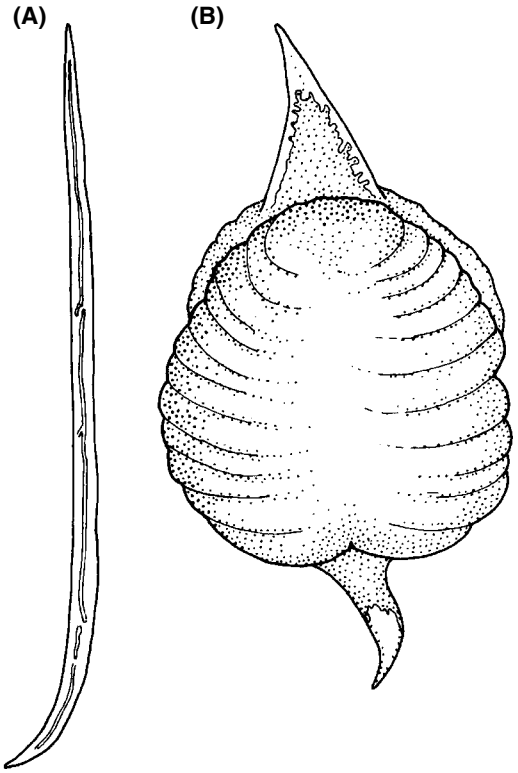


Figure 27.28 *Tetrameres americana*. (A) Male. (B) Female. (After Cram)

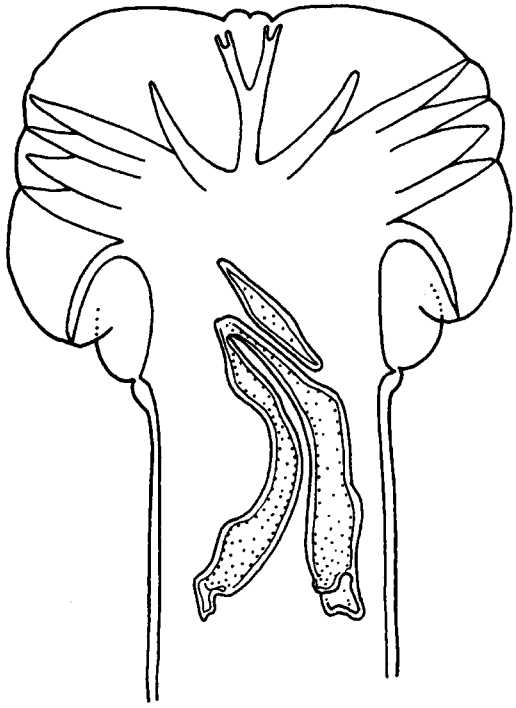


Figure 27.29 *Trichostrongylus tenuis*. Bursa of male. (After Railliet)

fasciatus and *Hyalella knickerbocki* (44). This worm is smaller than *T. Americana*.

Tetrameres fissispina. *T. fissispina* is reported from the chicken, turkey, duck, guinea fowl, goose, pigeon, and quail. It is common in ducks and geese and rare in other poultry. Intermediate hosts include amphipods, grasshoppers, earthworms, and cockroaches. Fish may serve as a transport host. Considerable tissue reaction occurs, with degeneration of the glandular tissue, edema, and extensive leukocyte infiltration (46).

Tetrameres pattersoni. *T. pattersoni* is found in the proventriculus of the Bobwhite quail and uses grasshoppers (*Melanoplus femurrubrum* or *Chortophaga viridifasciata*) or cockroaches (*Blatella germanica*) as intermediate hosts. Worms can be so numerous that the wall of the proventriculus is destroyed and death may result.

Trichostrongylus tenuis

Trichostrongylus tenuis is a parasite reported from most galliform birds, waterfowl, pigeons, and emu. These small, slender worms are found in the ceca and occasionally the intestine (Figure 27.29). These worms are highly pathogenic and were associated with the decline of the red grouse population in Scotland. Administration of as few as 500 larvae can cause mortality. The ceca become distended and congested, the mucosa inflamed and thickened. Heavy mortality occurs mainly in the fall in young birds from that year's hatch, and again in the spring. With emus, a bloody, mucoid diarrhea may be extensive. The life cycle is direct.

Vaznema zschokkei

Vaznema zschokkei parasitizes the proventriculus of the rhea. These are 16–23 mm in length.

Treatment and Control of Nematodes

Nematode control measures include sanitation, interruption of the life cycle, and use of anthelminthic products. Modern poultry practices such as confinement rearing of broilers and pullets and caging of laying hens have significantly decreased the variety of nematodes and generally the levels of infection. An increasing trend toward free-range rearing may lead to greater variety of worm species and increased risk, especially because these operations often dispense with the use of parasiticides and other drugs. This trend already has been recognized in the European Union (12, 33). Poultry such

as broiler breeder replacements, which are often raised in earthen-floored houses with restricted feeding, are likely to harbor the direct-life cycle intestinal worms *A. galli, C. obsignata*, and *H. gallinarum*. It is commonly thought that all broiler breeder farms are contaminated with these worms. Pen rearing of game birds allows parasitic worms to flourish, requiring a regular program of chemical treatment.

The life cycle of parasites is interrupted by confining the birds to keep them from contacting and consuming the intermediate host. Treating the soil with disinfectants is thought to be beneficial, but there is a dearth of published scientific evaluations of such programs. For small flocks, it is worthwhile to keep the birds off the ground with wire screen floors to reduce contact with worm eggs in the droppings.

Raising different species or ages of birds together in the same pens or in close proximity creates more opportunity for some parasites to prosper. For instance, turkeys should not be raised with chickens or any other birds likely to harbor the cecal worm (*H. gallinarum*) because they are likely to carry *H. meleagridis*, the agent of blackhead disease. Similarly, the chukar partridge is more susceptible to blackhead disease than the ring-necked pheasant, which also is a good host for cecal worms.

Chemotherapy

Only a few compounds have been tested for efficacy against roundworms in poultry, and only against the common worms *A. galli, H. gallinarum*, and *C. obsignata*. Some of the older drugs are not recommended because there is lack of sufficient efficacy or other problems associated with their use. For instance, piperazine, the only product approved for use in chickens, was tested at a wide range of dosages and found to be largely ineffective (19, 48). Haloxon, an organic phosphate, has toxicity issues in sheep and goats and is not commonly available in the United States. Other older drugs that are not commonly used include phenothiazine and santonin (extract of wormwood gall still available at natural remedy stores).

The benzimidazoles, represented by several products, are highly effective. Fenbendazole, approved for use in growing turkeys, is highly effective when given in the feed at 16 ppm for 6 days (5–10 mg/kg body weight). Fenbendazole was more than 98% effective in removal of adult and larval turkey ascarids (28, 51). Albendazole, cambendazole, levamisole (L-tramisole), and other compounds in this family are also highly effective (4, 5, 11, 32, 34). Some are available in a water-soluble formulation. Pyrantel pamoate was not effective in the treatment of *A. galli* in chickens (48).

Regular programs of worming are recommended for poultry that are to be kept for more than a few weeks. Turkeys, broiler breeder replacements, layer replacements, heavy broilers (roasters), and most game birds are reared for 8–20 weeks on litter, providing ample opportunity for worms to mature and contaminate the premises with more worm eggs. Of the worms found in poultry, *C. obsignata* is the most pathogenic. Aside from the potential damage caused by the worm itself, the main concern is that *H. gallinarum* carries the protozoan responsible for blackhead disease (*H. meleagridis*). Outbreaks are common in many types of poultry. For many years, blackhead disease was considered a minor nuisance in chickens. However, it is not uncommon for blackhead disease to cause 10% mortality, increased culling, and nonuniformity in breeder or layer flocks. Outbreaks may be seen at 8–10 weeks, but producers report problem farms where outbreaks occur at 4 weeks of age. Because there is no treatment for blackhead disease, it is important to remove the carriers by early and frequent deworming with a bendimidazole type dewormer, capable of killing the larvae. Deworming of turkeys is usually practiced on a regular basis but is of little value in preventing outbreaks of blackhead disease because of its easy lateral transmission in turkey flocks after introduction of infection from outside (20, 21).

Acanthocephalans in Poultry

The acanthocephalans (thorny-headed worms) resemble nematodes or other worms but many differences are apparent. They are named for the spiny proboscis at the anterior end, which bears a number of recurved hooks arranged in rows. The number, form, and arrangement of the hooks are used in species identification. Like tapeworms, this group of worms has no digestive tract, and absorbs nutrients directly through the body wall. The sexes are separate; the male is smaller than the female and often distinguished externally by a bell-shaped bursa surrounding the genital pore. Various arthropods, snakes, lizards, and amphibians serve as hosts of the larval stages (42). These worms are known from a wide variety of vertebrates but are uncommon in poultry. All known species of Acanthocephala require 1 or more intermediate hosts, a fact that limits the importance of this group in poultry reared indoors where the variety of invertebrates is limited. Only 4 species of Acanthocephala have been reported as parasites of domestic poultry in North America. Three of these were reported from immature forms and could have resulted from accidental infections.

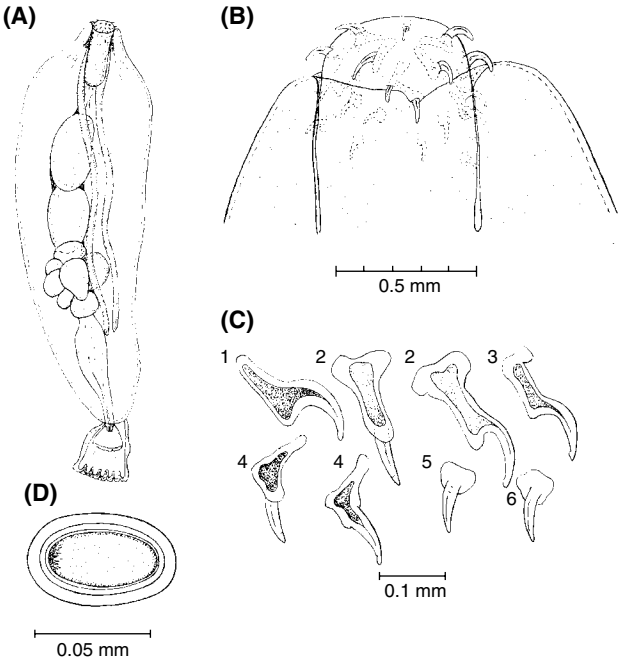


Figure 27.30 *Oncicola canis*. (A) Male showing reproductive organs. (B) Proboscis. (C) Hooks from proboscis (numerals indicate row). (D) Egg. (Price)

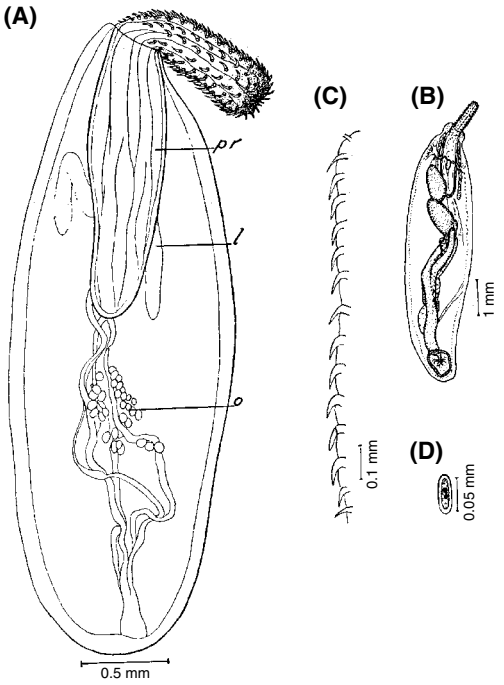


Figure 27.31 *Prosthorhynchus formosus*. (A) Young female (l, lemniscus; o, ovary; pr, proboscis receptacle). (Jones) (B) Male. (C) Hooks from proboscis. (D) Egg. (VanCleave)

Onicola canis

The *O. canis* (Figure 27.30) is reported from turkeys in Texas. Larval worms were found encysted under the epithelial lining of the esophagus, in some birds more than 100/bird, and were considered as possibly causing death. The adults normally occur in the dog and coyote.

Prosthoryhnchus formosus

Immature specimens of *P. formosus* (Figure 27.31) were reported from chickens at necropsy in New Jersey. Other bird hosts include the flicker and the robin (39).

Polymorphus boschadis

The *P. boschadis* (Figure 27.32) has been reported from ducks in Canada. It causes morbidity and mortality in domestic waterfowl, especially in young birds. It causes inflammation of the intestine, anemia, and cachexia (general ill health). Affected birds develop staggering gait and drooping head and wings.

Other Acanthocephalans in Poultry

Several other spiny-headed worms reported in other regions include: *Leiperacanthus gallinarum, Mediorhynchus gallinarum*, and *Neoschongastia gallinarum*

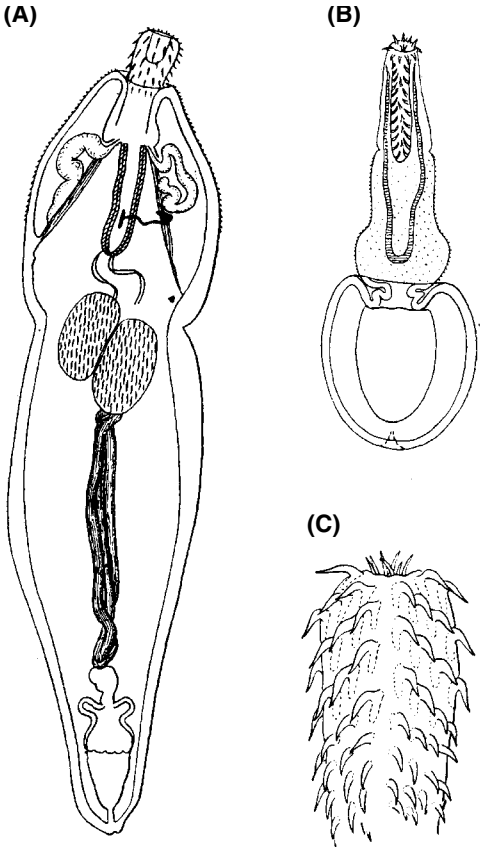


Figure 27.32 *Polymorphus boschadis*. (A) Male. (B) Larva from *Gamarus pulex*. (C) Proboscis of larva. (Luhe)

in Asia; *Macracanthorhynchus hirudinaceus* in chickens in Brazil; *Prosthorhynchus rhea* in South America; and *Prosthorhynchus transversus* in passerine birds, partridge, and pheasant in Europe.

Public Health Significance

None of the helminths discussed in this section pose a threat to public health. It is reportedly possible for larvae of *Strongyloides avium* to cause a creeping cutaneous eruption in accidental infections (larva currens), but the lesions are minor and leave no lasting pathology. There is no recent literature on this subject.

Acknowledgment

I acknowledge the contribution of previous authors of this chapter, particularly Thomas Yazwinski and Christopher Tucker, for their contributions to earlier versions of this work.

Cestodes and Trematodes

Introduction

Many species of worm parasites are seen during necropsy examination of the digestive tract or other internal organs of poultry. Some of these are large enough to cause intestinal blockage in severe cases. Others are so small that a hand lens may be required to distinguish them from intestinal contents. If flattened in shape, they are probably flatworms belonging to the phylum Platyhelminthes. Tapeworms are in the class Cestoda, and flukes are in the class Trematoda. Accurate identification is essential for effective control. Species identification may give direction to control measures aimed at eliminating the intermediate host, thus breaking the life cycle. Others may require treatment with anthelmintics.

Cestodes

Most birds are hosts to some species of cestodes or tapeworms (phylum Platyhelminthes/class Cestoda). A high percentage of chickens or turkeys may be infected with tapeworms if they are reared on a range or in backyard flocks. These parasites are found more frequently in warmer seasons, when intermediate hosts are abundant. Many species of tapeworms are now considered rare in intensive poultry-rearing regions because the birds do not come in contact with intermediate hosts. Beetles and houseflies inhabiting poultry houses still act as intermediate hosts for the 2 large chicken tapeworms known only by the scientific names *Raillietina cesticillus* and *Choanotaenia infundibulum*.

Some infections of the larger tapeworms may appear to block completely the intestine of an infected bird, but mortality from cestodiasis or long-term effects are rare. Different species vary considerably in pathogenicity, so species identification is worthwhile.

Diagnosticians are often satisfied with a diagnosis of cestodiasis or taeniasis without making further attempts at identification. However, prevention and control strategies may vary with each species of tapeworm. Only after the species has been determined can an assessment of flock damage and possible control measures be considered (Table 27.5). For identification of the less common species, specialized textbooks may be needed to supplement the keys and illustrations included in this text (8, 11, 15, 16).

Tapeworms or cestodes are flattened, ribbon-shaped, usually segmented worms. The term proglottid is used to describe these individual segments because the latter term is defined otherwise by classic zoologists (Figure 27.33). One to several gravid proglottids are shed daily from the posterior end of the worm. Each proglottid contains 1 or more sets of reproductive organs which may become crowded with a mass of eggs as the maturing proglottid becomes a gravid proglottid.

Tapeworms are characterized by complete absence of a digestive tract and obtain their nourishment by absorption from the gut contents of the host. Although the duodenum, jejunum, or ileum is the usual site for attachment, 1 species (*Hymenolepis megalops*) from ducks is found in the cloaca or cloacal bursa. Birds become infected by eating an intermediate host, thus allowing the larval stage of the tapeworm access to the intestine. This larval tapeworm is known as a cysticercoid (Figure 27.34C). The intermediate host may be an insect, crustacean, earthworm, slug, snail, or leech depending upon the species of tapeworm.

Most cestodes are host specific for a single or a few closely related birds. Identification of the parasite to genus and species helps pinpoint the intermediate host. The diagnostician then may be able to suggest practical control measures. Completion of a 2-host life cycle depends upon a unique set of ecologic conditions which juxtapose the host and the intermediate host.

Table 27.5 Tapeworms and hosts from poultry in the United States.

Tapeworm	Definitive Hosts (Occasional Hosts)	Intermediate Hosts	Degree of Pathogenicity
Amoebotaenia cuneata	Chicken (turkey)	Earthworm	Mild
Choanotaenia infundibulum	Chicken (turkey)	Housefly, beetle	Moderate
Davainea proglottina	Chicken	Slug, snail	Severe
Hymenolepis carioca	Chicken (turkey, bobwhite quail)	Stable fly, dung beetle	Unknown
H. cantaniana	Chicken (turkey, peafowl, Bobwhite quail)	Beetle	Mild or harmless
Raillietina cesticillus	Chicken (turkey, guinea fowl, Bobwhite quail)	Beetle	Mild or harmless
R. tetragona	Chicken (guinea fowl, peafowl, Bobwhite quail, turkey)	Ant	Moderate to severe
R. echinobothrida	Chicken (turkey)	Ant	Moderate to severe
R. magninumida	Guinea fowl (chicken, turkey)	Beetle	Unknown
Davainea meleagridis	Turkey	Unknown	Unknown
Drepanidotaenia watsoni	Wild turkey	Unknown	Unknown
Imparmargo baileyi	Wild turkey	Unknown	Unknown
Raillietina georgiensis	Wild turkey (domestic turkey)	Ant	Unknown
R. ransomi	Wild turkey	Unknown	Unknown
R. williamsi	Wild turkey	Unknown	Unknown
Metroliasthes lucida	Turkey (guinea fowl, chicken)	Grasshopper	Unknown
Diorchis nyrocae	Wild and domestic duck	Copepod crustacean	Unknown
Fimbriaria fasciolaris	Duck (chicken)	Copepod crustacean	Unknown
Hymenolepis anatina	Wild and domestic duck	Freshwater crustacean	Severe
H. compressa	Duck, goose	Unknown	Unknown
H. collaris	Wild and domestic duck (chicken)	Freshwater crustacean (snail = auxiliary)	Unknown
H. coronula	Duck	Crustacean, snail	Unknown
H. lanceolata	Goose, duck	Crustacean	Severe
H. megalops	Duck	Unknown	Unknown
H. parvula	Wild and domestic duck	Leech	Unknown

Thus, minor changes in flock management may cause a break in the life cycle and comprise an effective control measure.

History, Incidence, and Distribution

More than 4,000 species of tapeworms have been described from animals (14), with many of the earlier species bearing the genus name *Taenia*. Because no poultry tapeworms are currently listed in this genus, the term taeniasis is no longer appropriate, and the term cestodiasis would be a better substitute for infection with poultry tapeworms. Slender threadlike forms (*Hymenolepis carioca*) may require some magnification to distinguish individual proglottids, thus indicating that they are tapeworms. Some small forms (e.g., *Davainea proglottina*) are almost microscopic.

Classification

More than 1,400 species of tapeworms have been described from wild and domestic birds. Because most of them have no common name, they are best recognized by their genus and species names.

Three families (Davainidae, Dilepididae, and Hymenolepidae) and 10 genera (*Amoebotaenia, Choanotaenia, Davainea, Diorchis, Drepanidotaenia, Imparmargo, Metroliasthes, Raillietina, Hymenolepis, Fimbriaria*) are recognized here because they may appear in birds brought to diagnostic laboratories in the United States.

Morphology and Life Cycles

Adults. The anatomic features needed to identify poultry tapeworms are illustrated by describing *Davainea proglottina* (Figure 27.33). This species differs

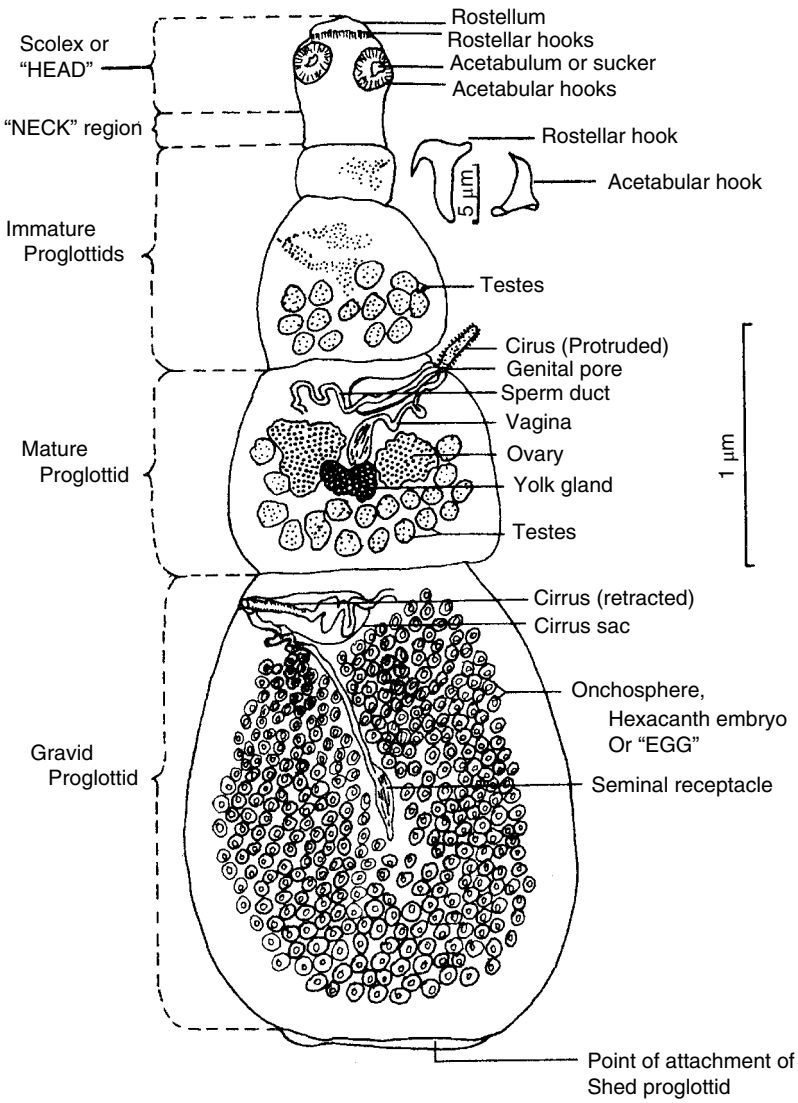


Figure 27.33 Adult tapeworm (*Davainea proglottina*). Although readily seen with the naked eye, this species has been called a microscopic tapeworm because it is small and often overlooked.

from most other tapeworms in possessing only 1 or 2 each of immature, mature, and gravid proglottids compared with dozens or hundreds in other species. The entire connected chain of proglottids is called a strobila. Besides the strobila, 2 other regions, the scolex and the neck, are recognized. Anchorage is accomplished by the scolex with the assistance of 4 pairs of suckers or acetabula, which may possess 1 or 2 rows of acetabular hooks. If hooks are present, the species is described as armed; if absent, it is unarmed. A plunger-shaped organ known as the rostellum is frequently present at the anterior end. The rostellum may assist in anchorage by means of 1 or 2 rows of rostellar hooks and by the suction created by partial withdrawal of the rostellum into the scolex. The neck is an undifferentiated area between the scolex and the strobila from which new proglottids proliferate.

A set of both male and female reproductive organs are found in each proglottid. Morphologic differences in size and location of these organs are used in taxonomic descriptions of different species. Older gravid proglottids containing numerous eggs are shed individually or in short chains late in the day after the worm has absorbed and stored nutrients from the gut contents of the host. *D. proglottina* generally sheds 1 gravid proglottid/day, and *R. cesticillus* may produce as many as 10–12.

Onchosphere. Within the uterus, the fertilized egg develops into a multicellular embryo called an onchosphere or hexacanth embryo. The onchosphere is a multicellular larva containing penetration glands and numerous muscular attachments to activate the hooks. Each gravid proglottid may contain several hundred of these multicellular embryos or eggs. Distinctive membranes (Figure 27.34A) surrounding the eggs may be useful in identifying the species.

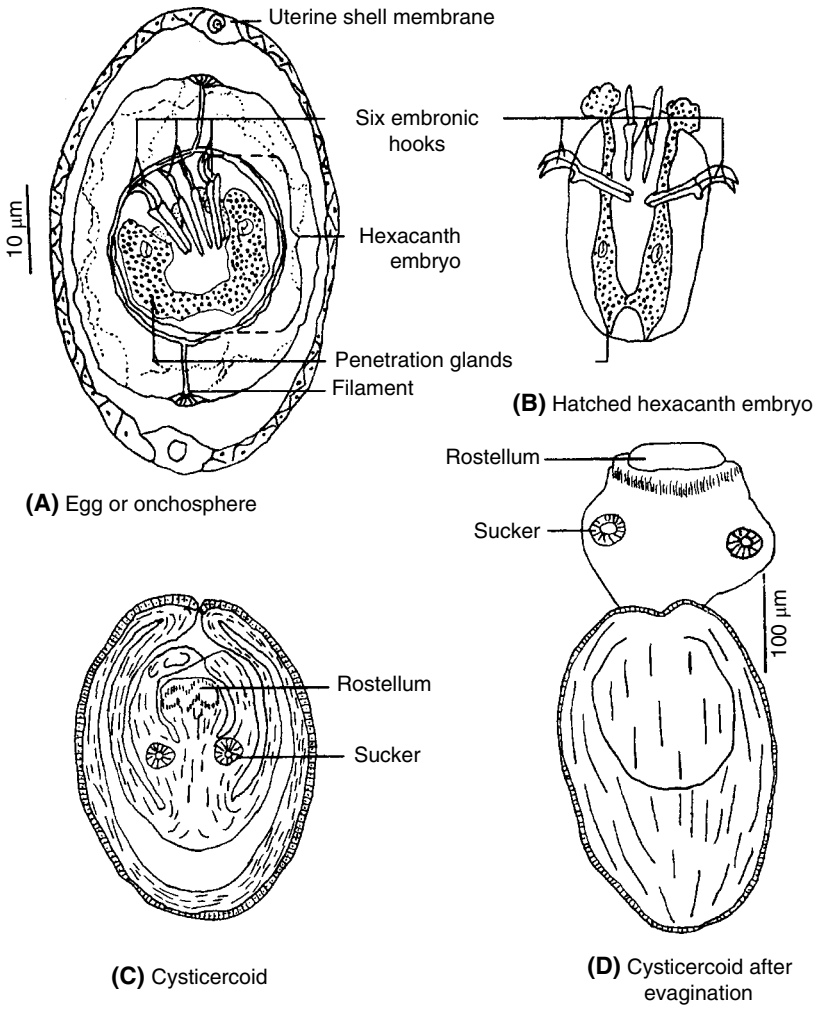


Figure 27.34 Larval stages of the chicken tapeworm (*Raillietina cesticillus*). (A) The egg is encapsulated by a membrane derived from the uterus wall. Eggs are occasionally found free in feces, but more often enclosed within a gravid proglottid. (B) Hexacanth embryos escape from shell membranes; active hooks and enzymes from secretory glands assist in penetration of gut wall of the beetle intermediate host. (C) Cysticercoid that has developed in the hemocoele of a beetle. (D) Scolex in the cysticercoid has evaginated after exposure to bile and enzymes in gut of the fowl.

Cysticercoid. Intermediate hosts such as beetles, houseflies, slugs, or snails become infected by swallowing individual eggs from the feces, or they devour the entire proglottid after being attracted by odor or movement. The 6-hooked embryo hatches from the egg in the gut of the intermediate host and penetrates the gut wall. The larva reorganizes and changes in polarity to become a cysticercoid in about 2 weeks (Figure 27.34C,D). The cysticercoid remains within the body cavity of the intermediate host until the latter is eaten by the bird host. In the digestive tract the cysticercoid is activated by bile and attaches to the intestine to begin the formation of a strobila. The first gravid proglottids appear in the feces 2–3 weeks after the cysticercoid is swallowed.

Diagnosis and Identification

Distinctive characteristics of tapeworms may best be demonstrated by examining (1) the scolex (Figures 27.33 and 27.35), (2) the eggs (Figures 27.34 and 27.37), or (3) individual proglottids of recently shed and whole live specimens (Figures 27.33 and 27.36) (11). Although differential staining can be used to show the internal organs of mature proglottids, this procedure is too slow for most diagnostic laboratories. Preservation in alcohol or formalin, although required before staining, often obscures useful characteristics needed for rapid identification. The intestine is best opened with scissors under water, thus permitting the strobila to float free, revealing the area to which the scolex is attached. Recovery of the scolex is worth considerable effort because its characteristics alone may indicate the species. Freeing the scolex may be accomplished by (1) teasing apart the mucosa with 2 dissecting needles, (2) cutting a deep gouge into the mucosa under the attachment point with a sharp scalpel, or (3) leaving the intestine submerged in saline for a few hours in the refrigerator. Wetmount preparations of the scolex examined under a coverglass with 3,100 or higher magnification may reveal sufficient characteristics to make a species identification. Hook characteristics may require measurement with an ocular micrometer under higher magnification. Semipermanent cleared preparations of scolices may be made by using a drop of Hoyer's solution (prepared by adding to 50 mL of

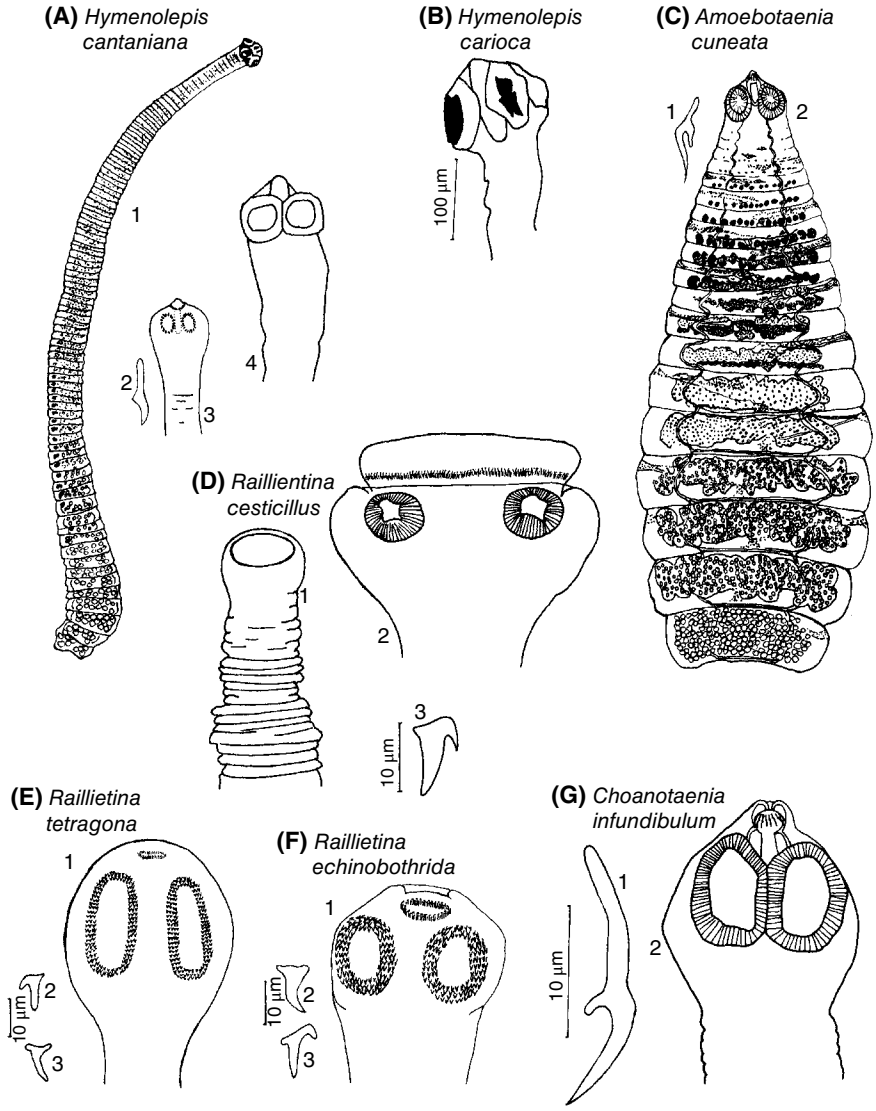


Figure 27.35 Tapeworms of chickens. Scolex characteristics. (A) *Hymenolepis cantaniana*: (1) scolex and strobilia (Ransom); (2) hook (Yamaguti); (3) scolex (Neveu-Lemaire); (4) scolex (Wehr). (B) *H. carioca* scolex. (C) *Amoebotaenia cuneata* (Monnig): (1) rostellar hook; (2) entire worm. (D) *Raillietina cesticillus*: (1) scolex (Ackert); (2) scolex (Monnig); (3) rostellar hook (Ransom). (E) *R. tetragona*: (1) scolex (Monnig); (2,3) rostellar and acetabular hooks (Ransom). (F) *R. echinobothrida*: (1) scolex (Monnig); (2,3) rostellar and acetabular hooks (Ransom). (G) *Choanotaenia* infundibulum: (1) hook (Ransom); (2) scolex (Monnig).

distilled water the following ingredients in this order: 30 g gum arabic flakes, 200 g chloral hydrate, and 20 g glycerin). Distinctive egg characteristics may be demonstrated by teasing apart a gravid proglottid under a coverglass (Figure 27.37). Wet preparations of mature or gravid proglottids under low magnification may reveal diagnostic characteristics such as the location, size, and shape of the cirrus pouch and the location of the genital pore and the gonads. If further details of the internal structure of the proglottid are required for identification, it may be necessary to kill, fix, stain, destain, dehydrate, and permanently mount the specimen (1).

Tapeworms of Chickens

A dichotomous key is given to the 8 species of tapeworms commonly found in chickens from the continental United States. In such keys, successive selections must be made between 1a and 1b, 2a and 2b, etc., until a species name is designated. After viewing a portion of the worm under the microscope, make a comparison of the appropriate figures organized under scolices (Figure 27.35), eggs (Figure 27.37), or proglottids (Figure 27.36). With rare species, additional descriptions from other texts may be required (16).

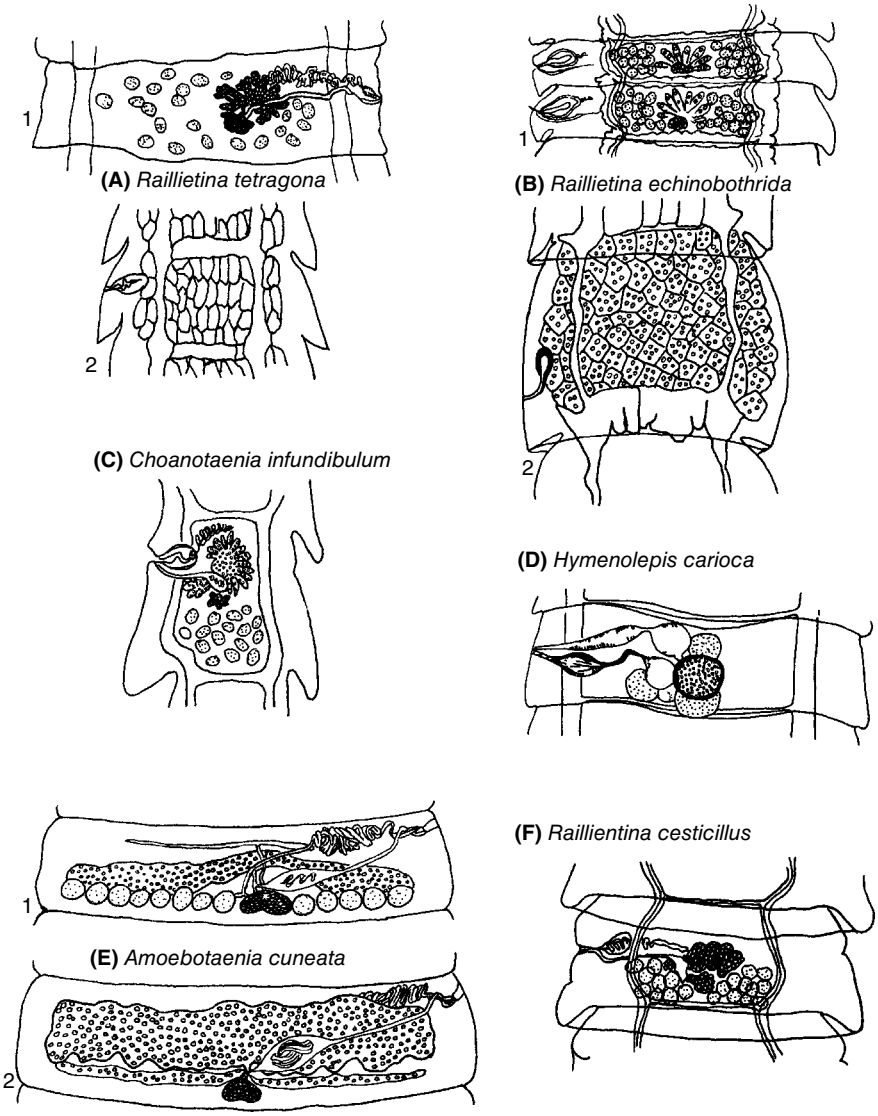


Figure 27.36 Mature and gravid proglottids of chicken tapeworms. (A) *Raillietina tetragona*: (1) mature proglottid (Ransom); (2) gravid proglottid showing egg capsules (Neveu-Lemaire). (B) *R. echinobothrida*: (1) mature proglottid (Fuhrmann); (2) gravid proglottid (Lang). (C) *Choanotaenia infundibulum* (Fuhrmann). (D) *Hymenolepis carioca* (Sawada). (E) *Amoebotaenia cuneata*: (1) mature proglottid; (2) gravid proglottid filled with eggs (Fuhrmann). (F) *Raillietina cesticillus*: mature proglottid (Monnig).

Key to Species

1a. Minute forms, less than 1 cm long. A very limited number of proglottids with the terminal proglottid being gravid with egg 2
1b. Longer than 1 cm 3
2a. Wedge-shaped worm. Contains about 20 proglottids. Posterior proglottids wide, short (Figures 27.35C, 27.36E, and 27.37) ..... *Amoebotaenia cuneata*
2b. Contains only 2–5 proglottids, rarely 9. Posterior proglottids as long as wide (Figure 27.33) ..... *Davainea proglottina*
3a. Threadlike, never more than 1.5 mm wide; fragile scolex is usually lost; often more than 100 worms in a single bird; proglottids short and wide, genus *Hymenolepis* 4
3b. Robust worms, gravid proglottids wider than 2 mm 5
4a. Mature worms with gravid proglottids present, less than 12 mm long (Figure 27.35A) ..... H. cantaniana
4b. Mature specimens with a total length including gravid proglottids of more than 12 mm (Figures 27.35B and 27.36D) ..... *H. carioca*
5a. 5–12 embryos enclosed in single capsule; verify by opening terminal proglottid; view under a coverglass (Figure 27.37F) 6
5b. Embryos in single egg capsules enclosed in distinct membranes (examine under high power) 7

6a. Cirrus sac small (75–100 mm long). Suckers markedly oval in shape (Figures 27.35E and 27.36A) ..... *R. tetragona*
6b. Cirrus sac large (130–180 mm). Suckers round (Figures 27.35F and 27.36B)..... *R. echinobothrida*
7a. Outer membrane prolonged in 2 elongated filaments (Figures 27.37B)..... *Choanotaenia infundibulum*
7b. Outer membrane smooth and round, 2 elongated filaments (Figures 27.34A and 27.37D) ..... *R. cesticillus*

Species descriptions are given for these 8 chicken tapeworms to assist in verifying tentative identifications.

Amoebotaenia cuneate (Linstow 1872)

Diagnostic Characteristics. This short (less than 4 mm, 25–30 proglottids) tapeworm may be recognized as whitish projections among the villi of the duodenum (Figure 27.35C); a triangular anterior end with a pointed scolex gives the entire worm a wedge-shaped anterior. Suckers unarmed, rostellum armed with a single row of 12–14 distinctive hooks 25–32 mm long, 12–15 testes located transversely in a single row across the posterior end of the proglottid (Figure 27.37E), genital pores usually alternate regularly, located at extreme anterior point of proglottid margin; 6-hooked single embryos, surrounded by a distinctive granular layer (Figure 27.37A); embryonal hooks, 6 mm.

Life History. Several species of earthworms belonging to the genera *Allotophora, Pheritima, Ocnerodrilus*, and *Lumbricus* act as intermediate hosts for this tapeworm. Literature descriptions of pathogenicity range from "comparatively slight" to "cause of death." No controlled experiments have been reported.

Choanotaenia infundibulum (Bloch 1779)

Diagnostic Characteristics. This large robust tapeworm, *C. infundibulum*, is extremely white and is readily seen attached to the upper half of the intestine. Mature worms up to 23 cm long; large rostellum armed with a single row of 16–22 large (25–30 mm) hooks, suckers

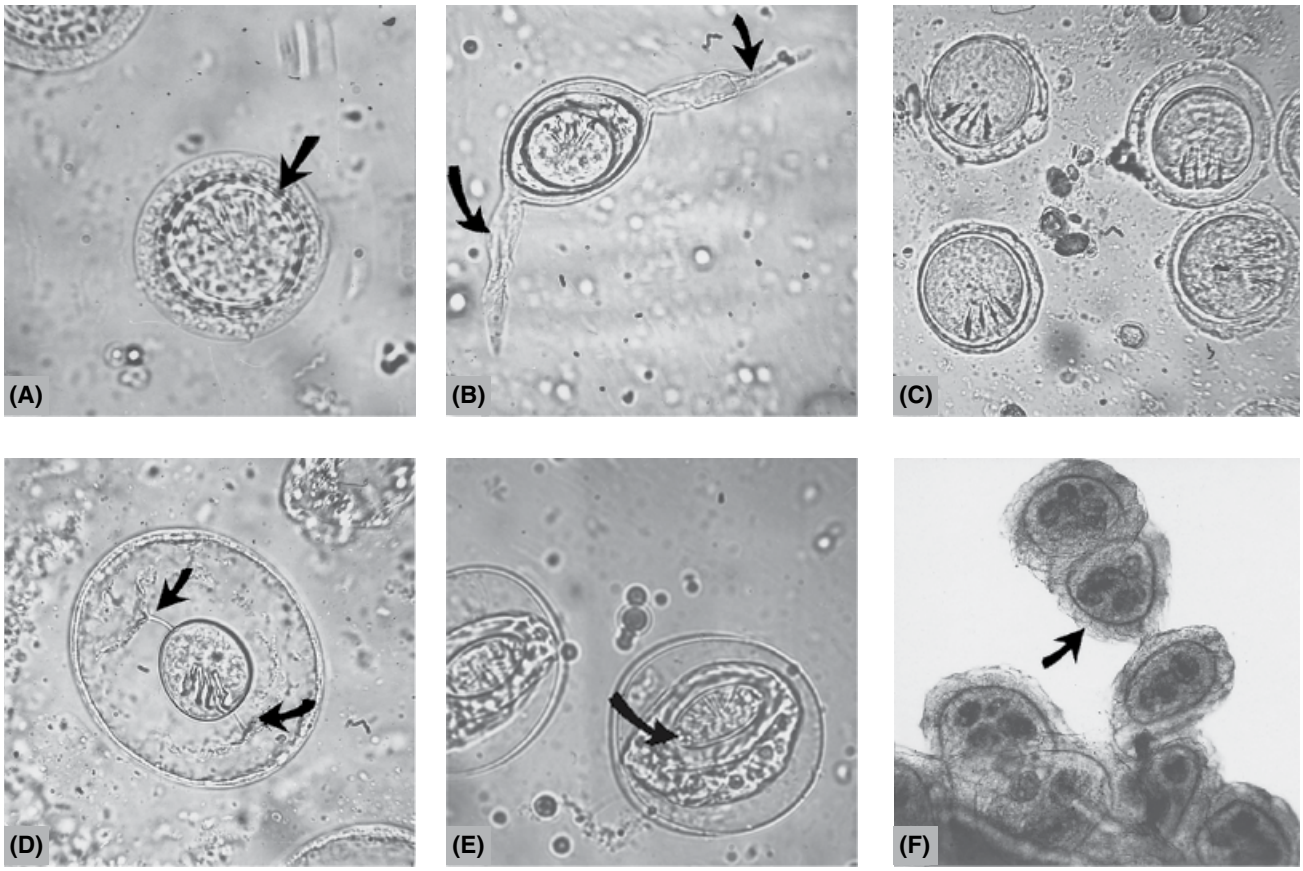


Figure 27.37 Eggs of chicken tapeworms (high power). (A) *Amoebotaenia sphenoides* showing distinctive granular layer. (B) *Choanotaenia infundibulum* with elongated filaments. (C) *Davainea proglottina*. (D) *Raillietina cesticillus*: gravid proglottid showing distinctive funnel-shaped structures between membranes. (E) *Hymenolepis carioca* or *H. cantaniana* showing football-shaped embryophore with granular accumulations at the poles. (F) Capsules containing 6–12 eggs. Found in the chicken (*Raillietina tetragona*, *R. echinobothrida*) and 2 turkey tapeworms (*R. georgiensis, R. williamsi*).

unarmed (Figure 27.35G); genital pores irregularly alternate; 25–60 testes are grouped in posterior portion of proglottid (Figure 27.36C); eggs are with distinctive elongated filaments (Figure 27.37B); and embryonal hooks are 18 mm long.

Life History and Pathogenicity. Houseflies and several species of beetles are proven natural hosts. Other insects including 9 families of beetles, grasshoppers, and termites are proven experimental hosts. Gravid proglottids are released 13 days after swallowing an infected fly. No controlled experiments testing pathogenicity have been reported.

***Davainea proglottina* (Davaine 1860)**

Diagnostic Characteristics. This microscopic *D. proglottina* tapeworm may be recognized in the duodenal mucosa by protrusion of the gravid proglottids above the villi if the open intestine is floated in water. Eggs are without distinctive membranes, but embryonal hooks are distinctive, 10–11 mm long (Figure 27.37C). Mature worms measure up to 4 mm long; never with more than 9 proglottids; suckers are armed with 3–6 rows of hooks (Figure 27.33); the rostellum is armed; genital pores regularly alternate and are located near the anterior margin; and the cirrus is disproportionately large.

Life History. Several species of slugs and snails host larval stages of this tapeworm. More than 1,500 cysticercoids have developed along the digestive tract of susceptible slugs, where they have remained infective for more than 11 months. Tapeworms may live as long as 3 years; more than 3,000 worms have been recovered from a single bird.

Pathogenicity. This parasite is one of the more harmful species in young birds. In controlled experiments, a 12% reduction in growth rate has been reported (5). Uncontrolled reports include emaciation, dull plumage, slow movements, breathing difficulties, thickened mucosal membranes that produce hemorrhage and fetid mucus, leg weakness, paralysis, and death.

***Hymenolepis cantaniana* (Polonio 1860)**

Diagnostic Characteristics. *H. cantaniana* is a short, hymenolepid tapeworm (maximum length 2 cm) that superficially resembles the longer *H. carioca*. It is usually listed as unarmed, but rostellar hooks have been described by European investigators (Figure 27.35A); the fragile rostellum is frequently lost; genital pores are unilateral, anterior to middle of proglottid; eggs are similar to those of *H. carioca*; embryonal hooks measure 13–14 mm.

Life History. Dung beetles (Scarabeidae) are intermediate hosts; each beetle may carry 100 or more cysticercoids. A unique larval development involves budding, which produces many cysticercoids from a single onchosphere. This tapeworm is considered relatively nonpathogenic, although no controlled experiments have been reported.

***Hymenolepis carioca* (Magalhaes 1898)**

Diagnostic Characteristics. Several thousand specimens of this extremely slender species have been found in the duodenum of a single chicken or turkey. The worm is so slender (about 1 mm in diameter) that the hundreds of inconspicuous proglottids look more like a thread than a worm. Suckers are unarmed; rostellar sacs are present; rostellum is rudimentary (Figure 27.35B); there are 3 testes, usually in a straight row; genital pores are unilateral, located anterior to middle of proglottid margin (Figure 27.36D); an inner membrane enveloping the onchosphere is elongated into a football shape with granular deposits at poles (Figure 27.37E); embryonal hooks measure 10–12 mm.

Life History. Twenty-six species belonging to 9 families of beetles and 1 species of termite are experimental or natural intermediate hosts; dung and ground beetles are the most common source of infection. Reports incriminating the housefly are probably erroneous.

Pathogenicity. Experimental infections establishing several hundred worms/bird had no effect on weight gains. These results indicate that this species is relatively nonpathogenic.

***Raillietina cesticillus* (Molin 1858)**

Diagnostic Characteristics. Scolex of this large robust tapeworm (up to 15 cm long) embeds deeply in the mucosa of the duodenum or jejunum. The distinctive, wide, flat, rostellum bears a double row of 300–500 hammer-shaped hooks. The flattened rostellum acts as a retractable piston drawing into an outer sleeve of the scolex, thus providing a firm grip on the mucosa (Figures 27.35D1,D2); there are 4 unarmed weak suckers; genital pores alternate irregularly (Figure 27.36F); there are 20–30 testes posteriad in proglottid; single eggs are encapsulated in uterine membranes; and mature eggs have 2 distinctive funnel-shaped filaments between the middle and inner membranes (Figure 27.37D).

Life History. More than 100 species of beetles belonging to 10 families are proven natural or experimental intermediate hosts. A minute histerid beetle (*Carcinops pumilio*) is the natural intermediate host in broiler houses. The darkling beetle (*Alphitobius diaperinus*), grasshoppers, ants, and lepidopterous larvae have proved negative as experimental hosts. As many as 930 cysticercoids have been found in a single ground beetle.

Pathogenicity. Early reports attribute this parasite with causing emaciation, degeneration, and inflammation of

villi, reduction of blood sugar and hemoglobin, and reduced growth rate. None of these early reports could be confirmed in extensive controlled experiments with broilers and layers maintained on optimum nutritional diets (2). Experimental infections (135 worms/bird) produced by feeding 300 cysticercoids caused no reduction in weight gain in broilers or reduced egg production in layers when compared with uninfected controls.

Raillietina tetragona (Molin 1858)

Diagnostic Characteristics. *R. tetragona* are moderately large tapeworms measuring up to 25 cm long × 3 mm wide. Scolex (Figure 27.35E1) anchors in the posterior half of the intestine; the rostellum is armed with 90–100 hooks, 6–8 mm long, arranged in a single or double row (Figure 27.35E2); suckers are oval-shaped, armed with 8–12 rows of minute hooks, 3–8 mm long (Figure 27.35E); genital pores are usually unilateral (Figure 27.36A); the uterus breaks up into capsules containing 6–12 eggs (Figures 27.36A2 and 27.37F), similar to *R. echinobothrida* from chickens and *R. williamsi* and *R. georgiensis* from turkeys; and the cirrus sac is small (75–100 mm long), more anterior in proglottid margin than with *R. echinobothrida*.

Life History. Several species of small ants that nest under rocks or boards act as intermediate hosts. The minimum prepatent period after feeding cysticercoids to chickens is 13 days.

Pathogenicity. Weight loss was demonstrated in controlled experiments (9) with white leghorns and hybrids infected with an average of 12–16 worms/bird. Decreases in egg production in 4 breeds of hens occurred after administering 50 cysticercoids/bird, causing reduced glycogen levels in livers and the intestinal mucosa of infected chickens.

Raillietina echinobothrida (Megnin 1881)

Diagnostic Characteristics. The *R. echinobothrida* species resembles *R. tetragona* but differs in the following characteristics: the strobila is larger (34 cm long × 4 mm wide); the scolex has rounded suckers containing 200–250 hooks, 10–13 mm long (Figure 27.35F) with 8–15 rows of hooks 5–15 mm long (Figures 27.35F2,F3); genital pores are in the posterior half of the proglottid (Figure 27.36B2); the cirrus sac is large (130–180 mm long); and gravid proglottids frequently loosen from each other in the center, making a window-like arrangement not found in *R. tetragona*.

Life History. As with *R. tetragona*, numerous species of ants have been found naturally infected with cysticercoids. Concurrent infections with both *R. echinobothrida* and *R. tetragona* cysticercoids have been found in ants.

Pathogenicity. *R. echinobothrida* is usually listed as one of the most pathogenic tapeworms because its presence has often been associated with nodular disease of chickens. Nadakal et al. (10) reported parasitic granulomas approximately 1–6 mm in diameter at the sites of worm attachment 6 months after experimental infection with 200 cysticercoids. The condition was associated with catarrhal hyperplastic enteritis as well as lymphocytic, polymorphonuclear, and eosinophilic infiltration.

Tapeworms of Turkeys

Six species of tapeworms from domestic and/or wild turkeys have been reported from the United States (12). Because these tapeworms are readily transferred between wild and domestic turkeys, wild turkeys provide a reservoir for these parasites of domestic birds. No controlled experiments on pathogenicity have been reported for any species. Descriptions included here are limited to the 2 species with known life cycles. Scolex (Figure 27.38) and proglottid characteristics (Figure 27.39) of different species are organized in separate figures to facilitate comparisons if complete specimens are unavailable.

Raillietina georgiensis (Reid and Nugara 1961)

Description and Diagnostic Characteristics. Rhw *R. georgiensis* species is a large (15–38 cm long × 3.5 mm wide) robust tapeworm from domestic and wild turkeys. Scolex (Figure 27.38A) is armed with a double row of 230 moderate length (12–23 mm) rostellar hooks and 8–10 circles of acetabular hooks, 8–13 mm long (Figures 27.38A2,A3); genital pores are unilateral, located in the middle of the proglottid (Figure 27.39A); eggs are in uterine capsules, similar to *R. tetragona* and *R. echinobothrida*.

Life History. A small brownish ant (*Pheidole vinelandica*) that frequents turkey ranges has been found naturally infected; gravid proglottids appear in droppings within 3 weeks after turkeys have fed on infected ants. This tapeworm was introduced to a domestic farm by wild turkeys.

Pathogenicity. Enteritis is present if parasites are found in large numbers. Some host damage is assumed on the basis of a close relationship to *R. echinobothrida* from chickens.

Metroliasthes lucida (Ransom 1900)

Description and Diagnostic Characteristics. *M. lucida* is a long tapeworm (20 cm) from turkeys and guinea fowl, rarely in chickens. There are unarmed scolex and suckers, 200–250 mm in diameter (Figure 27.38C); genital pores

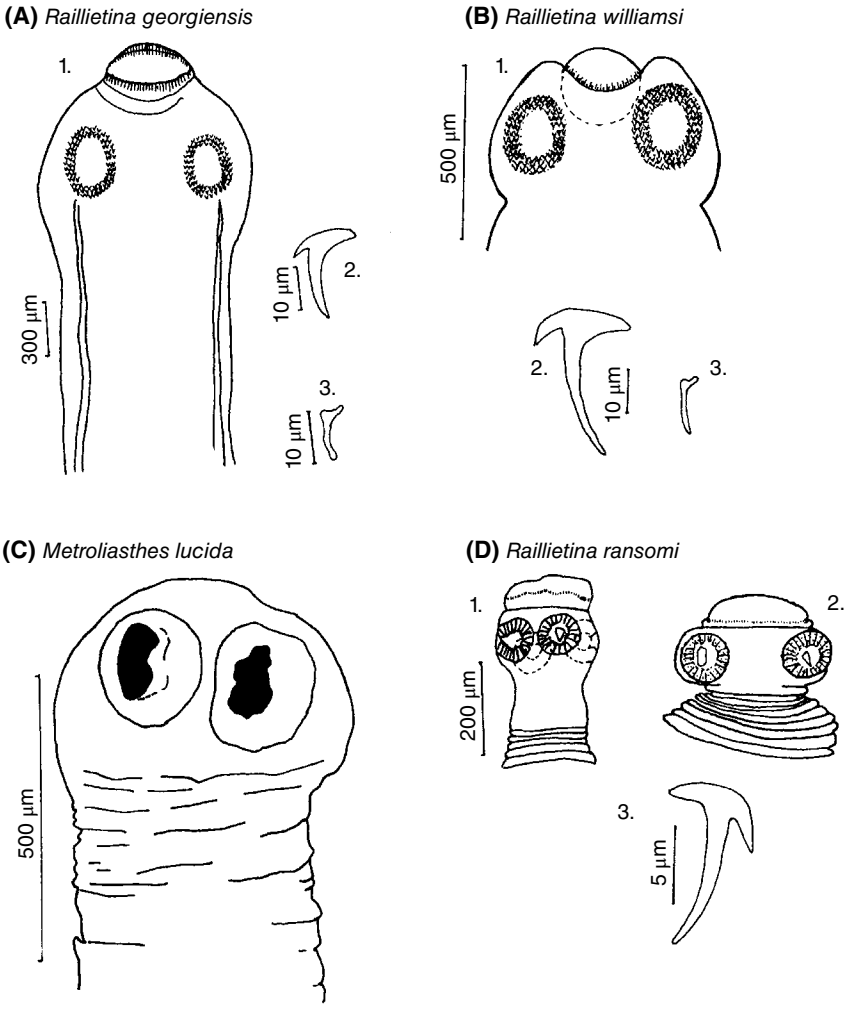


Figure 27.38 Scolices of turkey tapeworms. (A) *Raillietina georgiensis*: (1) scolex; (2) rostellar hook; (3) acetabular hook (Reid and Nugara). (B) *R. williamsi*: (1) scolex; (2) rostellar hook; (3) acetabular hook (Williams). (C) *Metroliasthes lucida* scolex (Ransom). (D) *R. ransomi*: (1,2) scolex; (3) rostellar hook (Williams).

irregularly alternate, near the middle of the margin in mature proglottids but posterior in gravid proglottids; the uterus consists of 2 sacs side by side, visible to the naked eye in gravid proglottids, and is known as the parauterine organ (Figures 27.39C2,C3); eggs have 3 membranes, 75 × 50 mm.

Life History. Several species of grasshoppers serve as intermediate hosts; cysticercoid development requires 15–42 days depending on temperature. The pathogenicity is unknown.

Tapeworms of Ducks and Geese

Domestic ducks and geese frequently become infected with numerous species of tapeworms introduced by wild ducks and geese. Some of these species have occasionally been reported in chickens. Two of the more common species are described in this section. Life cycles usually involve crustaceans or other aquatic invertebrates. No controlled pathogenicity studies have been made on any of these species.

Fimbriaria fasciolaris (Pallas 1781)

Description and Diagnostic Characteristics. This large (5–43 cm long × 1–5 mm wide) twisted tapeworm of ducks also occurs in chickens and 31 species of wild birds. This distinctive flaring anterior neck region is known as the pseudoscolex; strobila is unsegmented, but cross striations give the impression of segmentation (Figure 27.40A1); there are minute scolex (Figures 27.40A3,4) attached to pseudoscolex, 100–130 mm wide; suckers are unarmed; the retractile rostellum has 10–12 hooks 17–22 mm long (Figure 27.40A2); genital pores are unilateral and closely crowded together; onchospheres are 35–45 mm in diameter; hooks are 16 mm long.

Life History. Cysticercoids develop in copepod crustaceans (*Diaptomus* sp., *Cyclops* sp.); intermediate hosts are ingested with drinking water to infect the definitive host. The pathogenicity is unknown.

Hymenolepis megalops (Nitzsch, in Creplin 1829)

Description and Diagnostic Characteristics. *H. megalops*, a cosmopolitan tapeworm of waterfowl (Figure 27.40B),

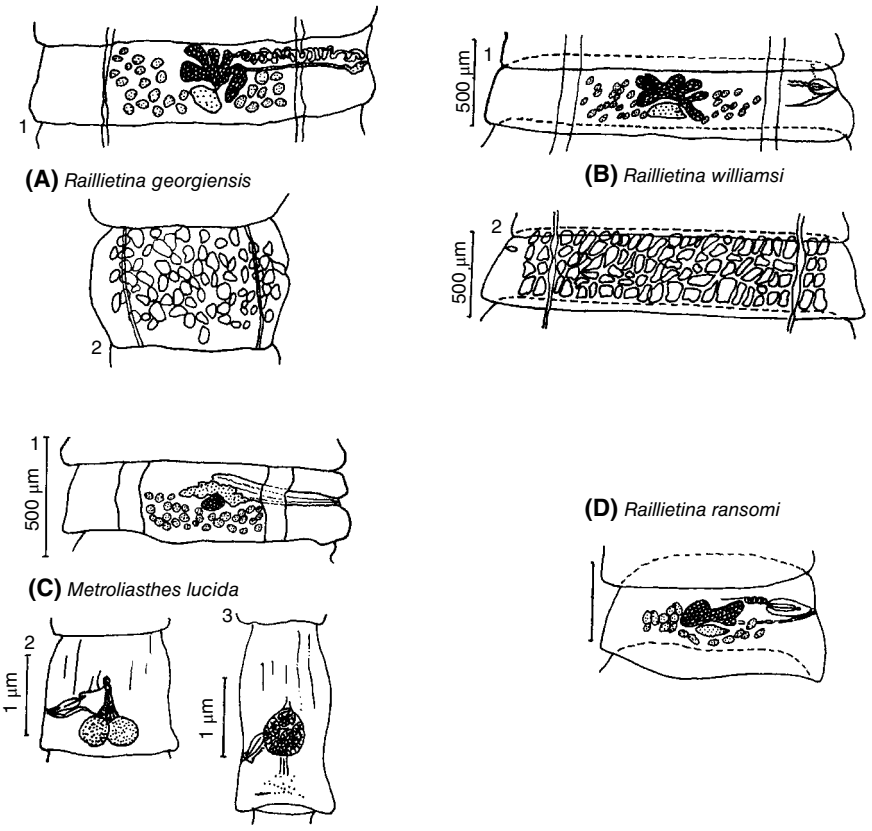


Figure 27.39 Mature and gravid proglottids of domestic and wild turkey tapeworms. (A) *Raillietina georgiensis* (Reid and Nugara). (B) *R. williamsi*: (1) mature proglottid; (2) gravid proglottid showing position of egg capsules, each containing several eggs (Williams). (C) *Metroliasthes lucida*: (1) mature proglottid; (2) proglottid showing 2-part uterus and developing parauterine organ; (3) gravid proglottid (Ransom). (D) *R. ransomi* mature proglottid (Williams).

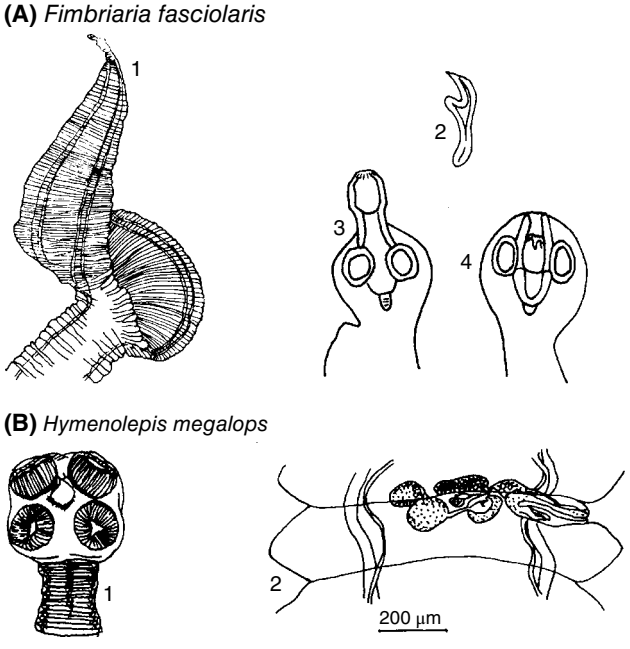


Figure 27.40 Tapeworms of ducks and geese. (A) *Fimbriaria fasciolaris*: (1) pseudoscolex showing irregular distension of the anterior end and the minute scolex (Todd); (2) rostellar hook (Fuhrmann); (3) scolex with rostellum extended; (4) scolex with rostellum withdrawn (Neveu-Lemaire). (B) *H. megalops*: (1) scolex; (2) mature proglottid (Yamaguti).

is 3–6 mm long and readily recognized by the large scolex (1–2 mm wide) attached to the cloaca or the cloacal bursa. Suckers and rostellum are unarmed, the latter containing a rudimentary central pit; eggs are not in capsules.

Life History. Onchospheres develop into cysticercoids after 18 days in ostracod crustacea. The definitive host is infected by eating ostracods.

Pathogenicity. Reports range from severe damage to mortality if other cestodes (*H. coronula, H. furcigera*) are also present.

Prevention and Control

The change in production methods in commercial poultry, from backyard or range management to confinement rearing in large houses, has brought on marked reductions in tapeworm infections in chickens and turkeys. These birds no longer have easy access to the required insect or other invertebrate hosts for most cestode and trematode parasites. *D. proglottina*, 1 of the most pathogenic species, was reported from 23% of the chickens submitted to the diagnostic laboratory in New York in 1932. No cases have been found in recent years, probably

because poultry no longer have easy access to garden slugs. We can expect a more diverse fauna of cestodes in birds reared under free-range conditions.

Prevention of contact with the intermediate host is the first step to consider in tapeworm control. Elimination of intermediate hosts may provide additional benefits besides tapeworm control. If *C. infundibulum* appears in a cage layer facility, housefly control will benefit the producer by preventing nuisance and public health complaints (Chapter 32). If *R. cesticillus* tapeworms appear in broiler houses, beetle control measures for the darkling beetle (*A. diaperinus*) also may reduce populations of the true intermediate host, *C. pumilio*, a minute histerid beetle. Identification of worm species will help to suggest control measures, where the control of the intermediate host is to be recommended.

Treatment

In the United States, there are no products for use in feed for treatment of tapeworms in poultry. Historically, butynorate (dibutyltin dilaurate) was used for treatment of 6 species of chicken tapeworms (*R. cesticillus, R. tetragona*, *C. infundibulum, D. proglottina, H. carioca*, and *Amoebotaenia sphenoides*) (3). Thus, all efforts toward control must be directed toward prevention by reducing populations of the intermediate hosts.

Trematodes

Trematodes (flukes) are flat, leaflike, parasitic organisms belonging to the phylum Platyhelminthes, class Trematoda. They differ from the cestodes (class Cestoda) in having a digestive system, and they do not form proglottids. The life cycle of all trematodes parasitizing birds requires a molluscan as an intermediate host; some species also use a second intermediate host. Because adult trematodes and larval metacercariae invade almost every cavity and tissue of birds, they may show up unexpectedly at necropsy.

More than 500 species belonging to some 125 genera and 27 families are known to occur in the 4 orders of birds most likely to be submitted to diagnostic laboratories as domestic or pet birds (4). Twenty of these flukes are considered potentially dangerous to poultry in the Western Hemisphere. These flukes belong to 4 orders: Anseriformes (ducks and geese), Galliformes (chickens and turkeys), Columbiformes (pigeons and allies), and Passeriformes (perching birds). Flukes are less host specific than tapeworms, so wild birds often introduce infection in areas where domestic poultry is reared. Because many snails live in ponds and streams, ducks and geese are the most frequently parasitized. The oviduct fluke (*Prosthogonimus* sp.), which is a frequent parasite of many species of wild birds, sometimes causes problems with ducks and chickens (6). This species will be used to illustrate fluke morphology and life history. *P. macrorchis* is the species name recognized in the United States, and this fluke is known as *P. ovatus* or by other specific names in other countries.

Morphology and Life History

The body of the adult fluke (Figure 27.41) is a flattened oval, and it bears 2 suckers. The digestive system consists of the mouth (within the oral sucker), the pharynx, a short esophagus, and 2 intestinal ceca. An anus is lacking in the trematodes. Two testes and 1 ovary are present in the same individual. After fertilization, the zygote is enclosed along with yolk cells from the vitellaria by an eggshell. Large numbers of eggs are stored in a prominent convoluted uterus. The excretory system, which originates in a series of flame cells bearing a tuft of cilia, drains with a series of collecting tubules that empty through an excretory pore near the posterior end of the parasite. The arrangement pattern of these collecting tubules is used as a family characteristic in the classification of flukes.

Life Cycle

Adult flukes continually shed eggs, which pass out with the feces of the host. These eggs contain an embryo that develops into a larval stage known as a miracidium. In this group of trematodes, the miracidium hatches after the egg is swallowed by a susceptible snail. Larval development continues within the snail

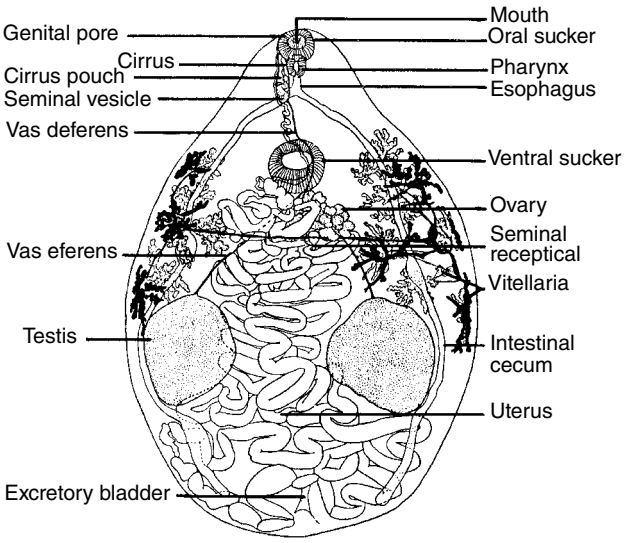


Figure 27.41 Morphology of an adult trematode (*Prosthogonimus macrorchis*). (Macy)

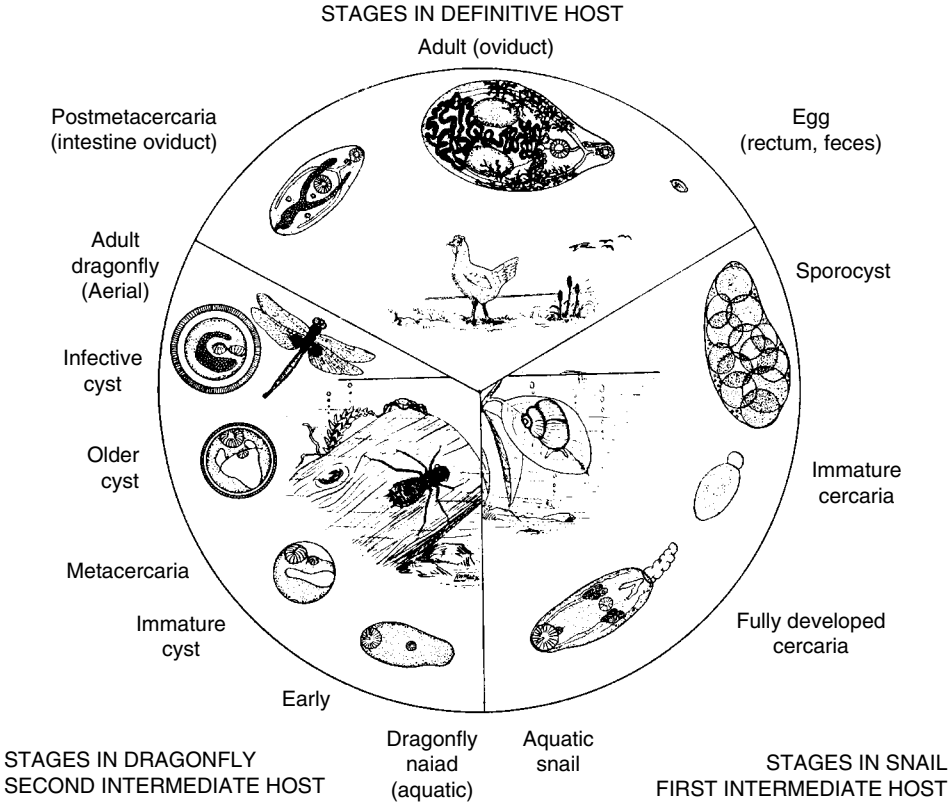


Figure 27.42 Life cycle of a typical digenetic trematode (*P. macrorchis*). (Macy)

through a succession of stages known as sporocysts and cercariae. The cercariae emerge from the snail and swim about in a lake or pond. Some are drawn into the brachial basket of a dragonfly naiad. The cercaria encysts (metacercaria) and remains in the insect until either the naiad or an infected adult dragonfly is eaten by a bird (Figure 27.42).

Identification

Twenty-four trematodes that are occasionally seen in birds at diagnostic laboratories have been described with keys by Kingston (4). More extensive listings of species are provided by Yamaguti (17), McDonald (7), and Schell (13). The latter text also describes methods of identifying, collecting, preserving, and staining trematodes with emphasis on North American families and genera.

Pathogenicity

Prosthogonimus sp., popularly known as the oviduct fluke, has caused economic losses to poultry producers by (1) drastically reducing egg production after a recent infection, and (2) occasionally being enveloped within a hen's egg and later discovered by a complaining customer. Other organs of the bird invaded by flukes include: (1) metacercarial cysts in the skin of chickens and turkeys (*Collyriclum faba*); (2) small adult flukes in the conjunctival sac of the eye (*Philophthalmus gralli*); (3) adults in the liver, pancreas, and bile duct of ducks and turkeys (*Amphimerus elongatus*); (4) adults in the collecting tubules of the excretory system of chickens, turkeys, and pigeons (*Tanaisia bragai*); (5) adults and eggs in the circulatory system of ducks by 3 species of blood fluke; and (6) 14 species of flukes that invade various areas of the digestive tract.

Control

If the life cycle is known and evidence of pathogenicity or economic loss is clear, changes in management are indicated. Efforts should be directed toward fencing poultry off from access to lakes or streams where dragonfly naiads, snails, and other aquatic intermediate hosts are abundant (6).

No chemotherapeutic products are available for use in poultry for control or prevention of trematode infections.

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28

Protozoal Infections

Introduction

Larry R. McDougald

Protozoa found in poultry are categorized within several taxonomic groups. Many are found in the phylum Apicomplexa (the coccidia) (3). These are intracellular parasites characterized by the presence of an apical complex in the sporozoite. Genera include *Eimeria*, *Isospora*, *Hemoproteus*, *Leucocytozoon*, *Plasmodium*, *Toxoplasma*, *Sarcocystis*, *Wenyonella*, *Tyzzeria*, and *Cryptosporidium*. A second phylum, Parabasalia, includes the flagellates and amebas. These include *Histomonas*, *Trypanosoma*, *Chilomastic*, *Entamoeba*, *Endolimax*, *Spironucleus*, and *Cochlosoma*. Some of these, such as *Cochlosoma* (1,2), are increasingly important. A third group, the Microspora, is represented by *Encephalitozoon cuniculi*, but little is known of its importance (5,6). Modern practices of poultry rearing minimize the impact of many parasites while increasing the risk from others. Occasionally, previously unknown protozoans are discovered by the use of modern diagnostic methods such as polymerase chain reaction (PCR), where it was not possible to discern different species by conventional methods (4).

Of the numerous protozoans known from chickens, turkeys, and gamebirds, only a few flourish under commercial conditions. Parasites with short, direct life cycles, such as coccidiosis, are favored, whereas others involving intermediate hosts are normally not a problem in poultry. An exception is blackhead disease (histomoniasis), which has a complicated life cycle involving intermediate hosts, but takes advantage of reservoir hosts (chickens) and is able to spread easily from bird to bird within a flock of turkeys. The importance of some of these parasites may change or increase with more emphasis placed on production in other systems, such as free-range.

Historically, producers have depended on anticoccidial drugs for control of coccidiosis in broiler chickens. However, there have been no new products in this area for several years, and it is unlikely that new products will be developed. With the emergence of drug resistance or tolerance to these drugs, more importance has been placed on vaccination with live vaccines. The use of such vaccines not only gives control of disease, but at the same time repopulates the production facilities with coccidia that are more sensitive to drugs and sometimes less pathogenic. With the introduction of attenuated coccidia in vaccines, this approach has become more important in disease control programs. Emphasis toward biological control and away from chemotherapy in production of 'all natural' or 'antibiotic-free' poultry favors this type of program. The growth of the gamebird industry has also created more need for tools for disease control. This industry has long been ignored in making modern drugs and vaccines available for disease control.

Blackhead disease remains a serious and enigmatic limit to production of poultry and gamebirds. In recent years there have been turkey companies experiencing massive losses over an entire growing season, sometimes with loss of entire flocks of 10–30,000 birds. At one time, highly effective drugs were available for treatment of outbreaks but these were deregistered in the 1990s leaving us without effective means of control. There are no products approved for treatment of outbreaks of blackhead disease, and no commercially available vaccines.

Diagnostic tools for protozoan diseases in poultry have been developed, particularly with coccidia and *Histomonas*. PCR-based tests are often used in research and may be useful to confirm species diagnosis. Enzyme-linked immunosorbent assay (ELISA), Western blot, and other tests are routinely used in research.

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Coccidiosis

Hector M. Cervantes, Larry R. McDougald, and Mark C. Jenkins

Summary

Agent, Infestation, and Disease. Poultry and gamebirds are susceptible to several species of *Eimeria*, which cause an enteric disease characterized by diarrhea, morbidity, lost weight gain, dehydration, and sometimes mortality. Coccidia are intracellular parasites, often resulting in disruption of the intestinal mucosa as they reproduce. The life cycle is complex, but there are no intermediate hosts. Lateral and vertical transmission is by mechanical contamination with the reproductive forms (oocysts).

Diagnosis. Diagnosis is by detection of characteristic gross lesions at necropsy and by detection of microscopic oocysts in fecal droppings.

Interventions. Control is largely by prevention, using anticoccidial drugs in the feed or live vaccines administered at the hatchery.

Introduction

Coccidiosis is a disease of universal importance in poultry production. The protozoan parasites of the genus *Eimeria* multiply in the intestinal tract and cause tissue damage, with resulting interruption of feeding and digestive processes or nutrient absorption, dehydration, blood loss, impaired skin pigmentation, and increased susceptibility to other disease agents. Historically, the spectacular onset of coccidiosis with bloody diarrhea and high mortality inspired awe and dread on the part of poultry growers and bird fanciers. Like many parasitic diseases, coccidiosis is largely a disease of young animals because immunity quickly develops after exposure and gives protection against later disease outbreaks. Unfortunately, no cross immunity exists between species of *Eimeria*, and later outbreaks may be the result of different species. The short, direct life cycle and high reproductive potential of coccidia in poultry often lead to severe outbreaks of disease in small backyard flocks or in the modern poultry house, where 20,000–60,000 chickens may be reared on new or reused litter.

Coccidiosis may strike any type of poultry in any type of facility. The disease may be mild, resulting from the ingestion of a few sporulated oocysts, and may escape notice, or it may be severe as a result of ingestion of millions of sporulated oocysts. Most infections are relatively mild or subclinical; because of the potential for a disastrous outbreak and the resulting financial loss, almost all young poultry are given continuous medication with low levels of anticoccidial drugs, which prevent the infection or reduce infections to a low, immunizing level. In the past, vaccines against coccidiosis have been used mostly in broiler breeder pullets and in turkey breeder replacements. Vaccination of broilers has rarely been practiced because even light infections with some species of coccidia can affect weight gain, feed conversion, and pigmentation of the skin. However, in recent years, consumer demand for birds raised without antibiotics (antibiotic-free, ABF) or drugs has increased significantly and new vaccines with improved administration techniques are targeting this growing market with encouraging results. For more details on the health challenges posed by ABF production refer to the new section on "Disease Prevention and Control in ABF Production."

Classification and Taxonomic Relationships

The biology and taxonomy of coccidia were reviewed by Davies et al. (28), Long (64), and Pellerdy (88). Coccidia are members of the phylum Apicomplexa, which is characterized by the presence of an apical complex in sporozoites. All apicomplexans are intracellular parasites. The genera *Eimeria*, *Isospora*, *Haemoproteus*, *Leucocytozoon*, *Plasmodium*, *Toxoplasma*, *Sarcocystis*, *Wenyonella*, *Tyzzeria*, and *Cryptosporidium* are found in poultry.

The most common apicomplexans in poultry belong to the genus *Eimeria*, described in this section. The oocyst, a thick-walled zygote shed in fecal matter by the infected host, is fairly distinctive and is often used in diagnosis and identification of some species. Oocysts have a thick outer shell and initially consist of a single fertilized cell. The process of sporulation begins immediately, if suitable conditions are present, yielding sporocysts and infective sporozoites in 48–72 hours. Sporulation is the process by which the oocysts become infective and it requires warmth (25–30°C), moisture, and oxygen (63). Infective (sporulated) oocysts contain 4 sporocysts, each of which contains 2 sporozoites (Figure 28.1).

The closely related parasites, *Sarcocystis*, *Toxoplasma*, *Cryptosporidia*, and *Plasmodium*, are discussed in the sections "Miscellaneous and Sporadic Protozoal Infections" and "Cryptosporidiosis."

When sporulated oocysts are ingested, the oocyst wall is crushed in the ventriculus (gizzard), releasing the sporocysts. The sporozoites emerge from the sporocysts by the action of chymotrypsin and bile salts in the small

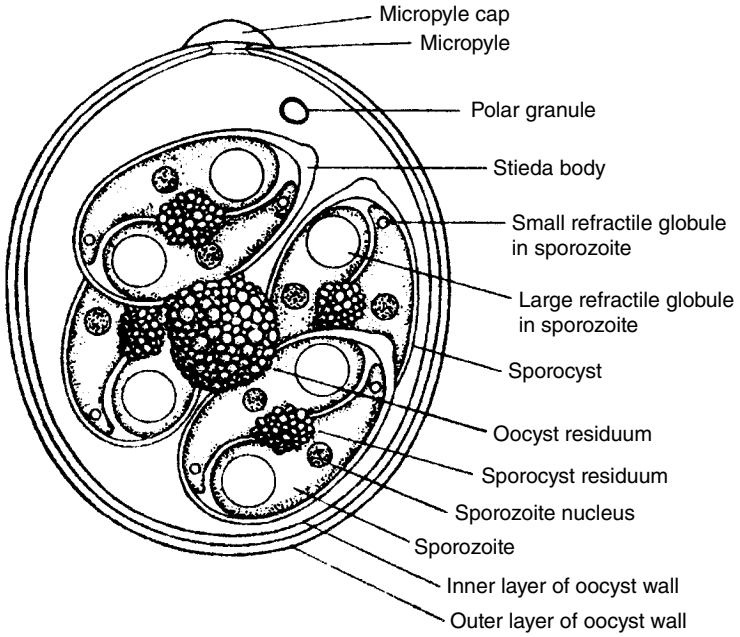


Figure 28.1 Diagram of sporulated oocyst of genus *Eimeria*. The oocysts of all *Eimeria* contain 4 sporocysts, each with 2 sporozoites, after sporulation.

intestine. Sporozoites enter epithelial cells or are taken into intraepithelial lymphocytes, where development begins. Species of coccidia are identified on the basis of: (1) oocyst morphology, (2) host specificity, (3) immune specificity, (4) appearance and location of gross lesions within the natural host, and (5) length of the prepatent period. The host specificity of *Eimeria* in birds and mammals is very strict, so that parasites from different species of birds or animals can be considered different species, even though they may have similar-appearing oocysts.

Biological characteristics useful in the identification of species are: (1) location of lesions in the intestine, (2) appearance of gross lesions, (3) oocyst size, shape, and color, (4) size of endogenous tissue stages (schizonts, merozoites, meronts, gametocytes), (5) location of the parasites within tissues, (6) minimum prepatent period in experimental infections, (7) immunogenicity in comparison with reference strains and more recently, and (8) sequences of DNA unique to each *Eimeria* species. Techniques of value include the electrophoretic pattern of metabolic enzymes (37) and polymerase chain reaction (PCR) (45, 55, 79, 83). Monoclonal antibodies are useful in experimental work but have not been developed to be specific for species identification. Digital image analysis (17) is useful for analysis of photographic imagery. For diagnostic purposes, the traditional biological characteristics are usually adequate. However, taxonomic difficulties are encountered in identification of species with morphologically similar oocysts which are found with overlapping tissue specificity. Species can be identified by comparison of isolates with several criteria listed in Tables 28.1 or 28.5, later.

Life Cycle

Coccidiosis differs from bacterial and viral diseases in the self-limiting nature of the infection. The life cycle of *E. tenella* (Figure 28.2) is typical of all *Eimeria*, although some species vary in the number of asexual generations and the time required for each developmental stage. After the oocyst wall is crushed in the ventriculus (gizzard) and the sporozoites are released from the sporocysts, the sporozoites enter cells in the mucosa of the intestine and begin the cell cycle leading to reproduction. At least 2 generations of asexual development (sometimes as many as 4) called schizogony or merogony give rise to a sexual phase, where male-line, small, motile microgametes seek out and unite with female-line, larger macrogametes. The resulting zygote matures into an oocyst, which is released from the intestinal mucosa and is shed in the feces, and under the right conditions of temperature, moisture, and oxygenation sporulates and becomes infective within 24–72 hours. With each species, the reproductive potential from a single ingested oocyst is fairly constant. The entire process takes 4–6 days, depending on species, although oocysts may be shed for several days after patency is reached. In some species (*E. tenella, E. necatrix*), the maximum tissue damage may occur when second-generation schizonts rupture to release merozoites. Other species may have small scattered schizonts, which cause little damage, but the gametocytes may elicit a strong reaction with cellular infiltration and thickened, inflamed tissues.

Relationship Between Coccidiosis and Other Poultry Diseases

The tissue damage and changes in intestinal tract permeability and function may allow colonization by various harmful bacteria, such as *Clostridium perfringens*, leading to necrotic enteritis (5, 107) or salmonellosis (6, 7). Cecal coccidiosis (*E. tenella*) may contribute to increased severity of the blackhead organism (*Histomonas meleagridis*) in chickens. Experimental infections with the 2 organisms were characterized by a higher incidence of hepatic disease, as compared with monoinfection with *Histomonas* (70).

Immunosuppressive diseases may act in concert with coccidiosis to produce a more severe disease. Marek's disease may interfere with development of immunity to coccidiosis (10), and infectious bursal disease may exacerbate coccidiosis, placing a heavier burden on anticoccidial drugs (71).

Coccidiosis in Chickens

Coccidiosis remains one of the most expensive and common diseases of poultry production in spite of advances in chemotherapy, management, nutrition, and genetics (18). The disease is often diagnosed in birds brought to diagnostic laboratories but the vast majority of cases are diagnosed in the field and handled by poultry service personnel. Regardless of the coccidiosis prevention program used, the prevalence of subclinical infections is such that nearly every broiler production company in the United States conducts broiler health surveys or "cocci checks" on a regular basis in order to monitor the incidence and severity of gross coccidial lesions and the quantity of oocysts of *E. maxima*, and in some cases, of other species, and assess the effectiveness of the coccidiosis prevention programs (19). Data from these surveys show that coccidiosis remains the most common subclinical disease of commercial broilers (18). In the most recent survey of broiler chicken production veterinarians practicing in the United States, coccidiosis was ranked as the most important disease of broiler chickens (13). Worldwide, the costs associated with coccidiosis including performance losses, mortality, prophylaxis, and therapy are estimated to exceed 3 billion US dollars (65).

Incidence and Distribution

Coccidia are found wherever chickens are raised. Their strict host specificity eliminates wild birds as sources of infection although they can be mechanical vectors. The most common means of spread of coccidia is mechanical, by personnel who move between pens, houses, or farms. Coccidial infections are self-limiting and depend largely on the number of sporulated oocysts ingested and on the immune status of the bird. Surveys in North and South America revealed coccidia to be present in almost all broiler farms (68, 73, 74). Very high percentages of positive flocks were also reported from Europe (12, 100, 104). These and other prevalence surveys have also confirmed the existence of the 7 species of *Eimeria* universally recognized (1, 4, 15, 31, 53, 54, 68, 79, 83, 100, 105). Oocysts in the litter or droppings of broiler chickens are usually most numerous between 3 and 5 weeks of age and often decline thereafter. Few oocysts are found after birds are removed from a farm because the parasites are killed by ammonia or composting heat in poultry litter or droppings. The ubiquitous and resistant nature of poultry coccidia seems to preclude the possibility of elimination or prevention of coccidia by quarantine, disinfection, or sanitation.

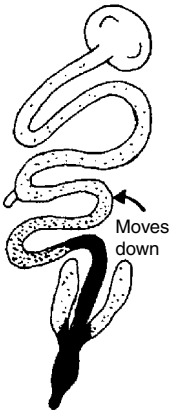
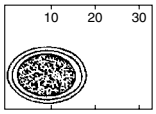
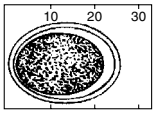
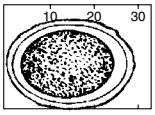
Etiology and Diagnosis

Although 9 species of *Eimeria* have been described from chickens, only 7 are universally recognized. From the beginning and up to the present time, the existence of *E. mivati* as a separate species has been questioned (85, 94, 96, 103). *E. hagani* (like *E. mivati*) has not been isolated in pure culture or detected in recent field surveys, despite the use of the most modern and highly sensitive molecular techniques. Recently, a field outbreak of *E. mivati* was reported (78) but no cultures were secured for DNA analysis. In one review article David H. Chapman writes "The description of *E. hagani* was brief and insufficient to justify consideration as a new species" (26). Therefore, the characteristics of the 7 universally recognized species of *Eimeria* are presented in Table 28.1. Through complete mitochondrial DNA genome analysis, Australian researchers (84) have found 3 additional isolate with operational taxonomic units (OTUs) referred to as X, Y, and Z, from Australian *Eimeria* that are closely related to *E. maxima*, *E. brunetti*, and *E. mitis*.

Concurrent infection with 2 or more species of coccidia is common (1, 4, 42, 54, 74). Each species causes separate and distinct, recognizable diseases, independent of the other species.

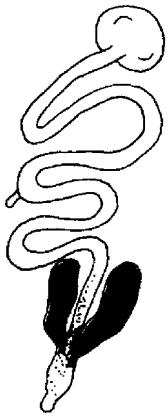
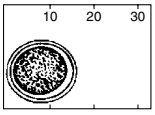
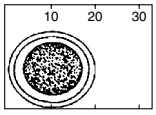
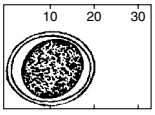
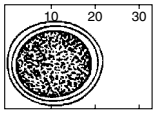
Characteristics useful in the identification of *Eimeria* species are listed previously under Classification and Taxonomic Relationships. However, for diagnostic purposes, the traditional characteristics are adequate, and a satisfactory diagnosis can be made from Table 28.1. If further confirmation is required, cross immunity and biochemical studies are required. Pure species isolates must be propagated from single oocysts. Analysis by molecular techniques (PCR) is useful to determine the species and relative importance of species (16). The severity of infection based on gross lesions is often graded on a scale of 0–4, as described by Johnson and Reid (57), where 0 is normal and 4 is the most severe. Lesions or fecal droppings may also be evaluated microscopically, by counting the number of parasite forms in a field.

Table 28.1 Diagnostic table of coccidia.

	Characteristics	*Eimeria acervulina*	*E. brunetti*	*E. maxima*
Macroscopic Lesions	Zone			
	Parasitized			
	Macroscopic Lesions	Light infection: whitish round lesions sometimes in ladder-like streaks. Heavy infection: plaques coalescing, thickened intestinal wall	Coagulation necrosis Mucoid, bloody enteritis in lower intestine	Thickened walls, mucoid, blood-tinged exudate, petechiae
Microscopic Characteristics	Millimicrons Oocysts Redrawn from Originals			
	Length × Width (μm) Length = Width =	AV = 18.3 × 14.6 17.7–20.2 13.7–16.3	24.6 × 18.8 20.7–30.3 18.1–24.2	30.5 × 20.7 21.5–42.5 16.5–29.8
	Oocyst Shape and Index Length/Width	Ovoid 1.25	Ovoid 1.31	Ovoid 1.47
	Schizont, Max in Microns	10.3	30.0	9.4
	Parasite Location in Tissue Sections	Epithelial	2nd generation schizonts subepithelial	Gametocytes subepithelial
Life History Characteristics	Minimum Prepatent Period (hour)	97	120	121
	Sporulation Time Minimum (hour)	17	18	30

[1] From Norton and Joyner (1980)

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Athens

E. mitis[1]	*E. necatrix*	*E. praecox*	*E. tenella*
			
No discrete lesions in intestine, mucoid exudate	Ballooning, white spots (schizonts), petechiae, mucoid blood-filled exudate	No lesions, mucoid exudate	Onset: hemorrhage into lumen. Later: thickening, whitish mucosa, cores clotted blood
			
15.6 × 14.2 11.7–18.7 11.0–18.0	20.4 × 17.2 13.2–22.7 11.3–18.3	21.3 × 17.1 19.8–24.7 15.7–19.8	22.0 × 19.0 19.5–26.0 16.5–22.8
Subspherical 1.09	Oblong ovoid 1.19	Ovoidal 1.24	Ovoid 1.16
15.1	17.3	20	54.0
Epithelial	2nd generation schizonts subepithelial	Epithelial	2nd generation schizonts subepithelial
93	138	83	115
15	18	12	18

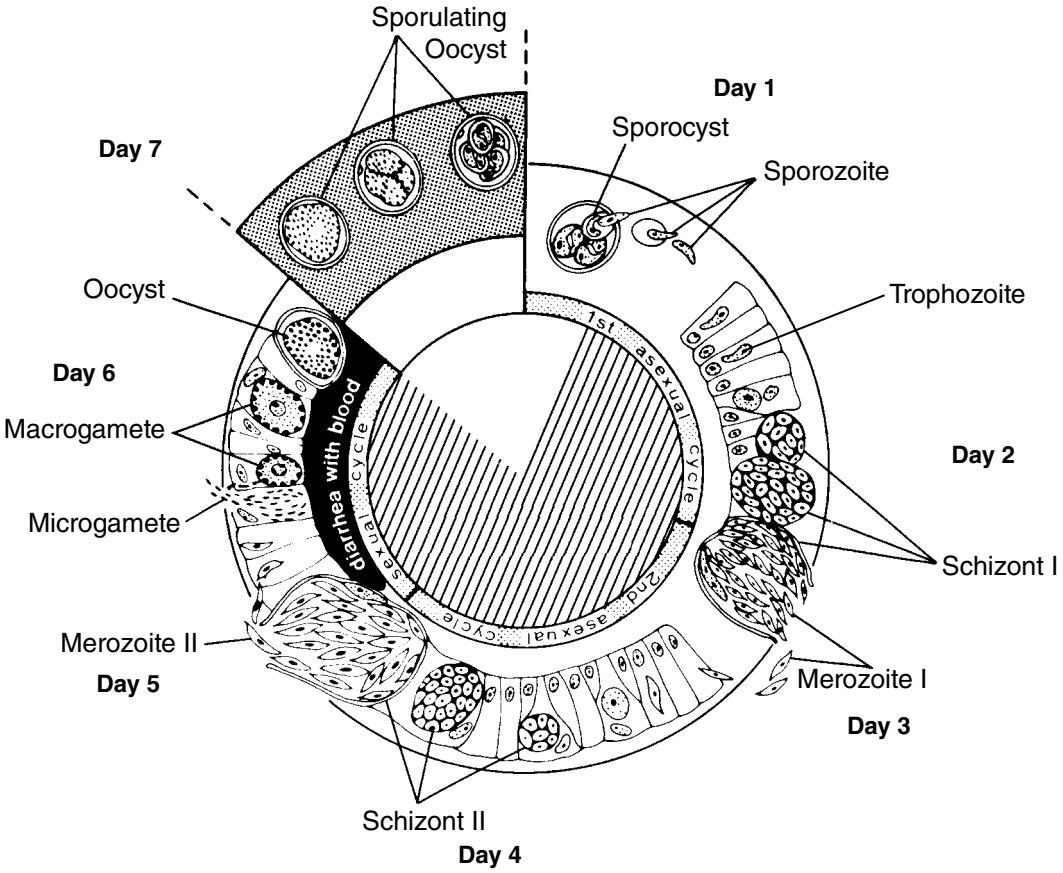


Figure 28.2 The 7-day life cycle of *Eimeria tenella* includes 2 or more asexual cycles and 1 sexual cycle during the 6 days after an oocyst has been swallowed by the host. The new generation of oocysts becomes infective to the next host after sporulation.

Description of Species

Eimeria acervulina Tyzzer 1929

Eimeria acervulina is the most frequently encountered species in commercial poultry in North and South America (31, 52, 54) and is commonly reported on other continents (1, 40, 42, 45, 62, 79, 83, 89, 99). Oocysts are ovoid and often show thinning of the shell at the small end. The average size of oocysts is 18.3 × 14.6 µm, but the range is 17.7–20.2 × 13.7–16.3 µm.

Pathogenicity. Severity of infection may vary with the isolate, the number of oocysts ingested, and the immune state of the bird. Ingestion of 10^3, 10^4, 10^5, or 10^6 sporulated oocysts by young chicks resulted in mild to severe coccidiosis, with lesion scores ranging from 1.1 (10^3 oocysts) to 4.0 (10^6 oocysts) (91). Reduction in rate of weight gain was also proportional to the infective dose. Watery and mucoid droppings may be seen as early as 4 days postexposure. Heavy infections often cause lesions to coalesce, and sometimes mortality may result. Light to moderate infections may produce little effect on weight gain and feed conversion but may cause loss of carotenoid and xanthophyll pigments from the blood and skin because of reduced absorption in the small intestine. The intestinal mucosa may be thickened, resulting in poor feed conversion (Figure 28.3B–E).

The intestine may be pale and contain watery and mucoid fluid. The gross lesion in light infections is limited to the duodenal loop, with only a few plaques/cm^2. In heavy infections, lesions may extend some distance through the small intestine, and plaques may overlap or coalesce. The plaques are generally smaller in heavy infections because of crowding. The lesions may be composed of schizonts, gametocytes, and developing oocysts. Microscopy of smears from intestinal lesions usually reveals numerous oocysts and gametocytes of varying stage of development.

Histopathology of the small intestine reveals the ovoid gametocytes in the mucosal cells lining the villi. In moderate to heavy infections, the tips of villi are broken off, leading to truncation and fusion of villi and thickening of the mucosa. Some epithelial cells may contain more than 1 parasite. Capillaries may be engorged with red blood cells and there is infiltration of granulocytes in the area parasitized. Schiff reagent will stain the macrogametes and developing oocysts a brilliant red, because of the polysaccharide used in oocyst wall formation.

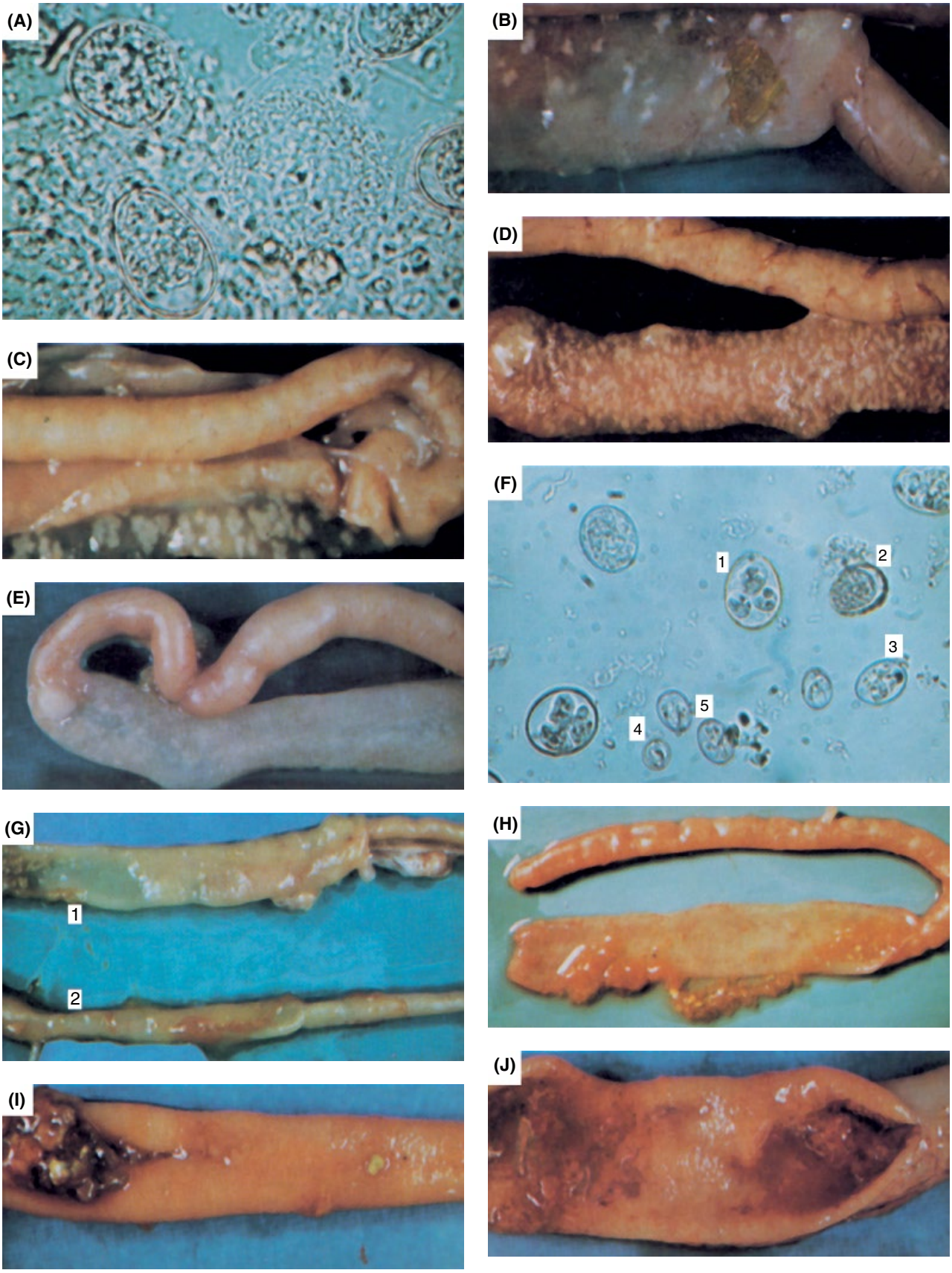


Figure 28.3 (A) Oocysts and a microgametocyte (center) of *Eimeria maxima*. (Long, P.L., L.P. Joyner, B.J. Millard, and C.C. Norton. 1976. A guide to laboratory techniques used in the study and diagnosis of avian coccidiosis. *Folia Vet Lat*. 6:201–217) (B) *E. acervulina* (+2). (C) *E acervulina* (+2). (D) *E. acervulina* (+3). (E) *E. acervulina* (+4). (F) 1. Sporulated *E. maxima* with distinctive brownish walls; 2. Unsporulated *E. maxima* showing roughened outer wall; 3. Probably *E. tenella*; 4. End view, probably *E. mitis*; 5. Side view, probably 2 *E. mitis*. (G) 1. Normal midgut; 2. *E. maxima* midgut (+1). (Long, P.L., L.P. Joyner, B.J. Millard, and C.C. Norton. 1976. A guide to laboratory techniques used in the study and diagnosis of avian coccidiosis. *Folia Vet Lat*. 6:201–217) (H) *E. maxima* midgut (+2 or +3). (I) *E. maxima* (+3). (J) *E. maxima* close-up view (+4). (*For color detail, please see the color section*.)

Eimeria brunetti Levine 1942

About 10%–20% of field isolates in surveys in the United States, South America, Australia, Brazil, and India contained *E. brunetti*, although often as a lesser component (1, 15, 40, 68, 79). The oocysts of *E. brunetti* average 24.6 × 18.8 µm and are easily confused with *E. tenella*. This species is found primarily in the lower small intestine, usually from the yolk sac diverticulum to near the cecal juncture. In severe cases, the lesion may extend from the gizzard to the cloaca and extend into the ceca (Figure 28.4E–H). Most field infections are difficult to recognize based on gross lesions but can be confirmed by observation of typically sized oocysts by microscopy. The average oocyst size is 24.6 × 18.8 µm, with a range of 20.7–30.3 by 18.1–24.2 µm. Oocysts are ovoid, with a length/width index of 1.31.

Pathogenicity. Although less serious than *E. tenella* or *E. necatrix*, *E. brunetti* can produce moderate mortality, loss of weight gain, poor feed conversion, and other complications. Inoculation with $1–2 \times 10^5$ sporulated oocysts frequently will cause 10%–30% mortality and reduced gain in survivors.

Gross Lesions and Histopathology. At early stages of infection, the mucosa of the lower small intestine may be covered with tiny petechiae and have some thickening, loss of color, and watery contents. In heavy infections, the mucosa is badly damaged, with coagulation necrosis appearing on days 5–7 postinfection (PI) and with a caseous eroded surface over the entire mucosa. Coagulated blood and mucosal casts are apparent in the droppings. Thickening of the mucosa and edematous swelling occurs in severe infections.

The asexual stages of first- and second-generation schizogony may be found in the upper small intestine. Histopathology on the fourth day of infection reveals schizonts, cellular infiltration, and damage to the mucosa. By the fifth day, tips of the villi are broken off. Merozoites invade the epithelium and develop into sexual stages in the lower small intestine and ceca. In severe cases, the villi may be completely denuded, leaving only the basement membranes intact.

Eimeria maxima Tyzzer 1929

The mid-small intestine is often parasitized with *E. maxima*. It is found around the yolk sac diverticulum, but in heavy infections the lesions may extend throughout the small intestine. *E. maxima* is an easy species to recognize because of the characteristic large oocysts, 30.5 × 20.7 µm (21.5–42.5 × 16.5–29.8), which usually have a distinctive amber color (Figure 28.3A,F–J). Oocysts have a shape index of 1.473. An abundance of yellow-orange mucus and fluid often is found in the midgut. This species can be differentiated from *E. necatrix* by the lack of large schizonts associated with the lesions and from *E. brunetti* by the larger oocysts and the appearance and location of the lesions.

Pathogenicity. *E. maxima* is moderately to highly pathogenic. Infection with $50–200 \times 10^3$ sporulated oocysts causes poor weight gain, morbidity, diarrhea, and sometimes mortality. Some isolates are capable of 30% mortality in 5-week-old chickens with 10^5 oocysts. There is often emaciation, pallor, roughening of feathers, and anorexia. Producers interested in maintaining good skin color in chickens must be concerned with subclinical infections because of the effect of this species on absorption of xanthophyll and carotenoid pigments in the small intestine. Infection with this species is the most common factor leading to necrotic enteritis (3).

Gross Lesions and Histopathology. Minimal tissue damage occurs with the asexual cycles, which develop superficially in the epithelial cells of the mucosa. When the sexual stages develop in deeper tissues on days 5–8 PI, lesions develop because of congestion and edema, cellular infiltration, and thickening of the mucosa. Foci of infection can be seen from the serosal surface because of microscopic hemorrhages. The intestine may be flaccid and filled with fluid, and the lumen often contains yellow or orange mucus and blood. This condition has been described as "ballooning." Microscopic pathology is characterized by edema and cellular infiltration, developing schizonts through day 4, and sexual stages (macrogametes and microgametes) in deeper tissues on days 5–8. In severe infections, considerable disruption of the mucosa occurs.

Eimeria mitis Tyzzer 1929

The lower small intestine is the normal site of this parasite, from the yolk sac diverticulum to the cecal necks. The lesions are normally indistinct with this species, but the potential for pathogenic effects on weight gain and morbidity is well documented (34). Oocysts average 16.2 × 16.0 µm (shape index 1.01), giving them a subspherical appearance.

Pathogenicity. Infection with $5 \times 10^5–5 \times 10^6$ sporulated oocysts will depress weight gain and cause morbidity and loss of pigmentation in broiler chickens. In layers, this species may affect egg production and induce a molt (35). The lack of distinct gross lesions causes this species to be overlooked or misdiagnosed in subclinical infections.

Gross Lesions and Histopathology. The gross lesion is very slight and can be easily overlooked. The lower small intestine appears pale and flaccid, and microscopic examination of smears from the mucosal surface may reveal numerous tiny oocysts (15.6 × 14.2 µm). The

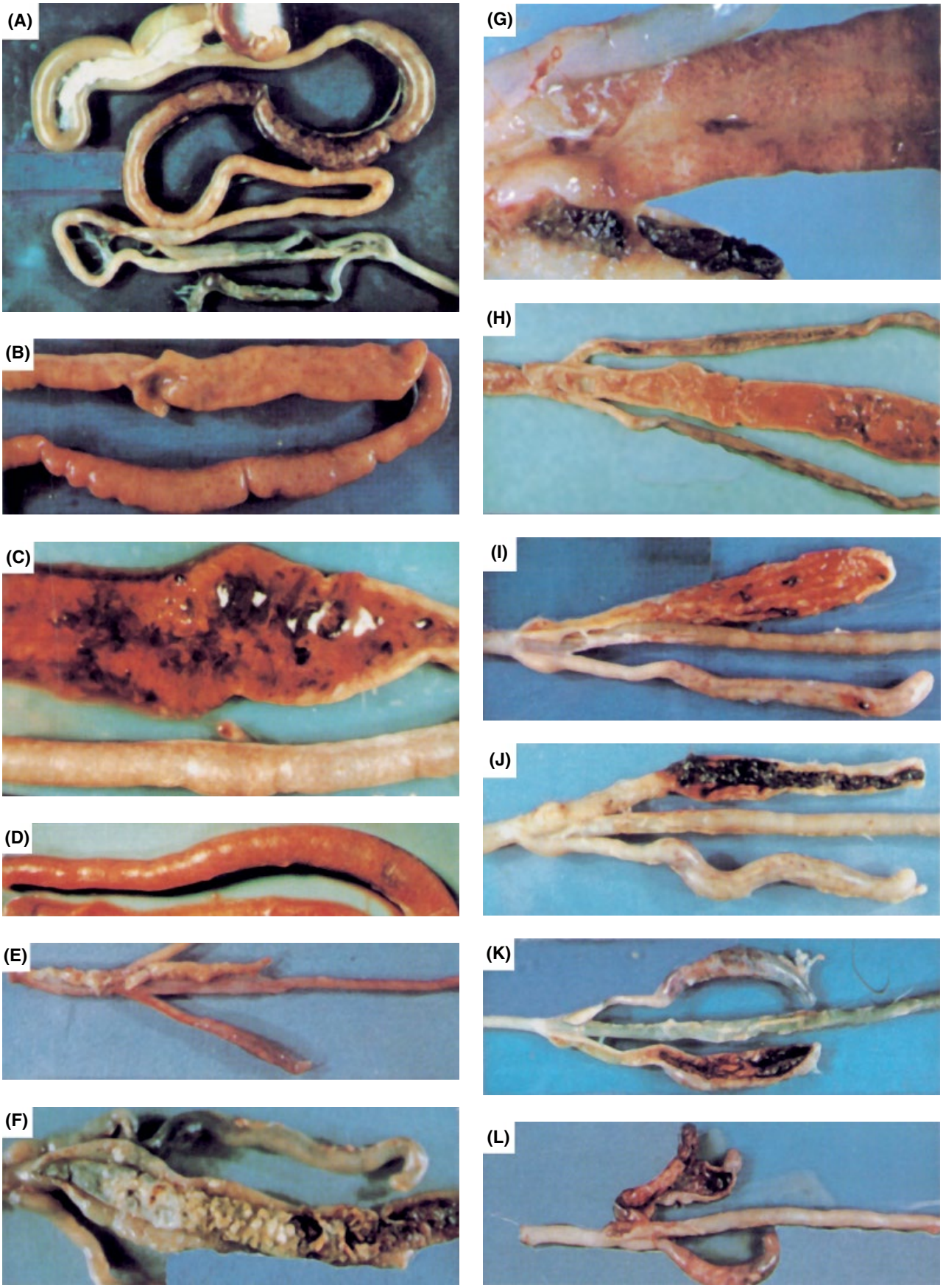


Figure 28.4 (A) *Eimeria necatrix* showing ballooning in midgut. (B) *E. necatrix* (+2). (C) *E. necatrix* (Long et al., 1976). (D) *E. necatrix* (4+). (E) *E. brunetti* (+4). (F) *E. brunetti* (+4). (G) *E. brunetti* (+3). (H) *E. brunetti* (+4). (Long, P.L., L.P. Joyner, B.J. Millard, and C.C. Norton. 1976. A guide to laboratory techniques used in the study and diagnosis of avian coccidiosis. *Folia Vet Lat*. 6:201–217) (I) *E. tenella* (+2). (J) *E. tenella* (+3). (K) *E. tenella* (+4). (L) *E. tenella* (+4) with cecal core. (*For color detail, please see the color section*.)

infection is distinguished easily from *E. brunetti* by the smaller, round oocysts. In light infections, the appearance of the gross lesion may be similar to *E. brunetti*. The gross lesions of this species are unremarkable because the developing parasites do not tend to localize in colonies as do other species, and the schizonts and gametocytes are superficial in the mucosa.

Eimeria necatrix Johnson 1930

Spectacular lesions in the small intestine caused this species to be one of the best known by early poultry producers. The lesions are found in the small intestine in approximately the same location as *E. maxima* (Figure 28.4A–D). Probably because of the low reproductive capability of *E. necatrix*, it is not able to compete with other coccidia and is diagnosed mostly in older birds such as breeder pullets or layer pullets aged 9–14 weeks old. The intestine often is dilated to twice its normal size (ballooning), and the lumen may be filled with blood and fluid laden with merozoites and clusters of large, mature schizonts. The oocysts are ovoid and average 20.4 × 17.2 μm, which is near in size to those of *E. tenella*. Curiously, the oocysts are found only in the ceca, rather than in the intestine, where lesions are found. The sexual stages (gametocytes) develop in the ceca and are scattered rather than clustered. *E. necatrix* is a poor producer of oocysts.

Pathogenicity, Gross Lesions, and Histopathology. *E. necatrix* along with *E. tenella* are the most pathogenic of the chicken coccidia. Infection with 10^4–10^5 sporulated oocysts is sufficient to cause severe weight loss, morbidity, and mortality. Survivors may be emaciated, suffer secondary infections, and lose pigmentation. Droppings of infected birds often contain blood, fluid, and mucus. Naturally occurring infections have caused mortality in excess of 25% in commercial flocks. In experimental infections, 100% mortality is possible. Layer pullets suffering outbreaks at 7–20 weeks of age may suffer mortality, morbidity, loss of uniformity, and decreased egg-laying potential. Gross lesions may be seen as early as 2–3 days PI, associated with first-generation schizogony, but the severe lesions at 4–6 days PI are caused by second-generation schizogony. The intestine may be ballooned, the mucosa thickened, and the lumen filled with fluid, blood, and tissue debris. From the serosal surface, the foci of infection can be seen as small white plaques or red petechiae. In dead birds, these lesions appear white and black, giving rise to the expression "salt and pepper" appearance. Smears examined microscopically on days 4–5 PI may contain numerous clusters of large (66 μm) schizonts, each containing hundreds of merozoites. Clusters of schizonts deep in the mucosa often penetrate the submucosa, damage the layers of smooth muscle, and destroy blood vessels. In these instances, the foci are large enough to be seen from the serosal surface. Later, scar tissue may be seen where epithelial regeneration is incomplete. Few pathogenic effects are seen with the invasion of the cecal mucosa by the third-generation schizonts and gametocytes because of the nonclustering nature of these stages. The third-generation schizonts produce only 6–16 merozoites.

Lesions may extend from the ventriculus (gizzard) junction to the ileo-cecal junction, causing dilation (ballooning) and thickening of the mucosa. The lumen may be filled with blood and pieces of mucosal tissue. Microscopic examination of smears from the mucosal surface reveals numerous clusters of large schizonts, which are characteristic for this species and distinguish it from others that overlap in habitat. Also, oocysts are found only in the ceca.

Histopathology of midgut from affected birds reveals a submucosa and lamina propria crowded with large clusters of schizonts. Often, large areas of the mucosa are sloughed off, and the lesion may extend through the muscle layers to near the serosal membranes.

Eimeria praecox Johnson 1930

Eimeria praecox is named from the short prepatent period (about 83 hours); hence a "precocious" parasite. Even though *E. praecox* is often overlooked because no prominent lesions exist, it is easily detected by timed infections of experimental birds. The oocysts are recognized easily because they are generally larger than those of other species found in the duodenum. At 21.3 × 17.1 μm, they are larger than *E. acervulina*, and *E. mitis*, and smaller than *E. maxima*. The shape index is 1.25.

Pathogenicity, Gross Lesions, and Histopathology. Heavy infections cause reduced weight gain, loss of pigmentation, dehydration, and poor feed conversion. The gross lesions consist of watery intestinal contents and sometimes mucus and mucoid casts. Most of the infection is confined to the duodenal loop. Small pinpoint hemorrhages may be seen on the mucosal surface on days 4–5 of infection. Recent studies suggest that this species may cause morbidity and reduced weight gain (2, 38, 92). Severe infections may cause dehydration. The epithelial cells of the sides of the villi (but not the tips) are most often infected. There may be several parasites in each cell. Three to four asexual generations are normal before gametogony. Infections with this species cause little tissue reaction.

Eimeria tenella (Railliet and Lucet 1891) Fantham 1909

Eimeria tenella is the best known of poultry coccidia because of the easily recognizable lesions and often spectacular losses it causes in commercial broilers or layer

pullets. This species inhabits the ceca (rarely adjacent intestinal tissues), causing a severe disease characterized by bleeding, high morbidity and mortality, loss of weight gain, emaciation, loss of skin pigmentation, and other signs. Oocysts are ovoid, averaging 22 × 19 µm (shape index 1.16). Diagnosis is dependent upon finding cecal lesions with prominent blood and often firm bloody cores and accompanying clusters of large schizonts and oocysts (Figure 28.4I–L).

Pathogenicity, Pathogenesis, and Epidemiology. Experimental inoculation with 10^4 or more sporulated oocysts can cause morbidity, mortality, and greatly reduced weight gain, making this one of the most pathogenic species in chickens. Inoculation with 10^3 sporulated oocysts is sufficient to cause bloody droppings and other signs of infection. The most pathogenic stage is the second-generation schizont, which matures at 4 days PI. Like *E. necatrix*, this species produces clusters of large schizonts, which may contain hundreds of merozoites. The schizonts develop deep in the lamina propria, so that the blood vessels are disrupted when the schizonts mature and merozoites are released. Onset of mortality in a flock is rapid. Most of the mortality occurs between days 5 and 6 PI, and in acute infections. Blood loss may reduce the erythrocyte count and hematocrit value as much as 50%. The maximum effect on weight gain is seen at 7 days PI. Some of the weight lost from dehydration may be regained quickly, but growth will always lag behind that of uninfected birds. In a few cases, death may result from gangrenous or ruptured cecal pouches. Extracts of infected cecal pouches produce acute blood coagulation and death when injected intravenously into other chicks (77).

Gross Lesions and Histopathology. Small foci of denuded epithelium are seen during maturation of the first generation of schizonts. Hemorrhage is apparent by day 4 PI, as a result of the maturation of second-generation schizonts in the lamina propria. The cecal pouches may become greatly enlarged and distended with clotted blood and pieces of cecal mucosa in the lumen. On days 6 and 7, the cecal core becomes hardened and drier, and may be voided in the feces. Regeneration of the epithelium is rapid and may be complete by day 10. The infection usually can be seen from the serosal surface of the ceca as dark petechiae and foci, which become coalesced in severe infections. The cecal wall is often greatly thickened because of edema and infiltration and later scar tissue.

Microscopically, the first-generation schizonts are widely scattered and mature at 2–3 days PI. Small focal areas of hemorrhage and necrosis may appear near blood vessels of the inner circular muscles of the muscularis layer. Heterophil infiltration of the submucosa proceeds rapidly as the large second-generation schizonts develop in the lamina propria. These are found in clusters or colonies that generally are progeny of a single first-generation schizont.

Epidemiology of Coccidiosis

Natural and Experimental Hosts

Host specificity is strict among the *Eimeria* infecting poultry. The chicken is the only natural host of the 7 species of *Eimeria* (*E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox*, and *E. tenella*) (Table 28.1). Reports of these species of *Eimeria* infecting other birds can be considered spurious. Cross-transmission of *Eimeria* spp. from chickens to other host species has been unsuccessful except where severely immunocompromised birds were used.

Naïve chickens of all ages and breeds are susceptible to infection. However, immunity develops after mild infections, limiting further infection. Newly hatched chicks often have high levels of maternal antibodies but it does not appear that this limits susceptibility. Outbreaks are common at 3–6 weeks of age. In special situations, infections may be seen as early as 1 week of age. Surveys of coccidia in broiler houses in Georgia demonstrated that oocysts of coccidia build up during the growth of a flock and then decline as the birds become immune to further infection (93). This self-limiting nature of coccidial infections is widely known in chickens and other poultry. There is no stimulation of cross-protective immunity between species of coccidia. Thus, more than 1 outbreak of coccidiosis is possible in the same flock, with different species involved in each. Breeder pullets and layer pullets are at greatest risk because they are kept on litter for 20 weeks or more. Normally, the infections with *E. acervulina*, *E. tenella*, *E. mitis*, *E. praecox*, and *E. maxima* are seen at 3–6 weeks of age and then *E. necatrix* at 8–18 weeks of age. *E. brunetti* is seen both early and late.

Coccidiosis rarely occurs in layers and breeders during the laying cycle because of prior exposure to coccidia and resulting immunity. If a flock is not exposed to a particular species early in life or if immunity is depressed because of other diseases, outbreaks may occur after layers are moved to production houses. Outbreaks of any species in layers can depress egg production.

Transmission and Vectors

Ingestion of sporulated oocysts is the only natural method of transmission. Once infected, chickens may shed oocysts in the feces for several days or weeks. The oocysts in feces become infective through the process of sporulation in about 2 days. Susceptible birds in the same flock may ingest the oocysts through litter-pecking or the contamination of food or water.

No natural intermediate hosts exist for the *Eimeria* spp., but oocysts can be spread mechanically by a multitude

of vectors, including insects, contaminated equipment, wild birds, and dust. Oocysts generally are considered resistant to environmental extremes and to disinfectants, although survival time varies with conditions. Oocysts may survive for many weeks in soil, but survival in poultry litter is limited to a few days because of the heat and ammonia released by composting and the action of molds and bacteria. Viable oocysts have been reported from the dust inside and outside broiler houses, as well as from insects in poultry litter (93). The darkling beetle (*Alphitobius diaperinus*), common in broiler litter, is a mechanical carrier of oocysts. Transmission from one farm to another is facilitated by movement of personnel and equipment between farms and by the migration of wild birds, which may mechanically spread the oocysts. New farms may remain free of coccidia for most of the first grow-out of chickens until the introduction of coccidia to a completely susceptible flock. Such outbreaks, often more severe than those experienced on older farms, are often called the *new house syndrome*.

Oocysts may survive for many weeks under optimal conditions but will be quickly killed by exposure to extreme temperatures or drying. Exposure to 55°C or freezing kills oocysts very quickly. Even 37°C kills oocysts when continued for 2–3 days. Sporozoites and sporocysts can be frozen in liquid nitrogen with an appropriate cryopreservation technique, but oocysts cannot be adequately infiltrated with cryoprotectorants to effect survival. The threat of coccidiosis is less during hot, dry weather and greater in cooler, damp weather.

Diagnosis of Coccidiosis

Coccidiosis can best be diagnosed from birds euthanized for immediate necropsy. The entire intestinal tract should be examined first from the serosal side and then from the mucosal side. A microscope should be available for viewing endogenous forms on questionable lesions. The finding of a few oocysts by microscopic examination of smears from the intestine indicates the presence of infection, but not a diagnosis of clinical coccidiosis. Coccidia and mild lesions are present in the intestines of birds 3–6 weeks old in most flocks despite the use of drugs to prevent coccidiosis. The gross lesions of the important species of coccidia are easily recognized by experienced diagnosticians. Questionable lesions should be examined by microscopy. Experimentally, the biological characteristics of *Eimeria* are adequate. Molecular diagnosis by PCR is often practiced when further confirmation is needed, or in surveys (see later). Under practical conditions, a diagnosis of coccidiosis is warranted when the gross lesions are serious. A diagnosis should be based on finding lesions and confirming microscopic stages on necropsy of representative birds from the flock, rather than from culls. Cryptosporidia may be found in chickens or turkeys but are easily differentiated because of their small size and location in the brush border of the mucosal cells (36, 48).

Microscopic Examination

Developing schizonts, gametocytes, and oocysts of coccidia may be seen in smears taken from the suspected lesion. A small amount of mucosal scraping should be diluted with saline on a slide and then covered with a coverslip. Oocysts or macrogametes are most easily seen, but in many cases, the lesion is caused by maturing schizonts. Diagnostic characteristics that are of value include the clusters of the large schizonts of *E. necatrix* and *E. tenella*, the small round oocysts of *E. mitis*, or the large oocysts and gametocytes of *E. maxima*. Presence of clusters of large schizonts in the midgut area is pathognomonic for *E. necatrix*, and a similar finding in the ceca indicates *E. tenella*. Oocysts associated with lesions in the duodenum include *E. acervulina*, or *E. praecox*, and oocysts associated with lesions in the lower gut are *E. mitis* or *E. brunetti*.

Oocyst size and shape are not useful as diagnostic characteristics, because of the extensive overlapping in size of the species. However, the combination of oocyst size, location in the gut, and appearance of the lesions gives considerable confidence in diagnosis. Measurement of 20–30 oocysts of the predominant type of oocyst usually gives a good indication of the size of the unknown species. This information is useful in conjunction with other observations in the identification of species in field cases.

Lesion Scoring

The severity of lesions is roughly proportional to the number of sporulated oocysts ingested by the bird and correlates with other parameters such as reduced weight gain, loss of skin pigmentation, and higher feed conversion. The most commonly used practice is based on the system devised by Johnson and Reid (57). In this technique, a score of 0–4 is assigned to a bird, where 0 = normal and 4 = the most severe lesion. This technique is most useful in experimental infections, where the dose of oocysts and medicaments are controlled, and the species are known. Even though the technique devised by Johnson and Reid was originally designed to score the severity of pure infections in a research setting, many veterinarians and parasitologists have adopted it to gauge the severity of natural infections in field work (21). Even though more than 1 species of coccidia may be present at some time, only 4 separate sections of the intestine are scored: (1) the duodenum (upper), with lesions of *E. acervulina*, (2) the midgut, from the duodenum past the yolk sac diverticulum, with lesions of *E. maxima*, *E. praecox*, *E. necatrix*, and *E. mitis*, (3) the lower small intestine from the yolk sac diverticulum to the cecal junctures, with lesions of *E. mitis*, *E. necatrix*, *E. maxima*, and *E. brunetti*, and (4) the ceca, where only *E. tenella*

lesions are found. The appearance of lesions from different species varies greatly.

Microscopic Scoring

As with lesion scores, the severity of coccidiosis can be judged by the number and appearance of parasite forms seen upon microscopic examination of smears from the mucosa, lumen, or feces. Microscopic scoring is particularly useful for detecting and rating species that do not produce easily seen gross lesions, such as *E. mitis*, and *E. praecox*. Diagnosticians using this technique often use a scale of 0–4 to indicate the number of oocysts seen/unit area.

Droppings Scoring

In laboratory infections, the droppings score may be used in the same manner as lesion score for a rapid and fairly reliable rating of the infection. The extent of abnormal droppings is rated on a scale of 0–4, where 4 = maximum diarrhea, with mucus, fluid, and/or blood. This technique has obvious complications where birds are infected with more than 1 species of *Eimeria*.

Histopathology Methods

Ordinary methods in histopathology are satisfactory for routine examination of tissues infected with coccidia. Staining of sections with H&E or other common histologic stains will demonstrate developing stages and a microscopic lesion scoring method was developed (41). Specialized techniques will identify specific stages: staining with Schiff reagent gives a brilliant red color with the polysaccharide associated with the refractile body and with wall-forming bodies in the macrogamete. Monoclonal antibodies conjugated with fluorescent markers such as fluorescein are very useful in research because specific stages of parts of cells can be readily identified.

Molecular Diagnosis of Coccidiosis

Sequences of DNA unique to each *Eimeria* species are used to design oligonucleotide primers thus allowing selective amplification by PCR. Molecular-based assays for detecting and differentiating *Eimeria* have been described that target genes such as the ribosomal RNA internal transcribed spacer regions 1 or 2 (ITS1, ITS2) (46, 55, 58, 61, 97, 98) or sequence characterized amplified regions (SCAR) (31, 32, 33), the latter identified through a technique termed random amplified polymorphic DNA. Multiplex PCR techniques have been described that combine all primers for each *Eimeria* species in a single tube (31, 58, 86). Newer technology includes real-time (quantitative) PCR (11, 60, 83, 102) and loop-mediated isothermal amplification (LAMP) (8, 9) as an alternative to gel electrophoresis.

The DNA extraction method is one of the most critical steps, whether the parasite source is intestinal tissue, fecal droppings, or litter samples. Several DNA extraction methods have been reported. An oocyst rupturing step is needed, whether by bead-beating or grinding, in a buffer containing EDTA (ethylenediaminetetraacetic acid) to prevent DNA degradation (46, 83, 109). The disrupted oocysts suspension is treated with phenol and chloroform followed by ethanol precipitation or use of commercial DNA extraction kits. The latter contain chaotropic agents to preserve DNA integrity and allow subsequent DNA purification by employing silica-containing mini-columns that selectively bind DNA. The objective in whatever DNA extraction method is chosen is to produce high quality DNA.

Species Composition in Chicken Fecal Droppings

The oocysts are concentrated by flotation in 1 M sucrose or saturated sodium chloride, then treated with 6.0% sodium hypochlorite (100% bleach) for 15 minutes at room temperature on an orbital rocker. The oocysts are then washed by suspension in deionized water and centrifugated at 1,400 *g* for 10 minutes at 4°C. This step is repeated 4–5 times to remove all residual bleach. The oocysts are then suspended in ASL buffer (Qiagen, Germantown, MD) and transferred to a bead-beater tube containing about 200 mg 0.5 mm glass beads. The oocysts are disrupted on a bead-beater (BioSpec Products, Inc., Bartlesville, OK) twice for 2 minutes with placement on wet ice between bead-beatings. *Eimeria* oocyst DNA is extracted using the QIAamp Fast DNA Stool Mini-Kit and then analyzed by ITS1 PCR using primers specific for *E. acervulina, E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox*, or *E. tenella* following described procedures (55). An internal standard is included in each amplification reaction. The *Eimeria* species in a sample are identified by the presence of a target band of predicted size (Figures. 28.5 and 28.6). A laboratory has used this procedure for over 10 years to characterize the species composition of *Eimeria* in litter from commercial broiler houses (55). Depending on location, the most prevalent species in broiler houses in the United States appear to be *E. acervulina* and *E. maxima*, followed by *E. praecox* and *E. tenella*.

Of interest is the discovery of genetic variants and cryptic species of *Eimeria*, termed OTU, that may exist and have escaped detection in traditional species-specific ITS PCR (14, 39, 50). Phylogenetic analysis using mitochondrial sequence data suggest that of these, OTU-X is related to *E. maxima*, OTU-Y is related to *E. brunetti*, and OTU-Z is related to *E. mitis* (84). One group has developed a generic primer pair to amplify a region of the mitochondrial DNA from *Eimeria* infecting chickens and, using this in capillary electrophoresis, can distinguish between all 10 chicken *Eimeria* (*E. acervulina*,

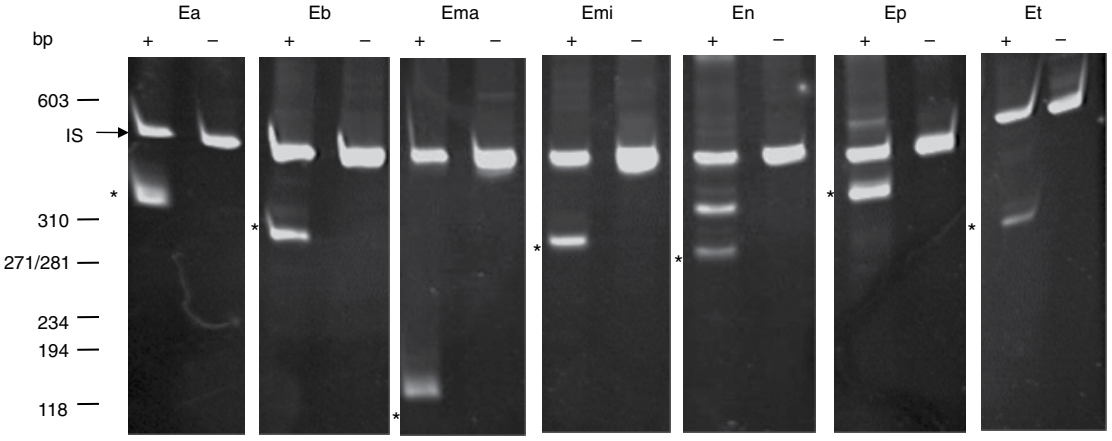


Figure 28.5 PCR amplification of ITS1 ribosomal DNA from positive control *Eimeria* spp. oocyst DNA samples in the presence of species-specific internal standards (IS). +, positive control *Eimeria* oocyst DNA present; –, H_2O control. Ea, *E. acervulina*; Eb, *E. brunetti*; Ema, *E. maxima*; Emi, *E. mitis*; En, *E. necatrix*; Ep, *E. praecox*; Et, *E. tenella*. bp, ψX174 HaeIII-digested DNA markers. Expected size of ITS1 rDNA amplification product for each species is indicated by an asterisk to the left of the respective lane. Reprinted with permission of the journal *Avian Diseases*.

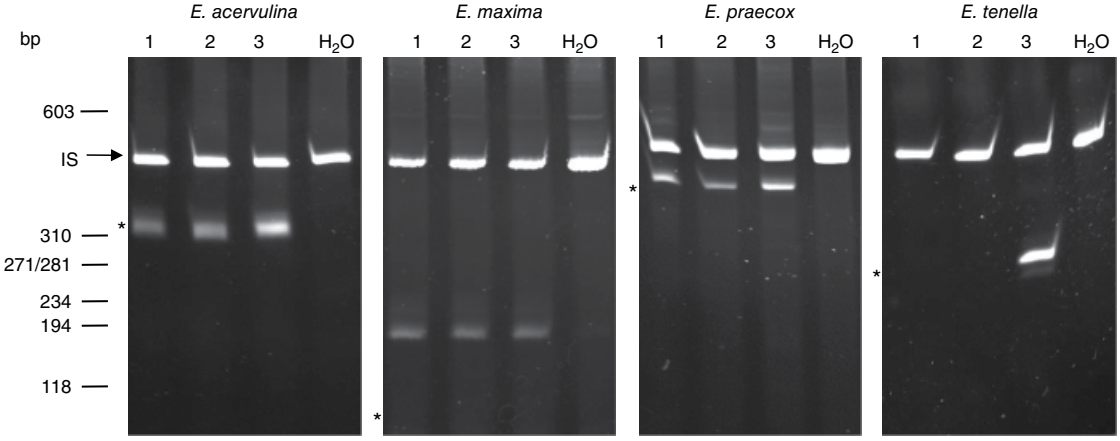


Figure 28.6 Determination of species composition of *Eimeria* oocysts recovered directly from poultry litter obtained from 3 commercial broiler houses by using ITS1 PCR in conjunction with internal species-specific standards (IS). 1–3, DNA from litter oocysts from poultry operations 1–3; H_2O, no DNA control. Headings indicate which *Eimeria*-species specific primers were used. bp, ψX174 HaeIII-digested DNA markers. Expected size of ITS1 rDNA amplification product for each species is indicated by an asterisk to the left of the respective lane. Reprinted with permission of the journal *Avian Diseases*.

E. brunetti, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox*, *E. tenella* and the 3 OTU variants) by size of the amplification product (84). A similar approach has been described for detecting 5–6 species of turkey *Eimeria* in litter from commercial turkey farms (43, 90). A laboratory utilizes 3 pairs of generic primers that amplify a region of *Eimeria* cytochrome c oxidase 1 (Cox1), cytochrome c oxidase 3 (Cox3), or cytochrome b (cytB) (Table 28.2, Figure 28.7). These primers can amplify the respective mitochondrial sequences in DNA extracted from a single isolate or from a mixture of *Eimeria* recovered from poultry litter. These amplicons can then be subjected to metagenomic analysis to identify all the *Eimeria* present in a population, including genetic variants such as OTU-X, -Y, and -Z.

Molecular analysis has the advantage that low populations of some species can be detected, even when gross lesions are not apparent. By targeting conserved regions of mitochondrial genes, one can be reasonably assured of identifying all *Eimeria* strains in a sample.

Procedures Used in Species Identification

Most of the species of coccidia are easily identified by attention to well-established biological characteristics (Tables 28.1 and 28.5). The largest oocysts belong to *E. maxima*, making it easily distinguishable from other species. Some species are identified easily by the location and appearance of gross lesions in concert with the size and location of oocysts or schizonts (*E. acervulina*, *E. maxima*, *E. necatrix*, and *E. tenella*). The lesions produced by

Table 28.2 DNA sequence and annealing temperatures of generic mitochondrial DNA primers useful for amplifying chicken *Eimeria* cytochrome oxidase 1 (Cox 1), cytochrome oxidase 3 (Cox 3), and cytochrome B (cyt B) using polymerase chain reaction (PCR).

MitoDNA Approximate PCR Target Product Size (nt)	Primer	Sequence (5′-3′)	Annealing Temperature (°C)[1]
Cytochrome 900 Oxidase 1 (Cox 1)	*Eimeria* Cox1-F	GTTTGGTTCAGGTGTTGGTTG	55
	Eimeria Cox1-R	ATCCAATAACCGCACCAAGAG	
Cytochrome 470 Oxidase 3 (Cox 3)	*Eimeria* Cox3-F	TTCAGAGAAGYYGGTACAAC	50
	Eimeria Cox3-R	CTACCTYTCCAGAAT	
Cytochrome 1000 B (Cyt B)	*Eimeria* cytB-F	ATGTCTCAAGTGAGATCTC	55
	Eimeria cytB-R	GAGGTAATTGAGMTCC	

[1] For metagenomic analysis using multiplex PCR in a single reaction tube, all 3 primer pairs can amplify the respective mitochondrial gene sequence using a 50°C annealing temperature.

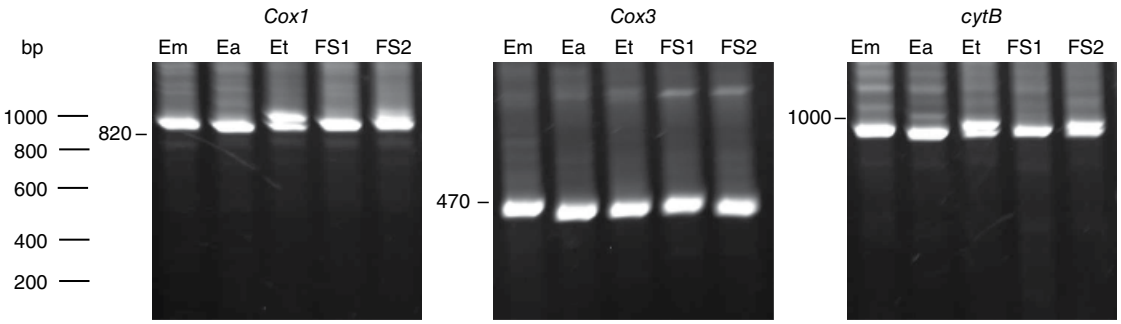


Figure 28.7 PCR amplification of mitochondrial DNA sequences cytochrome c oxidase 1 (Cox1), cytochrome c oxidase 3 (Cox3), or cytochrome B (cytB) of laboratory strains of *Eimeria maxima* (Em), *E. acervulina* (Ea), or *E. tenella* (Et), or a mixture of *Eimeria* spp. oocysts recovered from litter in 2 commercial broiler farms (FS1, FS2). bp, 100 bp DNA markers. Approximate size of amplification product for each mitochondrial DNA sequence is shown to the left of each respective gel image.

other species are not reliably distinct, and oocyst sizes overlap with those of other species. For *E. praecox*, the best method is determination of prepatent period by timed inoculation of birds in laboratory cages. Oocysts produced in less than 90 hours can only be *E. praecox*. *E. brunetti* oocysts are indistinguishable from those of *E. praecox*, *E. tenella*, and *E. necatrix* on size alone, but the location in the lower gut and appearance of the lesions are reliable indicators. *E. mitis* is located in the mid-lower gut, has small subspherical oocysts, and has a prepatent period of 99 hours, separating it from *E. brunetti*.

Poultry develop immunity to reinfection after inoculation with *Eimeria*, but there is no cross protection between species. This strict specificity of immunity has been exploited as a technique for distinguishing species of coccidia for taxonomic purposes. This test requires pure cultures of the test species and test animals reared in isolation for monoimmunization and challenge. When oocysts of 1 *Eimeria* species were used to immunize chickens, the resulting immunity protected against reinfection by the same culture but not against other species. Conversely, birds immunized with other species were not protected against infection with the culture of another species. Overall, the technique is time consuming and requires extensive laboratory isolation facilities and access to pure cultures of known species of coccidia, but may be useful as a research tool when used in concert with other tests or observations.

Preservation of Coccidia for Experimental Work

Droppings or litter collected in the field, or intestinal contents in the diagnostic lab, can be saved for isolation of coccidia in a solution of 2%–4% potassium dichromate. Aeration of oocyst suspensions is necessary to allow

sporulation. A good-quality aquarium pump is highly effective and can be regulated with valves and tubes to service several bottles at one time. For short-term storage, suspensions of oocysts may be refrigerated at temperatures above 4°C. Freezing temperatures quickly kill coccidian oocysts, as do elevated temperatures. Oocysts are quickly killed by storage at 37°C or higher.

Prevention and Control

Control of Coccidiosis by Chemotherapy

Early emphasis in chemotherapy was centered on the treatment of outbreaks with sulfonamides or other compounds as soon as signs of infection were apparent. The concept of preventive medication emerged with the realization that most of the damage is done by the time signs of coccidiosis are widespread in a flock. Today, almost all broiler flocks receive preventive medication (Table 28.3). Treatment is used as a last resort or when other programs have failed. The historical aspects of chemotherapy have been reviewed extensively by McDougald (69). For current information in the United States consult the Code of Federal Regulations (30) for up-to-date information on approved products at https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=558. For current information

Table 28.3 Preventive anticoccidials approved by the US Food and Drug Administration (FDA) for use in feed formulation. (Historical and scientific interest only. Not all products are available.)

Trade or Empirical Name, Approval Label (Manufacturer)	Trade Name	First Approval by FDA	Drug Withdrawal (Days before Slaughter)
Sulfaquinoxaline, 0.015%–0.025% (Merck)	SQ, Sulquin	1948	10
Nitrofurazone, 0.0055% (Hess & Clark; Smith–Kline)	nfz, Amifur	1948	5
Arsanilic acid or sodium arsanilate, 0.04% for 8 days (Abbott)	Pro-Gen	1949	5
Butynorate, 0.0375% for turkeys (Solvay)	Tinostat	1954	28
Nicarbazin, 0.0125% (Merck)	Nicarb	1955	4
Furazolidone, 0.0055%–0.011% (Hess & Clark)	nf-180	1957	5
Nitromide, 0.025% + sulfanitran, 0.03% + roxarsone, 0.005% (Solvay)	Unistat-3	1958	5
Oxytetracycline, 0.022% (Pfizer)	Terramycin	1959	3
Amprolium, 0.0125%–0.025% (MSD–AGVET)	Amprol	1960	0
Chlortetracycline, 0.022%	(American Aureomycin Cyanamid)	1960	(See feeding restrictions)
Zoalene, 0.004%–0.0125% (Solvay)	Zoamix	1960	(Higher levels, 5 days)
Amprolium, 0.0125% + ethopabate, 0.0004/0.004% (Merck)	Amprol Plus, Amprol Hi-E	1963	0
Buquinolate, 0.00825% (Norwich–Eaton)	Bonaid	1967	0
Clopidol or meticlorpindol, 0.0125%–0.025% (A. L. Laboratories)	Coyden	1968	0 days at 0.0125%; 5 days at 0.025%
Decoquinate 0.003% (Rhone–Poulenc)	Deccox	1970	0
Sulfadimethoxine, 0.0125% + ormetoprim, 0.0075% (Hoffmann–La Roche)	Rofenaid	1970	5
Monensin, 0.01%–0.0121% (Elanco)	Coban	1971	0
Robenidine, 0.0033% (American Cyanamid)	Robenz, Cycostat	1972	5
Lasalocid, 0.0075%–0.0125% (Hoffmann–La Roche)	Avatec	1976	3
Salinomycin, 0.004%–0.0066% (Agri-Bio)	Bio-Cox	1983	0
Halofuginone, 3 ppm (Hoechst–Roussell Agri-Vet)	Stenorol	1987	5
Narasin, 54–72 g/T (Elanco)	Monteban	1988	0
Maduramicin, 5–6 ppm (American Cyanamid)	Cygro	1989	5
Narasin + nicarbazin, 54–90 g/T (Elanco)	Maxiban	1989	5
Semduramicin, 25 ppm (Pfizer)	Aviax	1995	0
Diclazuril, 1 ppm (Schering-Plough)	Clinacox	1999	0

in the European Union consult the European Food Safety Authority at https://ec.europa.eu/food/safety/animal-feed/feed-additives/eu-register_en.

Characteristics of Anticoccidial Drugs

In spite of a higher demand for broiler chickens raised without antibiotics and/or drugs, fermentation-derived ionophore and chemically synthetized anticoccidials remain the backbone of coccidiosis control (20, 80). All types of drugs used for coccidiosis control are unique in their mode of action, the way in which parasites are killed or arrested, and the effects of the drug on the growth and performance of the bird. Following are the most important characteristics.

Spectrum of Activity. There are several important species of coccidia in chickens, several more in turkeys, and many others in other hosts. A drug may be efficacious against 1 or several of these parasites; very few drugs are equally efficacious against all.

Mode of Action. Each class of chemical compound is unique in the type of action exerted on the parasite, and even in the developmental stage of the parasite most affected. The chemical mode of action of some drugs is known to be a highly detailed event, and the action of other drugs remains a mystery. The sulfonamides and related drugs compete for the incorporation of para-aminobenzoic acid and metabolism of folic acid. Amprolium competes for absorption of thiamine by the parasite. The quinoline coccidiostats like clopidol inhibit energy metabolism in the cytochrome system of the coccidia. The polyether ionophores upset the osmotic balance of the protozoan cell by altering the permeability of cell membranes for alkaline metal cations.

Endogenous Stage Affected. The coccidia are prone to attack by drugs at various stages in the development in the host. Totally unrelated drugs may attack the same stage of parasite. The quinolones and ionophores arrest or kill the sporozoite or early trophozoite. Nicarbazin, robenidine, and zoalene destroy the first- or second-generation schizonts, and the sulfonamides act on the developing schizonts and on the sexual stages. Diclazuril acts in early schizogony with *E. tenella* but is delayed to later schizogony with *E. acervulina* and to the maturing macrogamete with *E. maxima*. The time of action in the life cycle has been construed as having significance in the use of drugs in certain types of programs in which immunity is desired, but there is no good evidence that this is the case under field conditions.

Coccidiocidal vs. Coccidiostatic Medications. Some drugs kill the parasite, but others only arrest development (80). When coccidiostatic medication is withdrawn, arrested parasites may continue to develop and contaminate the environment with oocysts. In such cases, a relapse of coccidiosis is possible. In general, the coccidiocidal drugs have been more effective than those that are coccidiostatic.

Effects of Drugs on the Target Animal. Most compounds used in animal feeds have good selective toxicity, providing toxicity for the parasite but being nontoxic to vertebrates. Unfortunately, toxicity and side effects of drugs on the host are possible where formulation errors lead to overdose. Sometimes, a drug may exhibit side effects at the recommended use level. Some of the toxicity may be the result of management, genetics, nutrition, or other interaction, and in other cases, the margin of safety is just too narrow. Environmental interaction is possible with nicarbazin, which interacts with high temperatures and high humidity to produce excess mortality. Nicarbazin also has adverse effects in layers, causing a bleaching of brown-shelled eggs, mottling of yolks, reduced hatchability, and reduced egg production. The ionophores are highly toxic at elevated doses, causing a transient paralysis in mild overdoses or a permanent paralysis and mortality in more severe cases. Monensin was once thought to interact with methionine to reduce feather growth, but this relationship is not clear. Under some conditions, lasalocid will stimulate water consumption and excretion, resulting in a wet litter. With slight overdoses, most of the ionophores depress weight gain under laboratory conditions. A withdrawal period of 5–7 days is often practiced to allow compensatory growth to make up for the lost gain (72). The ionophores are known for their toxicity to other animals. For example, monensin and salinomycin are highly toxic to horses. The LD_{50} for monensin in horses is about 2 mg/kg body weight. Salinomycin and narasin are highly toxic to turkeys and cause excessive mortality at the levels recommended for use in chickens, whereas monensin and lasalocid are well tolerated in turkeys at the level used for chickens.

Programs for Use of Anticoccidial Drugs in Broilers

The objective in broilers is to produce the maximum growth and feed efficiency with minimum of disease. In long-lived birds like table-egg layers and breeders kept on the floor, the objective is to protect against early acute infections and to provide long-lasting immunity. The choice of a product or program may depend on the season of the year or other factors which affect exposure. The following several types of programs are practiced.

Continuous Use of a Single Drug. Often, a single product will be used from day 1 to slaughter, or with a withdrawal period of 3–7 days. Most products are approved for use until slaughter, but producers withdraw medication for economic or other reasons, such as the compensatory gain previously mentioned when ionophores are withdrawn from the feed.

Shuttle or Dual Programs. The use of 1 product in the starter feed and another in the grower feed is called a shuttle program in the United States and a dual program in other countries. Some programs might contain as many as 3 drugs, with 1 drug in the starter, another in the grower, and yet another in the finisher. The shuttle program usually is intended to improve coccidiosis control. Intensive use of the polyether ionophore drugs for many years has produced strains of coccidia in the field that have reduced sensitivity to them. It is a common practice to use another drug such as nicarbazin, diclazuril, or clopidol in either the starter or grower feed to bolster the anticoccidial control and take some pressure off of the ionophore. In other cases, the order of these drugs is reversed. The use of shuttle programs is thought to reduce buildup of drug resistance. Historically, a high percentage of producers use some type of shuttle program.

Rotation of Products. It is considered sound management to make periodic changes in anticoccidial drug use. Most producers in the United States consider changes in the spring and fall. Rotation of drugs may improve productivity because of the build-up of isolates or species of coccidia that have reduced sensitivity after products have been used for a long time. Producers often notice a boost in productivity for a few months after a change of anticoccidial drugs. A similar effect has been demonstrated when live coccidiosis vaccines are used because all vaccines contain strains of *Eimeria* species susceptible to all the anticoccidial drugs currently on the market (66, 87). The seasonal rotation of products is intended to correspond with the intrinsic properties of the drugs. In the United States, nicarbazin is used principally in the cooler months of the year, which also corresponds with maximum coccidiosis challenge. In the summer months, coccidiosis challenge tends to be milder, so weaker anticoccidials or live coccidiosis vaccines are preferred.

Drug Resistance

The development of tolerance to drugs by coccidia after exposure to medication is the most serious limitation to the effectiveness of these products (23). Surveys reveal widespread drug resistance in coccidia in the United States, South America, and Europe (44, 51, 52, 59, 68, 74, 75). Even though coccidia develop less resistance to some drugs than others, long-term exposure to any drug will produce a loss in sensitivity and, eventually, resistance. Drug resistance is a genetic phenomenon, and when established in a line of coccidia, will remain for many years, or until selection pressure and genetic drift force return to sensitivity in the population. Drugs such as the quinolones like clopidol have a well-defined mode of action, and resistance develops quickly as coccidia are selected with cytochromes, which do not bind as readily to the drug. As an example, the emergence of resistance to decoquinate has been studied and documented in commercial broilers (108). The polyether ionophores, in contrast, have a more complicated mode of action involving the mechanisms of active transport of alkaline metal cations across cell membranes, and it has taken many years for coccidia to become tolerant, and in some cases, completely resistant. Many other drugs appear to be intermediate in selecting resistance in coccidia. The primary defense against drug resistance is the use of less intensive programs, shuttle programs, and frequent rotation of drugs and vaccines. Rotation of programs, used alone, will not prevent the development of resistance. In some instances, coccidia are able to become resistant to drugs after only a few months of use, and once developed, drug resistance is slow to dissipate. In recent years it has become a common practice to incorporate live coccidiosis vaccines in the rotation program, reasoning that the drug sensitive vaccine strains tend to replace the drug resistant wild types. This approach has had demonstrable effects on the drug sensitivity profile on farms where it has been practiced (66, 87).

Anticoccidial Drugs Used for Broilers in the United States

The products currently approved for use in chickens in the United States are listed in Table 28.3. Not all are still available commercially, but the approvals remain. Those used at present include monensin, narasin, salinomycin, semduramicin, lasalocid (polyether ionophores), diclazuril, nicarbazin, amprolium, decoquinate, clopidol, sulfadimethoxine/ormetoprim, and sulfaquinoxaline. A product combining narasin with nicarbazin is also used to take advantage of synergism between these molecules. Other products listed with approvals but lacking in significant activity include chlortetracycline and oxytetracycline. These products may prevent mortality from coccidiosis when given at high levels because of antibacterial activity but are not of much value in general use. The polyether ionophores became the drugs of choice for prevention of coccidiosis in 1972 and remain the most extensively used today. Other drugs, such as clopidol, diclazuril, halofuginone, nicarbazin, and robenidine, are used mostly in shuttle programs as an adjunct to the ionophores. In spite of the challenges posed by drug resistance anticoccidial drugs still remain the primary means of coccidiosis control worldwide (20, 80).

Unlike the European Union, in the United States ionophore anticoccidials are classified as antibiotics and therefore cannot be used in poultry sold with any claims to having been raised without antibiotics, raised without antibiotics (RWA), no-antibiotics ever (NAE), organic, etc. This creates a serious problem for the long-term prevention and control of coccidiosis in poultry because producers must rely exclusively on chemically synthetized anticoccidials and live coccidiosis vaccines.

Other diseases are made worse as a result, particularly necrotic enteritis. Chemically synthetized anticoccidials have no anticlostridial activity, and it is well known that live infection (even vaccination) with coccidian exacerbates clostridial infections. The issues related to coccidiosis control in ABF production were previously discussed (22). For additional review of the health challenges posed by raising birds without antibiotics please refer to the new section on "Disease Prevention and Control in ABF Production."

Immunization During Medication Programs in Broilers

Chickens develop immunity to coccidiosis after natural exposure and may even develop substantial immunity while receiving anticoccidial drugs (24, 47). The poultry industry has learned to take advantage of this phenomenon, practicing longer withdrawal programs of 2–3 weeks or even longer in some instances.

Coccidiosis Vaccines

Considerable research on coccidiosis vaccines in recent years has produced new live products. Table 28.4 lists the live coccidiosis vaccines currently approved for sale in broilers, breeders, and layers in the United States. Increasingly, these products are finding use in the broiler industry. When live sporulated oocysts of coccidia are given to chickens at an early age, immunity against the species contained in the inoculum is stimulated (95). The pathogenicity of coccidia in these vaccines is attenuated largely by the size of the dose and by the means of administration. Some vaccines sold in the United States or internationally contain modified live coccidian, attenuated by genetic selection for short life cycle development (precociousness). The use of coccidiosis vaccines in broilers has been limited by the possibility of adverse reactions, particularly a negative effect on feed efficiency. More recent advances in administration methods have overcome much of this limitation. The Coccivac products pioneered in this growing family now includes several other live vaccines produced by various manufacturers in many countries (Coccivac, Immucox, Hatch-pack Cocci-III, Paracox, Livacox, Bio-Coccivet, Advent, In-Ovo Cox, Eimeriavax, Evalon, and others). Some live vaccines have been prepared from attenuated lines of oocysts (e.g., Hatch-pack Cocci-III, Paracox 7, and Livacox 7). These vaccines normally contain 3 or more species of *Eimeria*, which are thought to be the most important. The *Eimeria* infecting poultry immunize only against themselves, so that the vaccine will only protect against the included species. In the case of broiler vaccines, these are *E. acervulina*, *E. maxima*, and *E. tenella*. The success of some vaccines may depend more on a novel administration technique rather than attenuation. One experimental product was encapsulated in alginate beads and then mixed into the starter feed for "trickle administration" (56). Other methods presently used are spray cabinet administration, direct eye-spray, *in ovo* inoculation, or spraying the oocysts directly into feed or mixing in water in the poultry house. One product was mixed into gels, which were placed into the chick boxes for the chicks to eat (27). Other experimental approaches include inoculation of parasites or antigens *in ovo* and inoculation via the yolk sac diverticulum.

Table 28.4 Live coccidiosis vaccines approved for sale in the United States.[1]

Vaccine	Target Bird	Manufacturer
Coccivac – B[2]	Broilers and roasters	Merck
Coccivac – B52	Broilers and roasters	Merck
Coccivac – D[b]	Layers and breeders	Merck
Coccivac – D2	Layers and breeders	Merck
Coccivac – T[2]	Turkeys	Merck
Immucox for Chickens I	Broilers and roasters	Ceva
Immucox for Chickens II	Layers and breeders	Ceva
Immucox for Turkeys	Turkeys	Ceva
Advent	Broiler chickens only	Huvepharma
Inovocox	Broiler chickens only	Huvepharma
Inovocox EM1	Broiler chickens only	Huvepharma
Hatch-pack Cocci-III	Broiler chickens only	Boehringer-Ingelheim

[1] All vaccines for broilers contain at least *Eimeria acervulina, E. maxima* and *E. tenella* and all vaccines for breeders contain at least *E. acervulina, E. brunetti, E. maxima, E. necatrix and E. tenella*.
[2] Currently not produced by the manufacturer.

Several reviews have been published that deal with the use of vaccines for the prevention of coccidiosis in poultry (25, 101, 106).

In the United States, administration of live coccidiosis vaccines worked well, particularly during the summer months when the arsenical compound, roxarsone, was available. Following the voluntary halt in sales of roxarsone by the manufacturer (at that time Pfizer, Inc.) over concerns of conversion of organic arsenic to inorganic arsenic (a recognized carcinogen) live coccidiosis vaccines struggled. Roxarsone is classified by the US FDA (Food and Drug Administration) as a nonantibiotic growth promoter with some anticoccidial activity against *E. tenella*. McDougald *et. al.* (76) demonstrated that roxarsone had good anticoccidial activity against *E. tenella*

and *E. brunetti* and that the performance improvements seen when roxarsone is administered to broiler chickens in combination with anticoccidals came primarily from its anticoccidial properties. Other research (49) has demonstrated that roxarsone also has significant anticlostridial activity. In order to remedy the performance issues encountered following the halt in sales of roxarsone, vaccine manufacturers and broiler producers have resorted to what it is now known as a hybrid program or a bio-shuttle program. In this program, an anticoccidial drug (an ionophore or a chemically synthetized anticoccidial in non-NAE production or a chemically synthetized anticoccidal in NAE production) is added to the feed at a low concentration for a specified period of time to reduce oocyst shedding and prevent adverse effects on performance (67). In order to prevent interference with immunity development the anticoccidial is generally added to the grower feed starting at 16–18 days of age.

Another approach to coccidiosis control includes the use of coccidian proteins, which have a protective effect when administered to chicks. These proteins can be made in quantity if the gene that encodes the protein is cloned into a bacterial cell. Research identified broad-spectrum antigens and appropriate routes of administration. One product based on this approach is CoxAbic, which is composed of an antigen developed from a monoclonal protein produced in the gametocyte of *E. maxima*. CoxAbic is given to hens in 2 doses to confer maternal protection during the first 3 weeks of brooding (110).

Control Programs Used in Breeders and Layers

Pullets started on the floor and later reared as caged layers are not as dependent on immunity to coccidiosis as are floor layers. Like broilers, they are often protected against coccidiosis with preventive medication, until they are moved to cages. Breeder pullets that will be kept on the floor during lay should have immunity to coccidiosis and may be vaccinated. Controlled exposure vaccination can be given by means of commercially produced live products (described above). Natural or accidental exposure assumes the presence of oocysts of important species. A broad-spectrum anticoccidial drug is sometimes given at the lowest approved level to provide protection for 6–12 weeks. Some producers reduce the level of the drug during the final 4 weeks in a step-down program, although as mentioned previously, chickens tend to develop immunizing infections despite the presence of the drug. This approach is aimed at allowing moderate numbers of coccidia to develop in the birds, stimulating the host immune system to protect against serious outbreaks. Such exposure usually is sufficient to protect against all species. Outbreaks of *E. necatrix* have sometimes occurred at 8–16 weeks, after all medication has been stopped. Climatic and seasonal conditions may add to the inherent uncertainties of this method.

Disinfection and Sanitation

Older recommendations for coccidiosis control often suggest directions for sanitation and disinfection to prevent outbreaks. Most of these are no longer considered valid because: (1) there have been too many failures in such programs; (2) oocysts are extremely resistant to common disinfectants; (3) complete house sterilization is never complete; and (4) an oocyst-sterile environment for floor-maintained birds could prevent early establishment of immunity and allow late outbreaks. In addition to disinfectants normally used in poultry houses, specific products have been used to target the oocyst for destruction.

Chickens reared in banks of cages often suffer outbreaks of coccidiosis. The concentration of susceptible birds in stacked laying cage batteries, and the presence of mechanical vectors, such as flies, make birds particularly vulnerable to infection.

Coccidiosis in Turkeys

Coccidiosis in turkeys is common but is often unrecognized because the lesions in turkeys are less distinctive than those in chickens. Several species infect turkeys, but only 4 are economically important. Typical signs of coccidiosis in turkeys are watery or mucoid diarrhea, blood-streaked feces, ruffled feathers, anorexia, and general signs of illness. Recovery is quick, so lesions could go undetected at necropsy. Several species have been found in commercial turkey farms throughout the United States (29, 90). Coccidia infecting domestic turkeys also infect wild turkeys. The common species of *Eimeria* found in commercial turkey operations are *E. meleagrimitis*, *E. adenoeides*, *E. meleagridis*, and *E. dispersa*. *E. gallopavonis* is seen in a low percentage of flocks. Range-rearing of turkeys can add significantly to the exposure of wildlife to coccidiosis and other diseases.

Turkeys of all ages are susceptible to primary infection, but birds older than 6–8 weeks are considered more resistant to the disease, presumably because they have acquired natural immunity. Older turkeys can suffer weight loss and morbidity but are not killed as easily as are younger birds. Reductions in the rate of weight gain are often unrecognized until adequate coccidiosis control measures have been instituted.

Etiology

Seven species of *Eimeria* have been described in turkeys in the United States. Identifying characteristics of each species are listed in Table 28.5. *E. innocua* and *E. subrotunda* have been so rarely recovered that further work will be required to re-establish the validity of these species.

Table 28.5 Diagnostic characteristics of *Eimeria* in turkeys.

Characteristics	Species						
	E. adenoeides	*E. dispersa*	*E. gallopavonis*	*E. innocua*	*E. meleagridis*	*E. meleagrimitis*	*E. subrotunda*
Lesions *Occasional lesions* *Parasites no lesions* *Species distinctive*							
Macroscopic lesions	*Liquid feces with mucus and flecks of blood,* loose whitish cecal cores	*Cream–colored serosal surface, dilation of intestine, yellowish mucoid feces*	*Edema, ulceration of mucosal ileum, yellow exudate, flecks of blood in feces*	*None*	*Cream–colored ceca, formation of caseous plug, a few petechial hemorrhages*	*Spotty congestion and petechiae from duodenum to ileum, dilation of jejunum, casts*	*None*
Length × width (μm) *Length =* *Width =*	*AV = 25.6 × 16.6* *18.9–31.3* *12.6–20.9*	*AV = 26.1 × 21.0* *21.8–31.1* *17.7–23.9*	*AV = 27.1 × 17.2* *22.7–32.7* *15.2–19.4*	*AV = 22.4 × 20.9* *18.57–25.86* *17.34–24.54*	*AV = 24.4 × 18.1* *20.3–30.8* *15.4–20.6*	AV = 19.2 × 16.3 15.8–26.9 13.1–21.9	*AV = 21.8 × 19.8* *16.48–26.42* *14.21–24.44*
Oocyst shape and index length/width (μm)	*Ellipsoidal* 1.54	*Broadly oval* 1.24	*Ellipsoidal* 1.52	*Subspherical* 1.07	*Ellipsoidal* 1.34	*Ovoid* 1.17	*Subspherical* 1.10
Minimum sporulation	*24 hours*	*35 hours*	15 hours	Under 45 hours	*24 hours*	18 hours	48 hours
Prepatent period (minumum)	*103 hours*	120 *hours*	105 *hours*	114 *hours*	*110 hours*	*103 hours*	95 *hours*
Refractile body	*Yes*	*No*	*Yes*	*No*	*Yes*	*Yes*	*No*
Pathogenicity	+ + + +	+	+ + + +	*None*	None	+ + + +	*None*

Besides the *Eimeria*, species reported from the turkey include *Isospora* and *Cryptosporidium* (see the next section). The *Eimeria* spp. are strictly intestinal, contrasting with *Cryptosporidium*, which may cause both respiratory and intestinal infection (48). The most pathogenic species of *Eimeria* are *E. adenoeides*, *E. meleagrimitis*, *E. gallopavonis*, and *E. dispersa*. Differentiation of oocysts of the pathogenic species from those of milder species is difficult because some of the species are poorly described. For instance, differentiation of *E. adenoeides* and *E. meleagridis* is difficult because they inhabit the ceca and have oocysts that are fairly similar.

Eimeria adenoeides Moore and Brown 1951

Gross lesions appear primarily in the ceca but extend to the lower small intestine and cloaca. Cecal contents are often hardened into a core consisting of mucosal debris. The cecal and/or intestinal wall is often swollen and edematous. Oocysts are ellipsoidal and have a high shape index length/width (5/1.54). The oocysts average 25.6 × 16.6 µm. Typical oocysts of *E. adenoeides* are more pointed at 1 end than other species, aiding in recognition.

Pathogenesis. *E. adenoeides* is considered the most pathogenic of the turkey coccidia. Experimental infections of 25,000–100,000 sporulated oocysts in young poults may produce mortality up to 100% on days 5 or 6 PI. Turkeys several months old may lose considerable weight after infection. Outward signs of infection are apparent after 4 days PI. Feces are frequently fluid, may be blood-tinged, and may contain mucous casts. White or gray caseous cores may be produced in the ceca. In mild to moderate infections the cecal contents may be viscous and filled with oocysts. The lesions heal quickly, so no evidence of infection may be seen soon after the acute phase unless the cecal core remains.

Gross Lesions and Histopathology. By day 4 PI, the intestine may suffer congestion, edema, petechial hemorrhage, and mucous secretion. Five days PI, the ceca contain white caseous material, which condenses into a core. The serosal surface of the intestine appears pale and may be edematous and dilated.

Invasion of the submucosa by heterophils occurs throughout the intestine, especially in the lower small intestine, ceca, and rectum. Epithelial cells at the tips of villi are most often invaded, but deep glands may also be parasitized. Edema is common deep in the muscular layers as the infection progresses. After day 5, regeneration of lost mucosa is rapid.

Eimeria dispersa Tyzzer 1929

The small intestine, principally the midgut region, is commonly parasitized, but some infection may occur in the cecal necks. Oocysts are large (average, 26.1 × 21.0 µm) and broadly ovoid (index = 1.24). Sporozoites lack a refractile body, and the oocyst wall is distinctively contoured and lacks the double wall common to other species. The prepatent period is 120 hours, longer than for other species.

Pathogenesis. Compared with some of the other species, the pathogenicity is low, but infection with 10^6–2×10^6 sporulated oocysts can cause reduction in rate of weight gain and diarrhea in young poults.

Natural and Experimental Hosts. The natural host of *E. dispersa* is apparently the Bobwhite quail, in which the parasite is more pathogenic than in turkeys. This is the only *Eimeria* in chickens or turkeys known to infect more than 1 species. Experimental inoculation has produced patent infections in domestic and wild turkeys, Hungarian partridge (*Perdix perdix*), ruffed grouse (*Bonasa umbellus*), sharp-tailed grouse (*Pediocetes phasianellus campestris*), Japanese and Bobwhite quail, and other pheasants. Infection in chickens often requires immunosuppression.

Gross Lesions and Histopathology. Three days PI, the duodenum appears cream-colored on the serosal surface. Later, the entire intestine may become dilated with thickening of the wall. Dilation continues on the fifth and sixth days, along with secretion of a cream-colored mucoid material containing denuded epithelium from the duodenum. Individual villi may become so dilated as to be visible to the naked eye.

The duodenum shows edema and progressively increasing congestion of capillaries. Separation of the epithelium and basement membranes may result in the lamina propria being exposed to a fibrin network or an open fluid-filled space. Necrosis is common on distal tips of villi. Parasites do not invade the glands.

Eimeria gallopavonis Hawkins 1952

Lesions of *E. gallopavonis* are restricted to the area posterior to the yolk sac diverticulum and tend to be most severe in the lower small intestine and large intestine. Some foci of infection may be seen in the ceca. Oocysts are elongate, averaging 27.1 × 17.2 µm (index = 1.52). Differentiation of this species from *E. adenoeides* may be difficult because their oocysts are similar in appearance.

Pathogenesis. Experimental infection with 5×10^4–2×10^5 sporulated oocysts causes mortality of 10%–100% in 2- to 6-week-old poults. Mortality occurs 5–6 days PI.

Gross Lesions and Histopathology. On days 4 and 5 postexposure, second- and third-generation schizonts are numerous in the ileum, necks of the ceca, and rectum.

By day 6 the rectum is parasitized with gamonts. Marked inflammation and edema are seen on days 5–6. By days 7–8, the lumen contains sloughed white caseous material containing oocysts and flecks of blood.

Eimeria meleagridis Tyzzer 1929

Oocysts of *E. meleagridis* are ellipsoidal, averaging 24.4 × 18.12 μm (index 1.34). The ceca are the site of infection, where they may be seen, associated with yellow-white caseous cores. However, this species causes little damage and would pass unnoticed. Oocysts resemble those of other species in the ceca, and differentiation is difficult without PCR or other tools.

Pathogenesis. Most studies have characterized this species as almost nonpathogenic.

Histopathology. Edema and lymphocytic infiltration may be seen histologically. First-generation schizonts develop in the surface epithelium of the small intestine, but later stages occur in the cecal epithelium.

Eimeria meleagrimitis Tyzzer 1929

Infection with *E. meleagrimitis* is primarily duodenal but may spread throughout the small intestine in heavy infections. This is the most pathogenic of the upper-intestinal coccidia in turkeys. The oocysts are small (average, 19.2 × 16.3 μm) and ovoid (index = 1.17).

Pathogenesis. Experimental infection of young poults produces morbidity and mortality, depresses weight gain, causes dehydration, and general unthriftiness. Inoculation of 2×10^5 sporulated oocysts produces some mortality and morbidity, but this species is not as pathogenic as *E. adenoeides*.

Gross Lesions and Histopathology. Infected birds show signs of dehydration. In the duodenum, enlargement and congestion are marked on days 5 and 6 of infection. Large amounts of mucus and fluid may be found in the lumen. Feces may contain occasional flecks of blood and mucous casts 5–7 days PI.

The tips of villi are most commonly parasitized, and the epithelium may be completely denuded, although hemorrhage is rare. Capillaries of the villi are markedly dilated and the tips edematous. Eosinophilic infiltration may begin as early as 2 hours PI and is extensive at the height of the infection.

Eimeria subrotunda Moore, Brown, and Carter, 1954

Poults inoculated with *E. subrotunda* produced no gross lesions and it was considered nonpathogenic (82). Parasites develop primarily in the upper small intestine anterior to the yolk stalk diverticulum and are located in the epithelial cells in the tips of the villi. Oocysts are subspherical (index = 1.099) and average 21.77 × 19.81 μm. Oocysts have no refractile granule.

Eimeria innocua Moore and Brown 1952

Eimeria innocua is said to produce no gross lesions and is considered nonpathogenic (81). The area parasitized is the small intestine, in the epithelial cells at the tips of villi. Oocysts are subspherical (index = 1.072), and average 22.4 × 20.9 μm. Oocysts have no polar granule. The prepatent period for oocyst production is 114 hours.

Undescribed Species

Several species of coccidia that do not fit descriptions of established species have been isolated from wild or domestic turkeys but have not been adequately described or named. Surveys are needed, with development of PCR primers, to address this issue adequately.

Prevention and Control of Turkey Coccidiosis

Treatment

Treatment drugs for use in turkeys include amprolium (0.012%–0.025% in water) or a sulfonamide (dosage depending on drug, often given 2 days on drug, 3 days off, and 2 days on, sometimes repeated a second week). The toxicity of sulfonamides limits their usefulness for turkeys.

Anticoccidial Drugs

Most producers use anticoccidial drugs continuously in the feed for at least 8 weeks, while poults are confined to a brooding facility. Drugs are often discontinued after the birds are moved to range or grow-out facilities. The drugs used in turkeys are the same as those used in chickens. However, the use levels are sometimes widely different. Consult a current Code of Federal Regulations (30) for available products and specific conditions of use.

Prevention with Planned Immunization

Turkey poults may be immunized against turkey coccidia using products containing a small number of live oocysts of the important species of *Eimeria*, although these products are not available in all countries. One product, Coccivac-T has been available in the United States, and another has been produced in Canada for the worldwide market (Immucox T CEVA). These products are sprayed onto the poults at the hatchery, causing a mild infection and resulting immune response.

Coccidiosis in Geese

Numerous species of coccidia have been described from domestic and wild geese. The most prevalent and damaging in commercial flocks are *E. truncata*, which causes

renal coccidiosis, and *E. anseris*, which causes intestinal coccidiosis. Renal coccidiosis may produce high mortality from the blockage of kidney function in young goslings. Coccidia may be introduced into domestic flocks by migrating and resident wild geese.

Eimeria truncata Raillet and Lucet 1891

Flock losses caused by renal coccidiosis have been reported as high as 87% in Iowa. Geese aged 3–12 weeks are affected, although the disease is most acute in goslings. Signs of infection include depression, weakness, diarrhea with whitish feces, and anorexia. Eyes become dull and sunken, and wings are drooped. Survivors may show vertigo and torticollis. Birds quickly develop immunity to reinfection.

Oocysts and endogenous stages of *E. truncata* are found only in the kidneys or cloaca near the junction of the ureters. Diagnosis of *E. truncata* is ensured by finding the distinctive oocysts in the kidneys and ureters. Oocysts average 21.3 × 16.7 µm and have truncated ends.

Natural and Experimental Hosts. Although thorough cross-infection experiments have not been performed in most cases, *E. truncata* has been reported from domestic and wild geese, ducks, and swans.

Gross Lesions and Histopathology. The kidneys may be enlarged and protrude from the sacral bed. The normal reddish brown is altered to light grayish yellow or grayish red. Pinhead-sized grayish white foci or hemorrhagic petechiae may be seen; they contain numerous oocysts and accumulations of urates. Invading and growing parasites may distort the kidney tubules to many times the normal size. Eosinophils and signs of necrosis are present in focal areas.

Eimeria anseris Kotlan 1933

The oocysts average 19.2 × 16.6 µm. Differentiation from the 14 species listed by Pellerdy (88) may be difficult.

Pathogenesis. *E. anseris* may produce anorexia, tottering gait, debility, diarrhea and morbidity, and sometimes mortality. The small intestine becomes enlarged and filled with thin, reddish-brown fluid. Catarrhal inflammatory lesions are most intense in middle and lower portions of the small intestine. There may be large whitish nodules or a fibrinous diphtheroid necrotic enteritis. Under dry pseudomembranous flakes, the oocysts and endogenous stages of the parasite are found in large numbers. Parasite stages invade epithelial cells of the posterior half of the intestine in closely packed rows. Developing gametocytes penetrate deeply into subepithelial tissues of the villi.

Treatment. Various sulfonamide drugs have been used in treatment of renal and intestinal coccidiosis of geese. Some studies indicated a favorable response, but unfortunately, there have been no controlled experiments.

Coccidiosis in Ducks

Coccidiosis in ducks is sporadic but is sufficiently frequent to warrant more attention from researchers. Cases involving moderate to heavy mortality have been reported on domestic duck farms in New York, New Jersey, Hungary, and Japan. Coccidia were recovered from every farm sampled on Long Island, New York. Clinical and subclinical coccidiosis appears to be common and can produce morbidity and mortality as well as poor performance.

Species of Coccidia and Descriptions

Although 13 species of coccidia have been reported from domestic and wild ducks, the descriptions are often insufficient to use in diagnosis. Many species will remain in doubt until further work is completed. Coccidia in ducks may be of *Eimeria, Wenyonella*, or *Tyzzeria*. The genus can be determined readily from the sporulated oocyst: *Eimeria* have 4 sporocysts, each containing 2 sporozoites; *Wenyonella* have 4 sporocysts, each with 4 sporozoites; and *Tyzzeria* have 8 naked sporozoites not contained within sporocysts.

Tyzzeria perniciosa Allen 1936, from domestic ducks in the United States, have thin-walled oocysts measuring 10–12.3 × 9–10.8 µm and sporulate to produce 8 free sporozoites.

Wenyonella philiplevinei Leibovitz 1968 is the best described of the coccidia from ducks. It is found in the lower intestine from the posterior jejunal annular band to the cloaca. The prepatent period is 93 hours. The oocysts have 3-layered walls, measure 15.5–21 × 12.5–16 µm (average, 18.7 × 14.4), and have a micropyle at 1 end, 1–2 polar granules, and no oocyst residuum. Sporulation results in 4 sporocysts/oocyst, each containing 4 sporozoites.

Pathogenesis of Duck Coccidiosis

Signs of infection with *T. perniciosa* usually include anorexia, weight loss, weakness, distress, morbidity, and up to 70% mortality. Hemorrhagic areas are common in the anterior portion of the intestine but may be found throughout. Bloody or cheesy exudate is common. The epithelial lining may be sloughed in long sheets. Parasite invasion may extend through the mucosal and submucosal layers as deep as the muscular layers. Acute hemorrhage as early as day 4 may be followed by death on days 5–6.

With *W. philiplevinei*, the effects are limited to 72–96 hours PI. Occasional petechial hemorrhages appear in the posterior ileal mucosa. Diffuse congestion is found in lower intestinal mucosa. In severe infections, mortality may occur on day 4.

Coccidiosis in Pigeons

Young pigeons suffer the greatest losses, but mortality may occur in birds as old as 3–4 months. The most frequently occurring species of coccidia in pigeons is *E. labbeana* (Labbe 1896, Pinto 1928). Oocysts are spherical or subspherical, averaging 19.1 × 17.4 µm.

Pathogenesis

Mortality of 15%–70% has been reported in young pigeons. Subclinical infections may persist in older birds for long periods. Immunity does not appear to be as long-lasting as reported for other hosts. Common signs of infection are anorexia, greenish diarrhea, marked dehydration, and emaciation. Droppings may be blood tinged, and the entire digestive tract may be inflamed. The common condition of 'going light' is frequently attributed to coccidiosis.

Treatment

A favorable response has been reported after the use of sulfonamides in drinking water at the same level or half the level recommended for chickens. Clazuril, a close relative of diclazuril (which is approved for use in chickens), was introduced in 1987 in France and Belgium for specific use in pigeons. This product is highly effective in treating coccidiosis in pigeons.

Cryptosporidiosis

Larry R. McDougald

Summary

Agent, Infestation, and Disease. The *Cryptosporidia* are coccidian-like parasites found in the intestinal tract, or sometimes in the respiratory tract. They have a short, direct life cycle, with oocysts excreted in the feces. These parasites are often missed on gross examination because of indistinct lesions and small oocysts. They are considered pathogenic, although opinions vary as to their importance. The most common species in chickens and turkeys are *C. baileyi* and *C. meleagridis*.

Diagnosis. Active infections in poultry, both respiratory and intestinal, can be diagnosed by identifying oocysts from fluids obtained from the respiratory tract or from the feces.

Interventions. There is no known method of prevention or treatment, other than sanitation.

Introduction

Cryptosporidiosis is caused by small coccidian parasites of the genus *Cryptosporidium*, which live within the microvillous region of epithelial cells of the respiratory and gastrointestinal tracts of vertebrates. Naturally occurring infections have been reported from at least 9 different avian hosts. In chickens, turkeys, and quail, these parasites are primary pathogens that can produce respiratory and/or intestinal disease, resulting in morbidity and mortality. Species of *Cryptosporidium* infecting mammals have received considerable attention in recent years because of the widespread increase in immunocompromised hosts (5). Several reviews of the biology of *Cryptosporidium* are available (11, 12, 30, 42).

Human Health Importance

Whereas cryptosporidiosis is important in humans and other animals, there is no evidence that *C. baileyi*, the avian species, causes any infection in other animals. Similarly, *C. parvum*, which is the predominant human pathogen, is not commonly seen in poultry. There is good evidence that *C. meleagridis*, an occasional but highly pathogenic species in turkeys, may actually be synonymous with *C. parvum*. Species reported from poultry are summarized in Table 28.6.

Table 28.6 Distinguishing features of *Cryptosporidium* spp. infecting poultry. See references 4, 22, 26.

Species	Host(s)	Site of Infection	Measurements of Oocysts (µm)
C. baileyi	Chicken, turkey, duck	Bursa of Fabricius, cloaca, respiratory epithelium	6.2 × 4.6 (mean), 6.3 – 5.6 × 4.8 – 4.5 (range)
C. meleagridis	Turkey, chicken	Small intestine	5.2 × 4.6 (mean), 6.0 – 5.6 × 4.8 – 4.5 (range)
Cryptosporidium spp.	Quail	Small intestine	Approximately 5

History and Taxonomy

The type species *C. muris* was described from laboratory mice by Tyzzer (39), who later also described many of the life cycle stages and named a second species, *C. parvum* (40, 41). Many other species were named from a variety of vertebrate hosts because researchers assumed an unwarranted degree of host specificity. Only a few are now considered valid. Two species (*C. baileyi* and *C. meleagridis*) infect chickens, turkeys, and quail (29). In chickens and turkeys, *C. baileyi* causes both intestinal (cloaca and cloacal bursa) and respiratory infections, and small intestinal infections of *C. meleagridis* infections are associated with diarrheal disease in turkeys and quail. An isolate that causes high mortality in quail, once thought distinct from *C. baileyi* and *C. meleagridis*, is now considered similar to *C. meleagridis* (29). However, as mentioned above, *C. meleagridis* may be a synonym of *C. parvum*.

Life Cycle and Morphology

Taxonomy of the coccidia is based on the differences in oocyst structure, sequence similarities in the 18S RNA gene, the heat shock gene (HSP-70), host specificity, and site of infection (4, 29, 41). In contrast to other coccidia found in poultry, *Cryptosporidium* spp. oocysts do not have sporocysts surrounding the sporozoites, 4 of which lie naked within the oocyst wall (Figure 28.8). *C. baileyi* shows little host specificity among birds.

The life cycle of *Cryptosporidium*, like other true coccidia belonging to the suborder Eimeriorina, can be divided into 6 major developmental events (Figure 28.9): excystation (release of infective sporozoites), merogony (asexual multiplication within epithelial cells), gametogony (formation of male and female gametes), fertilization (union of gametes), oocyst wall formation (to produce an environmentally resistant form), and sporogony (the formation of infective sporozoites within the oocyst wall).

The life cycle differs in several respects from that of *Eimeria* spp. infecting poultry (8). The intracellular stages of *Cryptosporidium* spp. are confined to the microvillous region of the host cell. Oocysts sporulate within the host cell and are infective when released in the feces. Oocysts are of 2 types: thin walled or thick walled. Thin-walled oocysts are not environmentally resistant and contain sporozoites surrounded by a single unit membrane. Upon release from the host cell, the sporozoites invade adjacent host cells. Thick-walled oocysts have a multilayered wall and are passed through the feces to infect other hosts. The majority of oocysts are the thick-walled form. In mammals, the thin-walled, autoinfective oocysts and type I meronts (asexual stages) cause reinfection

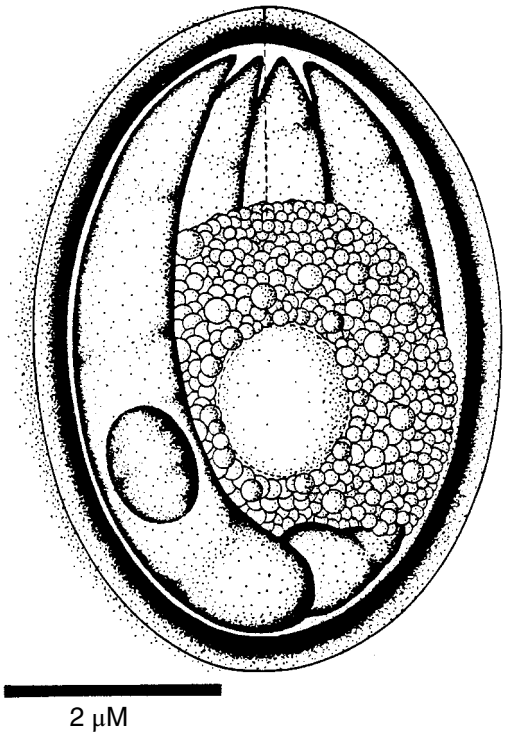


Figure 28.8 Composite line drawing of an oocyst of *Cryptosporidium baileyi*. Note the 4 sporozoites surrounding the oocyst residuum and the suture in the 2-layered oocyst wall (8). Reproduced with permission of John Wiley and Sons.

within the same host, allowing severe infections to build up after ingestion of a small number of ingested oocysts. This is particularly important in immune-deficient hosts and may lead to a chronic life-threatening disease. Another feature of *Cryptosporidium* spp., which differs from *Eimeria* spp. in mammalian and avian hosts, is the frequent establishment of infections in the mucosal epithelium of a wide variety of organs. *C. baileyi* can infect the cloaca, the cloacal bursa, the upper and lower respiratory tracts, and the eyelids.

Diagnosis of cryptosporidiosis is difficult because of the diminutive size of *Cryptosporidium* spp. and their location at the brush border of the epithelial cell. The tiny oocysts are difficult to see with light microscopy because they are only a fraction of the size of other coccidian oocysts and have no features to make them stand out against a light background. For the same reason, they can be missed even in histopathology. Phase contrast and interference contrast microscopy are useful in wet preparations. Oocyst morphology may be useful for species identification (Table 28.6). Only *C. baileyi* can be identified on the basis of morphology alone because it is larger and more ovoid than *C. meleagridis* from turkeys or quail. *Cryptosporidium* isolated from quail will not infect chickens or turkeys. Thus, the species infecting quail can only be distinguished from *C. meleagridis* on

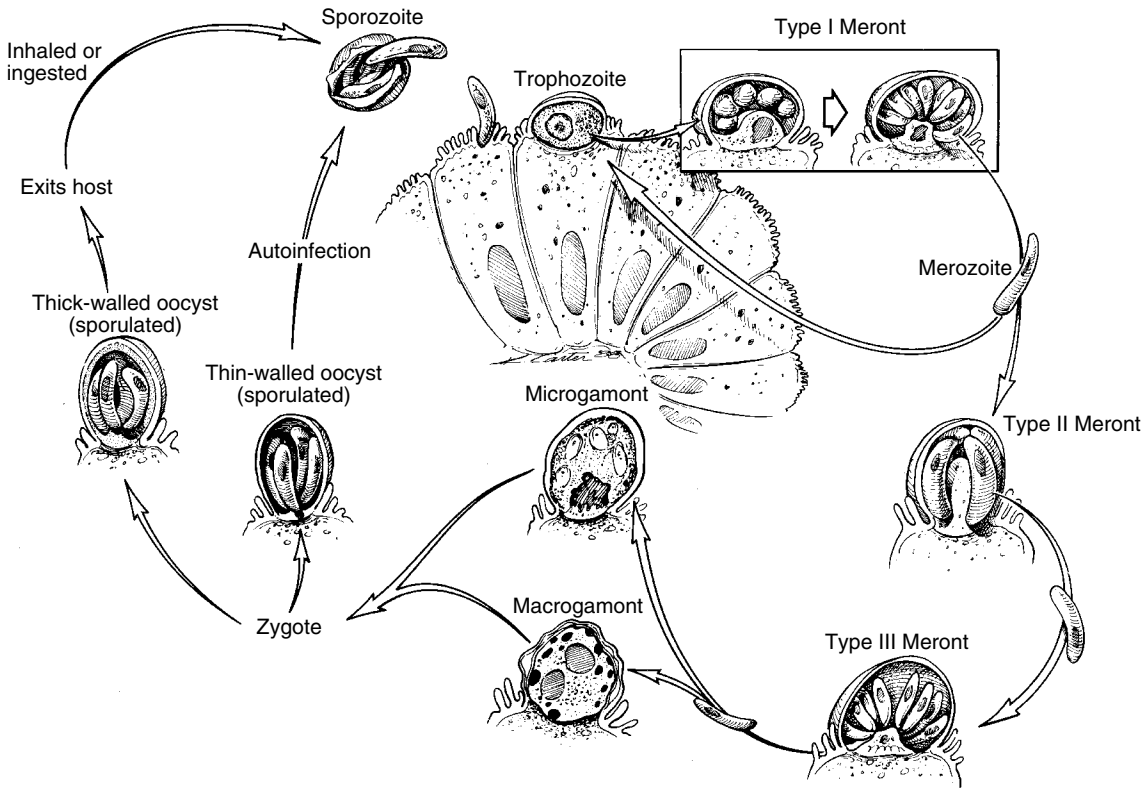


Figure 28.9 Life cycle of *Cryptosporidium baileyi* as it occurs in the mucosal epithelium of the intestine (cloacal bursa and cloaca) and the respiratory tract of broiler chickens.

the basis of host specificity. Oocyst walls of all forms are about 0.5 µm thick, colorless, and have no micropyle (Figure 28.8).

Incidence and Distribution

Cryptosporidium spp. are prevalent in domesticated, caged, and wild birds, reported from 30 bird species. The reported worldwide distribution of *Cryptosporidium* spp. in avian hosts corresponds to the regions in which poultry health specialists and biologists have used appropriate diagnostic tools and will continue to expand as awareness of their importance as primary pathogens increases.

Cryptosporidiosis in Chickens

Cryptosporidium (probably *C. baileyi*) was diagnosed in 6.8% of 1,000 consecutive histology cases of chickens in Georgia (16). In North Carolina, *Cryptosporidium* spp. oocysts were found in the feces of 9 (27.3%) of 33 broilers, 3 (10%) of 30 broiler breeders, and 1 (5.9%) of 17 layers (25). Using an enzyme-linked immunosorbent assay (ELISA), 22% of 454 broiler flocks in the Delmarva region were found to have birds that were seropositive for *Cryptosporidium* spp. when they were processed (6, 35). The number of positives among different companies sampled ranged from 2.8% to 40%. These investigations did not distinguish between intestinal and respiratory infections. Goodwin found respiratory cryptosporidiosis widespread in farms under contract to a broiler complex in North Georgia (16). The factors responsible for clinical expression of respiratory cryptosporidiosis are not understood but may cause high mortality and morbidity, with subsequent lower weight gains and higher feed/gain ratios (10). Experimentally induced respiratory and intestinal infections in broiler chickens have established the pathogenic potential of *C. baileyi* (2, 28). These and other data indicate that *Cryptosporidium* spp. are common in broiler chickens and could have a significant impact on productivity and performance.

Pathogenesis and Epidemiology

Oocysts are picked up from heavy fecal contamination of the litter or cages. *C. baileyi* generally invades the epithelium of the cloaca and cloacal bursa. Respiratory infections apparently result from the inhalation or aspiration of

oocysts that are present in the environment. As few as 100 oocysts can result in intestinal infections when given orally, or in respiratory infections when inoculated intratracheally. Oocysts of *C. baileyi* are infective at the time they are passed in the feces, and no vectors have been identified. Because *C. baileyi* can infect a variety of avian hosts, it is possible that wild birds may serve as carriers. Although *C. baileyi* is not infective for mammals, it is possible that rodents (mice and rats) and insects can serve as mechanical carriers (18).

Mild to heavy intestinal and respiratory signs can be seen as early as 3 days after inoculation of oocysts. Intestinal disease is usually mild. No overt signs of gastrointestinal disease occur in chickens receiving oocysts by gavage into the crop.

Signs of respiratory disease may appear within the first week after intratracheal (IT) inoculation of *C. baileyi* oocysts into 7- or 9-day-old broiler chickens, sometimes with severe morbidity and mortality (2, 9, 26). Oral inoculation of broilers with 4×10^5 oocysts produced only asymptomatic intestinal infections.

Respiratory signs of sneezing and coughing occur in most IT-inoculated chickens by 6 days postinfection (PI). By 12 days PI, respiratory signs are more severe, and many of the birds extend their heads to facilitate breathing. Severe respiratory signs are present for about 3–4 weeks PI, after which there may be gradual improvement. Weight gains were depressed with respiratory infection but not with intestinal infections (9). Chickens were more resistant to IT inoculation at 28 days than at 7 or 14 days of age (28).

Airsacculitis and pneumonia can occur as early as 6 days but are more common 12–28 days following IT inoculation of *C. baileyi* oocysts. Early in the disease process, posterior thoracic air sacs are slightly thickened and contain foamy, clear to white or gray fluid. By day 12, air sacs may become very thick and contain white caseous exudate. The lungs of birds with severe airsacculitis are almost always affected and exhibit focal consolidation (10%–80%), particularly in the ventral region. Abdominal air sacs may also be affected.

Histopathology of IT-inoculated chicks shows large numbers of parasites throughout the microvillous region of the epithelium lining the trachea and bronchi (15). Cilia are lost by replacement with developing parasites by 4 days PI (Figure 28.10). By 12 days, almost all cilia may be replaced by developing parasites, and the mucociliary elevator function ceases in affected trachea and bronchi. Histologic lesions include epithelial cell hyperplasia, thickening of the mucosa by mononuclear cell infiltrates with some heterophils, loss of cilia, and discharge of mucocellular exudate into the airways. There is accumulation of mucus, sloughed epithelial cells, lymphocytes, macrophages, and parasites in the tertiary bronchi and atria of the lungs. Affected lobules are expanded by accumulation of exudate and infiltration of mononuclear cells (Figure 28.11). Affected air sacs lined with respiratory epithelium also contain large numbers of parasites and suffer similar changes.

Intestinal (cloaca and cloacal bursa) cryptosporidiosis in chickens (produced by *C. baileyi*) may result in histologic lesions but does not usually result in gross lesions or overt signs of disease. Several reports suggest, however, that performance of broilers can be adversely affected. An unusually high mortality was associated with *C. baileyi* infection in the cloacal bursa, and there were lower pigmentation scores when inoculated birds were compared with noninfected controls (2, 19).

Interaction of *C. baileyi* and other respiratory pathogens predisposes birds to secondary invasion by *Escherichia coli* because of the disruption of the mucociliary elevator (9). Infectious bronchitis virus and *E. coli* also enhance the severity of *C. baileyi*-induced respiratory disease in chickens.

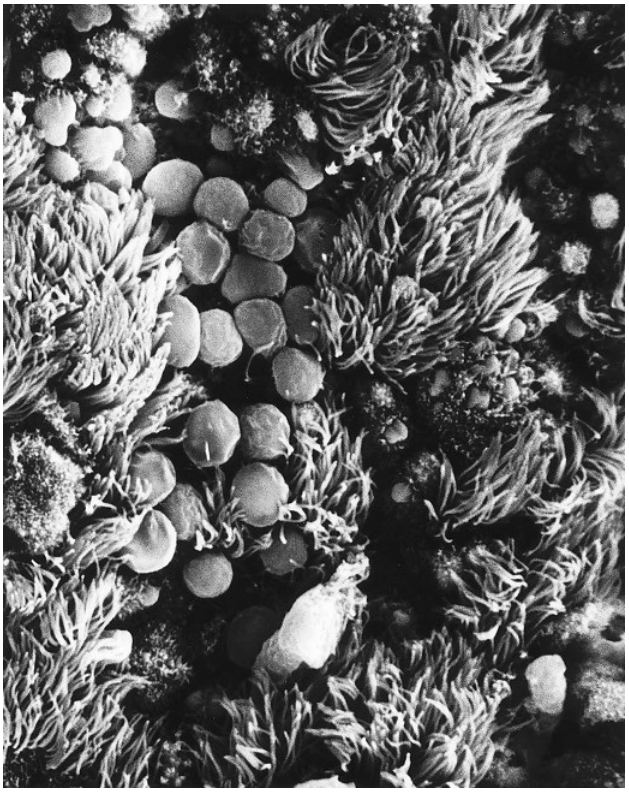


Figure 28.10 The mucosal surface of the primary bronchi obtained from a broiler chicken 4 days after intratracheal inoculation of *Cryptosporidium baileyi*, as shown by scanning electron microscopy. Some developmental stages of the parasite can be seen among the cilia of the respiratory surface. At this stage of infection, the mucociliary elevator is probably still functional, and the bird would not have overt signs of respiratory distress. On days 10–18 after intratracheal inoculation, developmental stages of the parasite form a virtual monolayer on the respiratory surface. Few or no cilia can be found. (S.L. White)

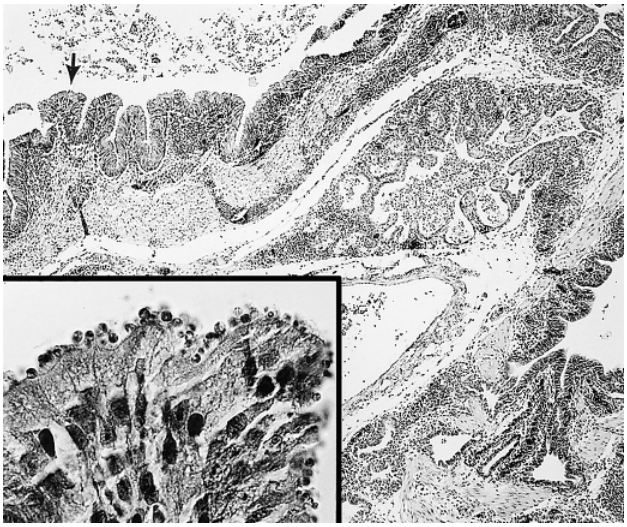


Figure 28.11 *Cryptosporidium baileyi* in the lungs of a broiler chicken. Accumulation of lymphoid cells around bronchi 6 days after intratracheal inoculation of *C. baileyi* oocysts. H&E-stained histologic section. Inset: a higher magnification of the villus (arrow) showing the numerous developmental stages of the parasite on the epithelial surface.

Cryptosporidiosis in Turkeys

Two species of *Cryptosporidium* found in turkeys are *C. meleagridis* (35) and *C. baileyi*. However, the description of *C. meleagridis* is indistinguishable from that of *C. parvum*. The intestinal (cloacal bursa and cloaca) and respiratory infections produced by *C. baileyi* are similar to those described previously for chickens (8, 9, 27).

Slavin (34) reported small intestinal cryptosporidiosis caused by *C. meleagridis* in a flock of 10- to 14-day-old turkey poults. Illness was associated with diarrhea, unthriftiness, and moderate mortality. More than 30 years later, several outbreaks of this disease were reported (17, 43), although the number of reported cases is low.

Turkey poults infected with *C. meleagridis* may develop severe diarrhea. Numerous parasites are seen lining the brush border of the mucosa of the middle and lower small intestine. The gut becomes pale and distended with cloudy mucoid fluid and gas bubbles. Villi in the affected regions become atrophic, crypts become hypertrophic, and large numbers of lymphocytes, heterophils, and some macrophages and plasma cells accumulate within the lamina propria (17).

There are several case reports of severe respiratory cryptosporidiosis in commercial turkeys caused by *Cryptosporidium* spp. (probably *C. baileyi*) (14, 22, 32, 37). The disease may have upper or lower respiratory involvement. Upper respiratory infections may cause acute bilateral swelling of infraorbital sinuses, similar to that reported for birds infected with *Mycoplasma* spp., and serous conjunctivitis (14, 22). Case reports of lower respiratory tract infections reported signs including rattling, coughing, sneezing, and gasping (32, 37). The trachea and bronchi were colonized, with concomitant airsacculitis and pneumonia. Microscopic lesions of the infected tissues included deciliation of the epithelium and inflammation.

Intratracheal inoculation with *C. baileyi* from the intestinal tract of broiler chickens into the trachea of turkeys produced respiratory signs similar to those observed in natural outbreaks (27).

Although there are reports of clinical outbreaks, the importance of *Cryptosporidium* spp. in commercially reared turkeys is not clear.

Cryptosporidiosis in Quail

Both respiratory and intestinal cryptosporidiosis have been reported in commercially grown quail, but the species involved has not been adequately described. Field reports suggest similar respiratory disease and low mortality similar to that seen with cryptosporidiosis in chickens (38). Histologic examination revealed parasites in the microvillous region of epithelial cells lining the nasal cavity, trachea, bronchi, salivary glands of the roof of the mouth, esophageal glands, and cloacal bursa. Pathologic changes in the respiratory mucosa were similar to those described previously for chickens infected experimentally with *C. baileyi*. In another spectacular case of cryptosporidiosis, 5 successive hatches of 25,000 young quail (*Colinus virginianus*) developed severe, fatal intestinal cryptosporidiosis (23). Diarrhea developed 4–6 days after hatching, and mortality soon exceeded 90%. At necropsy, numerous developmental stages of the parasite were observed in the microvillous border of the small intestine. No parasites were observed in the cecum, colon, cloacal bursa, respiratory tract, or other tissues. Oocysts, obtained from the intestines of these infected quail, were not infective to day-old broilers. Based on recent work, this isolate was probably *E. meleagridis* (29).

A similar outbreak was reported from young quail caused by a combination of *Cryptosporidium* spp. and a reovirus isolated from intestinal contents (33). Subsequent laboratory studies (20) suggested that the *Cryptosporidium* and not the reovirus was responsible for the intestinal disease.

Prevention and Control

There are no effective anticryptosporidial drugs or vaccines, and other approaches to the control are still experimental. Sanitation or disinfection may provide some help, but no proven programs can be recommended.

Sanitation

The oocysts of *Cryptosporidium* spp. infecting poultry are remarkably resistant to chemical agents that readily kill most viral, bacterial, and fungal pathogens. Destruction of oocysts in commercial production facilities is not considered practical. In the laboratory, oocysts remain viable for months when stored at 4°C in a solution of 2.5% potassium dichromate. Oocyst viability is also maintained after a 10- to 15-minute incubation in 25% commercial bleach (sodium hypochlorite). Incubation of *C. baileyi* oocysts for 30 minutes at room temperature in each of 9 commonly used disinfectants mixed with water at the highest concentration recommended by the manufacturers had little or no effect on viability (36). Incubation in 50% ammonia resulted in the greatest reduction in excystation, and 50% commercial bleach destroyed many of the oocysts. Steam cleaning is a safe and effective means of disinfecting contaminated laboratory cages because oocysts are destroyed by temperatures greater than 65°C.

Immunity

A single intestinal and/or respiratory infection with *C. baileyi* can stimulate an immune response in broiler chickens of sufficient magnitude to clear the parasite from the infected mucosae and to protect the host against reinfection of the same species (6, 9). Experience with cryptosporidiosis in other animals suggests that immune protection may be short-lived. Oral or IT inoculation of oocysts into 8- to 14-day-old broilers results in heavy infections of the exposed mucosae for 14–16 days and then a rapid clearance of the parasite. High titers of circulating antibodies specific to *C. baileyi* can be detected after primary infections, and the birds exhibit a delayed hypersensitivity reaction to *C. baileyi* oocyst antigens. Data from laboratory studies and from a serologic survey suggest that acquired immunity may be important in the protection of broilers from cryptosporidiosis during the last several weeks of grow-out. Studies are needed to identify antigens of *Cryptosporidium* spp. that may be candidates for use as vaccines.

Diagnosis and Culture

Active infections in poultry, both respiratory and intestinal, can be diagnosed by identifying oocysts from fluids obtained from the respiratory tract or from the feces. Identification of *Cryptosporidium* spp. oocysts differs somewhat from techniques used for the oocysts of *Eimeria* spp. For viewing, oocysts are concentrated and observed by standard bright field or phase contrast microscopy (7), acid-fast staining (13, 31), negative staining (4, 21), and staining with auramine-O for examination by fluorescence microscopy (25). These techniques allow one to readily distinguish *Cryptosporidium* spp. oocysts from yeast cells or Blastocystis that are often present in specimens.

Fecal or respiratory specimens can be collected and submitted fresh, in 10% formalin or in an aqueous solution of 2.5% potassium dichromate. A highly effective method of obtaining specimens in the field and in the laboratory is with moist cotton-tipped swabs. Vigorous swabbing of the tracheal or cloacal epithelium will remove oocysts from the microvillous border. The swabs are placed in a tube containing 1 mL of water or fixative for transportation to the laboratory.

Cryptosporidium infection also can be detected by demonstrating other stages of the life cycle from fresh or stained mucosal scrapings from the mucosa (24). Abbassi (1) described a semiquantitative microscopic slide flotation method that was reliable for *C. baileyi* in feces and organs of chickens. These parasites also appear in histologic sections stained with hematoxylin and eosin as 2- to 6-µm basophilic bodies within the brush border of the epithelial cells. Because of the small size of these parasites, transmission electron microscopy is useful to reveal developmental stages and oocysts within the host cells. Inoculation of chicken embryos (10 days) with oocysts of *C. baileyi* is a good method for propagation of this species in the laboratory, providing about 50% of the number of oocysts obtained from chickens (44).

Previous exposure to the parasite can be demonstrated by testing for serum antibodies specific to *Cryptosporidium* by ELISA or other immunologic tests (6, 35).

Amplification of DNA sequences with polymerase chain reaction (PCR) is a useful tool for identifying some species of *Cryptosporidium* (29), but studies with 8 DNA loci revealed homologies between *C. meleagridis* and the human pathogen *C. parvum*, which could not be resolved by PCR (3).

Histomoniasis (Histomonosis, Blackhead Disease)

Michael Hess and Larry R. McDougald

Summary

Agent, Infection, and Disease. The protozoan parasite *Histomonas meleagridis* is the etiological agent of histomoniasis, commonly known as blackhead disease. All gallinaceous birds are susceptible to the protozoan and its intermediate host, the cecal worm (*Heterakis gallinarum*).

Diagnosis. Necrotic foci in the liver, along with engorged ceca are easily recognized and strongly indicative. Turkeys severely infected will excrete 'sulfur-yellow droppings.' Diagnosis can be confirmed by histopathology. Presence of *H. meleagridis* and/or its DNA can be confirmed by polymerase chain reaction using samples from infected birds or the environment.

Intervention. In most countries no specific prophylactic or therapeutic drug is registered, and there are no vaccines. This frequently leads to loss of entire flocks or euthanasia to relieve suffering.

Introduction

Definition and Synonyms

Histomoniasis (syn. histomonosis, blackhead disease, enterohepatitis) caused by the protozoan *Histomonas meleagridis* is mainly characterized by ulceration and inflammation of the cecal walls, engorgement of the ceca with large caseous casts, inflammation of the mesenteries, and extensive focal necrosis of the liver. The roles of the cecal worm (*Heterakis gallinarum*) as an intermediate host, earthworms as accessory hosts, and the ability of *H. meleagridis* to colonize new hosts by direct transmission comprise one of the most intriguing relationships in parasitology. Tyzzer (60, 61) conducted extensive studies on the biology of *H. meleagridis* and described it as a simple cell with flagella as well as pseudopodia. The complicated interrelationship of *H. meleagridis* with bacteria was demonstrated by Reid and his students, using germ-free and mono-contaminant techniques (44, 46, 55). The presence in the gut of certain species of bacteria, including *Escherichia coli*, *Staphlococcus aureus*, and *Clostridium perfringens*, were necessary for virulence and production of histomoniasis (46). This interaction, together with the presence of other protozoa displaying certain morphological similarities with *H. meleagridis*, caused some to question the etiology of blackhead disease for a time (29, 55).

Economic Significance

There are no public records on the annual losses in different host species and geographic areas. With the ban of drugs for prevention or treatment, the number of cases in turkeys increased dramatically in Europe, the United States, and South America. About 100 outbreaks are reported/year in Europe and more than 120 cases/year in the United States. In turkey flocks, mortality can reach 100%. Killing of suffering animals and diseased flocks is also an important welfare issue. Although histomoniasis is usually less severe in chickens, losses from morbidity and mortality might be greater in chickens than in turkeys because of the frequency of occurrence and the numbers of birds involved. Histomoniasis is prevalent in broiler breeder chickens in most areas of the United States and Europe because of the widespread prevalence of the cecal worm, *H. gallinarum*.

Public Health Significance

Histomoniasis has no public health significance because it affects only birds.

History

Histomoniasis in turkeys was first described in 1895. The literature on certain aspects of the disease and/or the pathogen has been reviewed in depth providing a good resource for older references (24, 28, 41, 44, 46, 55). The finding that chickens display a much lower mortality and often remained carriers provided the first useful recommendation for control: turkeys should not be kept together with chickens and their inhabited areas remain as a source of infection because of the presence of intermediate hosts (*H. gallinarum* and earthworms).

Research on histomoniasis became neglected after the discovery of highly effective control measures in the late 1960s, leaving considerable basic biological and biochemical work undone. However, since a thorough review in 2005, more than half as many research papers have appeared since that date driven by therapeutic bans (41).

Etiology

Classification

The only agent causing blackhead disease is the protozoan, *H. meleagridis* (Figure 28.12). The organism belongs to

the phylum Parabasalia, class Tritrichomonadea, order Tritrichomonadida, family Dientamoebidae and genus *Histomonas*. This positioning is also supported by proteomic studies (3, 36, 52). Considerable variation in the species was suggested by several studies but more investigations are needed to propose additional species (15, 23, 42, 64). Application of a multilocus approach on 3 different genes revealed the existence of 2 genotypes (4). A larger (17 μm), nonpathogenic, 4-flagellated protozoan parasite found in the cecum belongs to a separate genus, named *Parahistomonas wenrichi*. Other agents, such as trichomonads and fungi (*Candida albicans*), have been rejected as agents of blackhead disease, because these are now recognized as causing other, similar-appearing, diseases (29, 55).

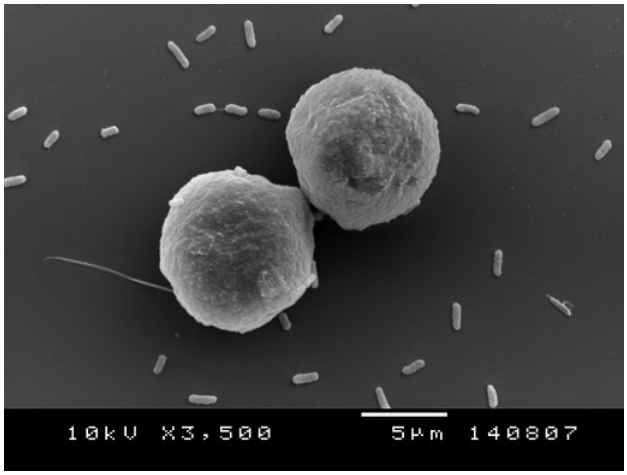


Figure 28.12 Scanning electron micrograph of *in vitro* cultivated histomonads with flagellum. (M. Hess).

Morphology

Histomonas meleagridis in its nonamoeboid state is nearly spherical and about 10 μm (3–16 μm) in diameter (Figure 28.13). The amoeboid phase is highly pleomorphic. Pseudopodia may be observed by examining live parasites on a warm microscope slide. The organism may form a pseudocyst with a double membrane, but there is no evidence that these forms affect survival outside the host (71). Cecal lumen forms have a single flagellum 6–11 μm in length. A pelta and an axostyle are wholly contained within the cell. The parabasal body is V-shaped and anterior to the nucleus. The nucleus is

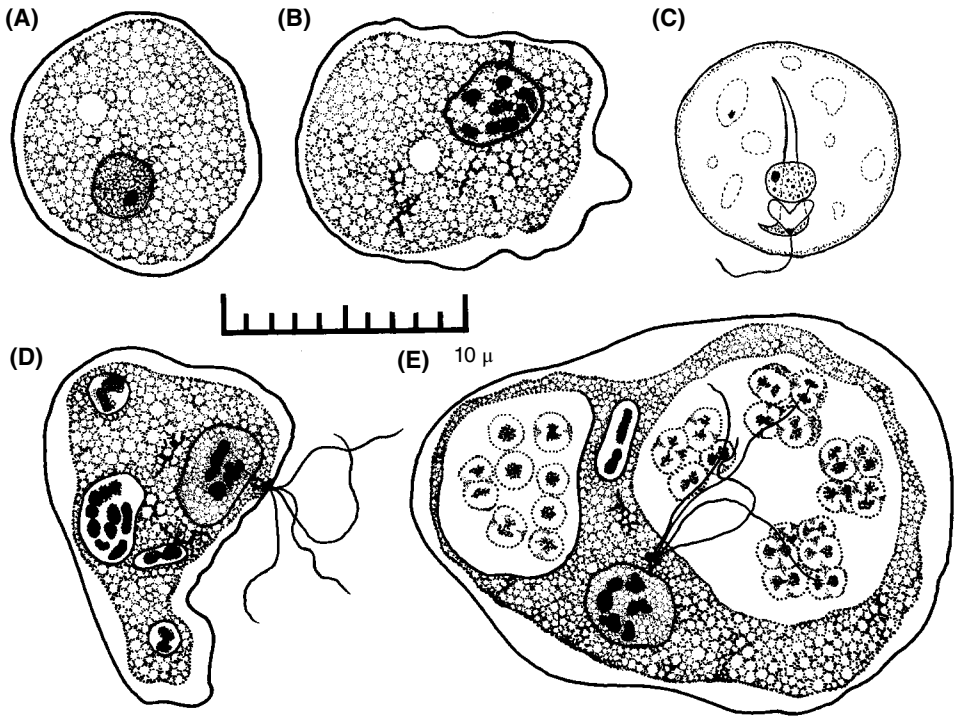


Figure 28.13 Examples of *Histomonas meleagridis* (A–C) compared with *H. wenrichi* (D,E) showing variations for each species. (A) Tissue type *H. meleagridis* in fresh preparation from liver lesion, viewed with phase-contrast microscopy. (B) *H. meleagridis*. Transitional stage with pseudopodia but no flagellum, in lumen of the cecum. Distribution of chromatin suggests the beginnings of binary fission. (C) An organism from culture, with free flagellum typical of lumen dwelling forms. (B.M. Honigberg and G.F. Bennett) (D) Small *H. wenrichi*, structurally distinguishable from *H. meleagridis* by its larger size and by presence of 4 flagella. (E) *H. wenrichi* as viewed in stained smear from cecum in which packets of Sarcina were abundant. Drawn from living specimens (A,B,D, and E) or tissue sections. Reproduced with permission of Wiley.

spheroid to ellipsoid or ovoid and averages 2.2 × 1.7 μm. *H. meleagridis* lacks mitochondria, instead relying on other organelles (hydrogenosomes) for energy metabolism.

Tissue forms usually lack flagella and exist in several different forms: (1) parasites in the invasive stage at the peripheral areas of the lesions are 8–17 μm in size, amoeboid, and appear to form pseudopods, (2) a vegetative stage is larger (12–21 μm) and more numerous and is clustered in vacuoles in degenerating tissue, and (3) a third stage present in older lesions is eosinophilic and smaller and may represent a degenerating form.

Susceptibility to Physical and Chemical Agents

The naked protozoan cell is not resistant to environmental conditions outside the bird. Temperature, drying, and the presence of oxygen are critical factors limiting viability outside the host (16, 43).

Much attention is given to the control of the intermediate host, the cecal worm (*H. gallinarum*) which is common in chickens but rare in turkeys. Instead, outbreaks in turkeys occur after accidental introduction of worm eggs onto a turkey farm by a vector. Numerous vectors are suspected, but more work is needed. Worm eggs could be brought to a farm on contaminated equipment, on the clothes of workers, or by insects (flies and beetles). Control of worms in chickens (and thus, blackhead disease) depends on frequent application of benzamidazole-type wormers. Treatment for 2–3 days is necessary for a complete worm control. Some veterinarians recommend litter or soil treatment to reduce contamination by worm eggs, but there is no experimental evidence that this is effective.

Pathogenicity

Severity of infections varies greatly between host species. Studies of field isolates have shown little variation in the virulence of field isolates, but it is well known that outbreaks vary greatly in the field. More studies are needed in this area.

All gallinaceous bird species can become infected with blackhead disease, but the turkey is considered the most susceptible. A high percentage of infected turkeys suffer morbidity and death whereas chickens often have a milder form of the disease, characterized by some mortality, extensive runting and culling, and unthriftiness. High losses have been reported in peacocks and farm-reared Bobwhite quail (48). Variation in susceptibility among different breeds, strains, and age of chickens was reported (24). Chickens 4–6 weeks old may be more susceptible than older birds. In field cases in hens, egg production was depressed, accompanied by some mortality (12, 13, 40). Clinical outbreaks in broiler breeders prior to production reduce flock uniformity and later productivity (12). Bacterial flora in the gut is important in the development of blackhead disease, shown by studies in germfree birds (14), but the course of infections is little affected by administration of antibiotics. Experimentally, cecal coccidiosis in chickens (*E. tenella*) interacts with histomoniasis to increase the number of birds showing liver lesions, as well as the severity of lesions (45).

Isolates of *H. meleagridis* grown *in vitro* frequently lose pathogenicity in successive passages, sometimes also modifying morphology and growth behavior (21, 41).

Pathobiology and Epizootiology

Incidence and Distribution

Various case reports in turkeys and chickens underline the importance of the disease in these species. The number of cases reported in European countries increased after drugs were banned. Mortality was variable; French turkey flocks were mostly below 10%, whereas outbreaks in Germany and Holland were as much as 50%–100% (9). Serological data indicate a widespread prevalence in chickens, and the prevalence in different husbandry systems is variable (19, 66). However, presence of *H. meleagridis* on Vietnamese chicken farms did not correlate with the type of farms, age of birds, and seasonality (51). Chickens on the production farm may be infected by access to outside areas. Game birds (pheasants, partridges) reared in captivity or in the wild also serve as reservoirs.

Natural and Experimental Hosts

Numerous gallinaceous birds are reported as hosts for *H. meleagridis* (46). The turkey, chicken, chukar partridge, peacock, pheasant, and ruffed grouse may be severely affected, whereas guinea fowl, Bobwhite quail, and ostrich have a milder form of the disease. Ducks may become asymptomatic carriers and act as a carrier (8). The coturnix quail can be infected experimentally, but it is a poor host.

Transmission, Carriers, and Vectors

The Role of *Heterakis gallinarum*

The survival and transmission of *H. meleagridis* is very much associated with the cecal nematode *H. gallinarum* (60). The female worms probably become infected with the histomonads during copulation and incorporate the protozoan into eggs before shell formation.

In the ceca of its host, the histomonad leaves the worm larva and multiplies in the lumen and mucosa. Within 2–3 days the tissue forms enter the bloodstream and are

carried to the liver by the hepatic–portal system. In the cecal tissues and in the liver the cells divide and grow, forming necrotic areas that are visible on gross inspection. Interaction between *H. gallinarum* and *H. meleagridis* can be influenced by nonstarch polysaccharides in the feed, favoring the establishment of the cecal worm (11).

The Role of Earthworms

Earthworms can serve as transport hosts in which heterakid eggs hatch and survive (46). The earthworm, thus, serves as a means for collection and concentration of heterakid eggs from the poultry yard environment. On range or pastures, where climate and soil types favor survival of heterakids and earthworms, the latter must be considered in attempts to control a recurrent histomoniasis problem.

Transmission by Direct Contact

Transmission of blackhead disease within a turkey flock occurs readily by direct contact between susceptible and infected birds or fresh droppings and does not require an intermediate host (31, 47). Depending on the experimental setting and the detection method, transmission between infected and in-contact turkeys occurs 1–3 days PI, but this process is much less efficient in chickens (25, 32, 37). Feces containing excreted histomonads may contribute to the spread of the parasite but the efficacy is much lower than with bird to bird contact (1).

Vectors

The common cecal worm, *H. gallinarum*, is the only worm known to serve as an intermediate host for blackhead disease (60). Even closely related nematodes are unable to serve as hosts. Worm ova are resistant to environmental conditions and may remain infective for 2–3 years. Most gallinaceous birds are host to the cecal worm and wild populations may serve as reservoirs. The common earthworm has been shown to consume and harbor infective larvae of the cecal worm, thus serving as a vector. Although poorly documented, arthropods such as flies, grasshoppers, sowbugs, crickets, and snails may serve as mechanical vectors. Diagnosticians are sometimes confused by the inability to find cecal worms in birds with histomoniasis. Some of the reasons for this are: (a) the infection within a flock may pass from bird to bird without cecal worms, (b) the cecal worm remains in larval form for 2–3 weeks and is very small (2–3 mm), and (c) the development of histomoniasis worsens the environment for the worm, so that many are killed or expelled.

Incubation Period

Disease is caused when histomonads penetrate the cecal wall, multiply, enter the bloodstream, and eventually parasitize the liver. Overt signs of histomoniasis are apparent from 7–12 days and death may occur 11–14 days postinfection (PI) (24). Gross lesions begin with reddening and thickening of the cecal mucosa within 3 days. The incubation period varies with the size of the infective dose and the route of infection. Infections from worm eggs require longer than those from direct bird to bird contact. Experimentally, cecal and liver lesions in turkeys develop about 3 days earlier with cloacal inoculation compared with infection via heterakid eggs. Experimental infection in chickens demonstrated that lesions peaked between 10 and 14 days PI (70).

Clinical Signs

Signs of histomoniasis in turkeys include yellow feces, drowsiness, dropping of the wings, walking with a stilted gait, closed eyes, head down close to the body or tucked under a wing, and anorexia (Figure 28.14). Sick birds tend to huddle together. By 6–12 days PI, turkeys become emaciated. Infections in chickens may be mild and go



Figure 28.14 *Histomonas meleagridis* infected turkey with yellow diarrhea. (M. Hess)



Figure 28.15 Multiple necrotic nodules in the liver and caseous cores in ceca of a turkey infected with *Histomonas meleagridis* (experimental infection). (M. Hess)

unnoticed or may be severe and cause high mortality. Sulfur-colored droppings are seen in the later stages of disease when liver function is severely damaged and bile pigments are excreted through the kidneys. In chickens, the main signs are drooping feathers, morbidity, closed eyes, and a drop in egg production in layers. Cecal discharges may contain blood and caseous cecal cores are common. Sometimes gross pathology of blackhead disease in chickens may resemble cecal coccidiosis.

Morbidity and Mortality

Turkeys seem to be more uniformly affected, with flock mortality up to 80%–100%, but outbreaks with low mortality are also reported (9). Mortality can decline to normal after the acute phase, but the stress of moving sometimes causes another mortality peak (20). In chickens the disease may be diagnosed in young pullets or in layers early in production. Although losses from histomoniasis in chickens are generally low, mortality has exceeded 30%. Increased culling is common prior to egg production.

Pathology

The heavy destruction of the liver influences various clinical chemistry values. Despite this, the measurement of these changes never became routine in experimental studies. In chickens the number of circulating heterophils decreases in early stages of the infection, and there is a sharp increase of macrophages/monocytes (49).

Gross

The primary lesions of histomoniasis develop in the ceca and liver (Figure 28.15). Lesions are also reported from other organs, such as the spleen, the bursa of Fabricius, pancreas and kidneys, indicating a wide spread of the pathogen within the host (Figure 28.16) (56). First lesions are observed in the ceca. After tissue invasion by histomonads, cecal walls become thickened and hyperemic. Serous and hemorrhagic exudate from the mucosa fills the lumen of ceca and distends the walls with a caseous or cheesy core. Ulceration of the cecal wall may lead to perforation resulting in generalized peritonitis.



Figure 28.16 Multiple areas on necrosis in pancreas of a turkey infected with *Histomonas meleagridis* (experimental infection). (M. Hess)

Liver lesions in turkeys are often apparent a few days after infection and are highly variable in size, number, and appearance. Most common are multifocal circular depressed areas of necrosis with varying size circumscribed by a raised ring. In rare cases of recovery, lesions leave purulent scars on the surface of the liver. Lesions in lung, kidney, spleen, pancreas, and mesenteries are sometimes recognized as white, rounded areas of necrosis.

In chicken layers histomoniasis is often accompanied with colibacillosis (Figure 28.17). Case reports in turkeys and quails described high mortality in birds where lesions were nearly exclusively confined to the caeca (20, 48).

Microscopic

Invasion of the cecal wall results in hyperemia and heterophil leukocyte infiltration, probably a combined response to bacteria, histomonads, and heterakid juveniles. Within 5–6 days, numerous histomonads are visible as pale, weakly staining, ovoid bodies within lacunae in the mucosa. Large numbers of lymphocytes and macrophages have infiltrated tissues by this time, and the heterophil population has also increased. Cecal cores are composed of sloughed epithelium, fibrin, erythrocytes, and leukocytes along with trapped cecal ingesta. By 12–16 days, giant cells appear in the tissues of the cecum. Coagulation necrosis and histomonad invasion extend well into the muscular tunic, extending nearly to the serosa. In survivors, histomonads are scarce within the tissues by 17–21 days and are mostly concentrated near the serosal layers. Large numbers of giant cells form and may appear grossly as granulomata bulging upon the serosal aspect of the cecum. Old lesions, after recovery, are characterized by lymphoid centers scattered throughout the cecal tissue. Expulsion of cores and the regeneration of epithelium may occur, particularly in chickens, but the cecum may suffer permanent damage.

The liver has microscopic lesions visible by 6–7 days PI and consists of small clusters of heterophils, lymphocytes, and monocytes near portal vessels. Histomonads are difficult to visualize in these areas. After 10–14 days, the lesions are enlarged, becoming confluent in some areas. There is extensive lymphocytic and macrophage infiltration, and heterophils are present in moderate numbers. Hepatocytes in centers of the lesions degenerate. Many individual or clustered histomonads are visible in lacunae near the periphery of lesions. From 14–21 days PI, necrosis becomes increasingly severe, resulting in large areas consisting of little more than reticulum and cellular debris. Histomonads at this stage are present mostly as small bodies in macrophages. If recovery occurs, foci of lymphoid cells remain, along with areas of fibrosis and regenerating hepatocytes.

Immunity

Chickens and turkeys produce antibodies against *H. meleagridis* antigens prepared from infected livers and ceca, after natural or experimental infections. In turkeys such antibodies persist at least for up to 16 weeks PI (39). The immune response in chicken ceca is characterized by an increase of IgG, IgM, and IgA and an effective innate immune response, characterized by expression of IL-1β, CXCLi2, and IL-6 mRNA which altogether may contribute to the higher resistance noticed in chickens (54, 68).

Active Immunization

Early work suggested that a partial immunity developed after infections, which might or might not be adequate to protect against reinfection. Until recently, data about the vaccination of turkeys against histomoniasis were inconclusive and even contradictory. However, complete protection of turkeys has been achieved by vaccination with an attenuated clonal culture containing clonal histomonads attenuated by serial passage *in vitro* (27, 39, 41, 49, 50). Efficacy in turkeys could also be demonstrated against various field isolates. Reversion to virulence was not noticed following 5× back passages



Figure 28.17 Chicken layer with caseous cores in the ceca (*Histomonas meleagridis*) and fibrinous coelomitis (*Escherichia. coli*). (M. Hess)

(57, 58). Using the same strain, the drop in egg production in chicken layers could be severely limited by a single vaccination (40). Vaccination with attenuated histomonads obviously limits the infiltration of various immune cells into liver and caeca by recalling a primed immune response (49).

Passive Immunization

Early attempts at passive transfer of immunity were unsuccessful. Serum from immune chickens repeatedly inoculated into the peritoneum of naïve birds offered no protection. Killed histomonads used as a vaccine also failed to stimulate protection in turkeys (6, 27).

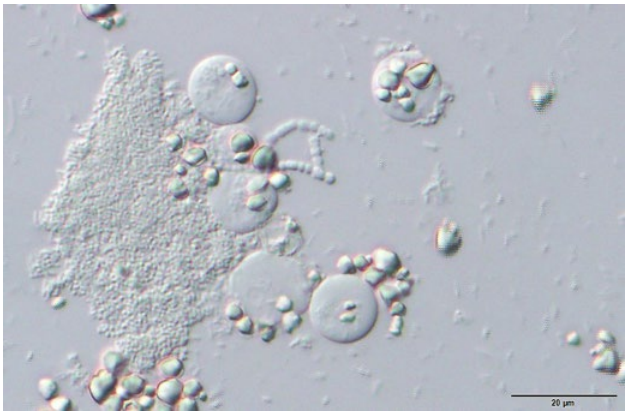


Figure 28.18 Phase contrast micrograph of *in vitro* cultivated histomonads, 100×. (M. Hess)

Diagnosis

Most experienced poultry workers make a field diagnosis in turkeys on the basis of gross appearance of lesions. Laboratory confirmation is sometimes necessary to rule out concurrent infections with other agents that affect the cecum or liver (coccidiosis, salmonellosis, aspergillosis, and trichomoniasis) (62). Chickens are more likely to have lesions in the ceca that can be confused with other diseases, particularly cecal coccidiosis.

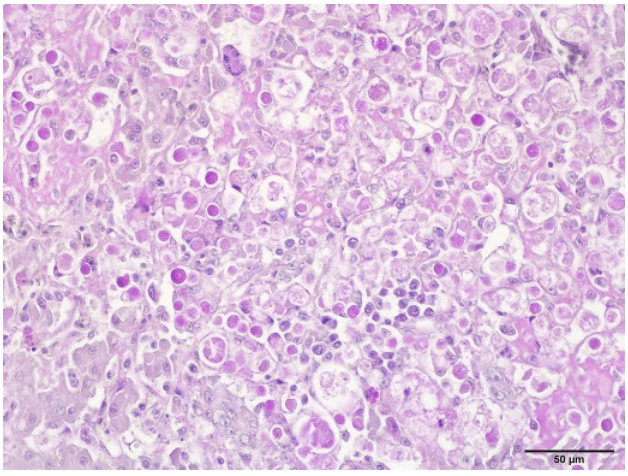


Figure 28.19 Periodic acid-Schiff staining of histomonads in the liver, 40×. (M. Hess)

Isolation and Identification of Causative Agent

Identification of histomonads by microscopy adds confidence to the diagnosis. The organisms can be observed with warmed phase-contrast microscopy with fresh specimens (Figure 28.18) (16). The parasites can be isolated and cultured *in vitro*. A commonly used medium contains mainly Medium 199 rice flour and horse serum, and it is balanced with Hank or Earle salts (25, 26, 63). Microaerophilic conditions must be maintained. The fluid medium can also be stored in dry form, until needed for field sampling, and reconstituted with water (2). Cultured histomonads tend to become attenuated after repeated subculture (21).

In recent years various polymerase chain reaction (PCR) methods were described, some of which were used to confirm the presence of the parasite in fecal or tissue samples and to quantify histomonads (5, 17, 22, 33, 34, 37, 69). For routine diagnostic histopathology, any of several stains, including hematoxylin and eosin or periodic acid-Schiff (PAS), may be used (Figure 28.19). Excellent cytologic preparations have been made from fresh cultures using Hollande cupric picroformol and a protein-silver stain. Tissue forms are unambiguously identified by *in situ* hybridization or immunohistochemistry (Figures 28.20 and 28.21) (38, 56).

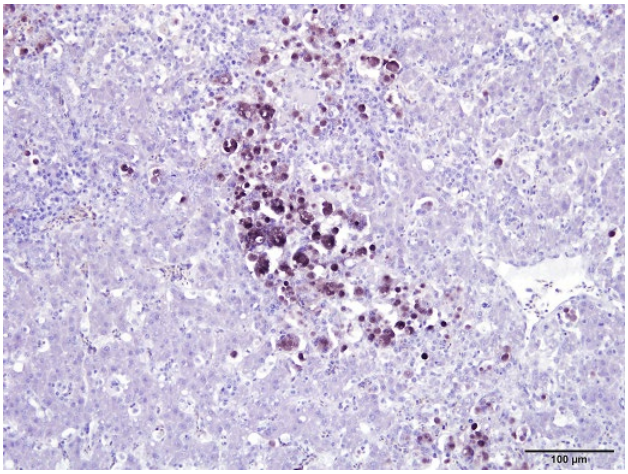


Figure 28.20 *In situ* hybridization of histomonads in a turkey liver, 20×. (M. Hess)

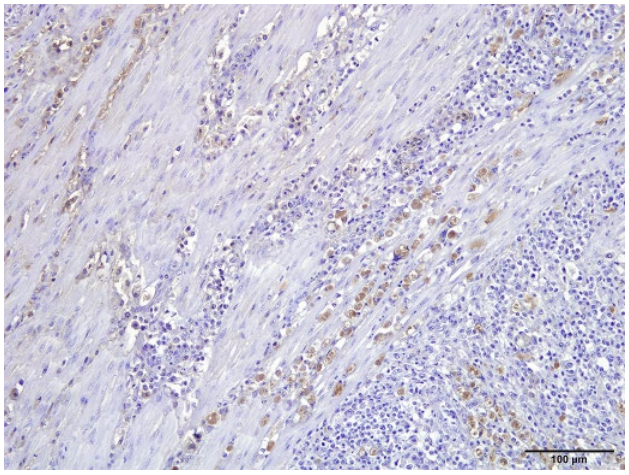


Figure 28.21 Immunohistochemical demonstration of histomonads within the muscularis layer of the cecum, 20×. (M. Hess)

Differential Diagnosis

For differential diagnosis other agents that affect the cecum or liver (coccidiosis, salmonellosis, aspergillosis, and upper digestive tract trichomoniasis) should be considered. Chickens are more likely to have lesions in the ceca that can be confused with other diseases, particularly cecal coccidiosis.

Intervention Strategies

As there are no chemotherapeutic products available for treatment of infections, and there is no commercial vaccine for histomoniasis, control measures are mainly focused on prevention (41). The primary reservoir of infection is the cecal worm egg. Thus, prevention is largely based on quarantine and avoidance of contact of susceptible birds with sources of cecal worm eggs.

For the protection of turkeys, exclusion of chickens from any contact is essential, because chickens may often harbor large numbers of egg-laying cecal worms. Outdoor turkey ranges can become contaminated with heterakid eggs, creating a situation in which histomoniasis recurs in turkey flocks for many years. Because of the longevity of infectious eggs, range rotation is not practical as a solution.

Rearing turkeys indoors tends to reduce outbreaks of blackhead disease as hygiene increases, but exacerbates the extent and severity of outbreaks (10). Wet litter may also support the spread of histomonads which would explain the beneficial effect of changing the litter after the first clinical signs are noticed. Water acidification has no preventive effect and does not minimize the presence of *H. meleagridis* (10).

It is most likely that outbreaks arise by the introduction of a small number of cecal worm ova into the growing facility. After introduction, the infection spreads throughout the flock by direct contact. The recent observation that histomoniasis cannot spread within a flock without direct contact between birds offers a potential method for containing outbreaks (53). If the growing facility is divided into subunits, even by netting or other barriers, the outbreak might be limited to the contaminated units.

Leghorn pullets and broiler breeder pullets often become infected in problem houses where worm eggs have built up in number for several years. In some areas histomoniasis is reportedly common in broilers. In some instances, disinfection may have value in killing worm eggs, but there is no experimental work to support this conclusion.

Management Procedures

Management practices alone are rarely adequate to keep the disease at a low level in commercial flocks.

Treatment

Preventive chemotherapy and treatment are not possible in Europe, North America, and many other countries because there are no licensed products. Regulatory action has removed the most useful drugs from the market in the United States, the EU, and some other countries (41).

An inhibitory effect of paromomycin, an aminoglycoside antibiotic, on the progression of histomoniasis was described in the 1960s. The preventive effect is dose dependent; the drug has no therapeutic effect caused by its pharmacology because it is not absorbed from the gut (7, 67). The selection for some antibiotic resistance during application of the drug raises concerns about the usage (35).

Recent studies on phytomedical substances showed a positive effect *in vitro* with some of these substances, but efficacy has not been confirmed *in vivo* (18, 59, 65).

Because of the close association of *H. meleagridis* with bacteria, it has become common practice to treat outbreaks with antibiotics. Although it is generally beneficial to administer antibiotics to combat secondary infections, there is no evidence that such treatments have a direct effect on histomoniasis (30).

Worm control is considered a central part of blackhead disease control programs for chickens. Frequent worming with benzamidazole type anthelmintics is known to reduce exposure to both worms and histomonads. It is important to administer wormers at least 1 week prior to the usual expected time of outbreaks, based on the history for each farm.

Miscellaneous and Sporadic Protozoal Infections

Robert Beckstead

Summary

Agent, Infestation, and Disease. An assortment of protozoa has been identified in various bird species. Clinical signs and pathogenicity vary widely between these parasites with the impact of many on poultry production still unknown. Current housing practices in the United States, Canada, and Europe for production birds have decreased the incidences of infection for many of these parasites, although they are still common in certain parts of the world.

Diagnosis. Diseases caused by miscellaneous protozoa are usually diagnosed by microscopic visual examination of the organism isolated from the site of infection. However, most parasites discussed in this chapter have polymerase chain reaction-based diagnostic tests.

Prevention. No treatment strategies are available for these protozoa; therefore, prevention is necessary. Knowing the route and source of protozoan infection is an important factor in developing biosecurity measures to decrease potential encounter with the bird and to break the parasites life cycle.

Cochlosoma anatis

Cochlosoma anatis was originally isolated from the intestines of the European domestic duck (110), but has since been reported from other poultry. The disease significance of *C. anatis* remains uncertain, but recent reports suggest *C. anatis* can cause limited pathology in the gut of both turkeys and ducks resulting in diarrhea and stunting (11, 13, 19, 106). Direct association of *C. anatis* with clinical signs has been difficult, because other protozoa, viruses, and pathogenic bacteria may be present.

Etiology and Classification

Cochlosoma was first described in 1923. In 1930, Tyzzer described 2 similar genera from the intestines of the ruffed grouse. He erected a new family, Cochlosomidae, to include the type genus *Cochlosoma* Kotlan, which is the same as *C. anatis* (89, 110). Microscopic studies of *C. anatis* documented the presence of a pelta, axostyle point, parabasal apparatus and costa substantiating that *C. anatis* should be classified with trichomonads (65). Classification of this genus within the order Trichomonadida Kirby, 1947 and family Cochlosomatidae Tyzzer, 1930, was further supported by ultrastructural homology between *C. anatis* and *Trichomonas* (66, 89). These taxonomic relationships have been confirmed by the phylogenetic analysis of the small-subunit rRNA gene (47).

Morphology

By light microscopy, *C. anatis* is 6–12 µm long and 4–7 µm wide with a characteristic adhesive disc on the anteroventral surface of its pyriform body (Figure 28.22). The parasite has a single nucleus and can be distinguished readily on Giemsa- or trichrome-stained impression smears (19). In wet mount preparations, the trophozoites move in a characteristic jerking motion as a result of the flagella that causes it to rotate around its long axis (81). Scanning electron microscopy (Figure 28.23) shows a prominent lateral groove, an undulating membrane, 6 flagella, and an axostyle (71). On the left side, the disc is interrupted by a lateral groove, which extends along the length of the body (89). Four anterior flagella emerge as 2 pairs, just above the lateral groove, on the left wall of the ventral disc (89, 106). The recurrent flagellum arises with the 4 anterior flagella, is associated with the undulating membrane, and trails beyond. The sixth flagellum arises to the left of the body midline on the dorsal surface. The thin axostyle projects from the posterior end of

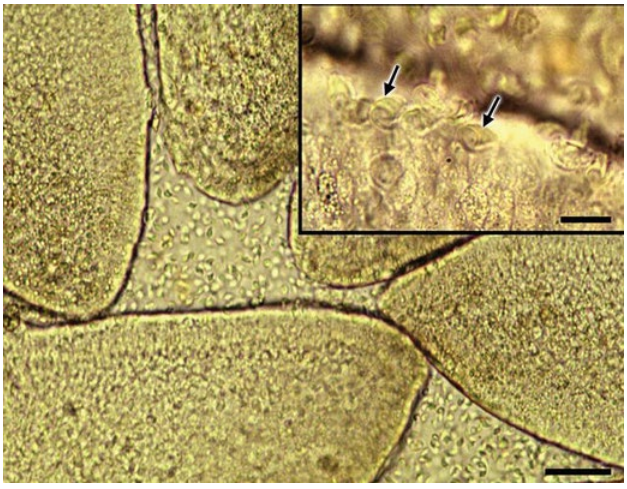


Figure 28.22 *Cochlosoma anatis* as viewed by light microscopy in a wet mount preparation of mucosa of the jejunum of a turkey. Numerous protozoan cells are evident between the intestinal villi. Bar = 80 µm. Inset: higher magnification; distinct ventral disc indicated by arrows. Bar = 10 µm.

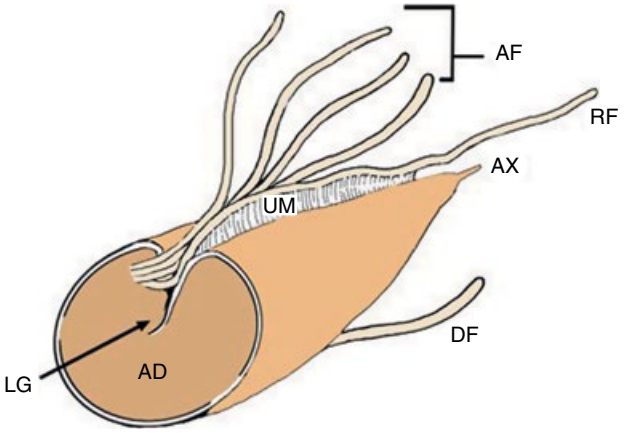


Figure 28.23 *Cochlosoma anatis*: prominent external features, ventral view. Ventral adhesive disc (AD), recurrent flagellum (RF), undulating membrane (UM), anterior flagella (AF), axostyle (AX), and anterior opening to the lateral groove (LG). The lateral groove runs the full length of the trophozoite adjacent to the undulating membrane. Not visible is the origin of the single dorsal flagellum (DF).

the trophozoite. The organelle structure of *C. anatis* has been extensively characterized by transmission electron microscopy, revealing the presence of a hydrogenosome-like structure (89).

Transmission, Incubation Period, and Life Cycle

Transmission of *C. anatis* can occur either by oral or cloacal inoculation with fecal material containing the trophozoites or intestinal scrapings from infected birds (11, 71). Forty percent of directly infected turkeys were positive for *C. anatis* in intestinal mucosal scrapings at 4 days post inoculation (PI), whereas all poults were positive by 6 days PI (71). Ducks shed trophozoites in their feces by 7 days PI (11). Lateral transmission was observed when naïve turkeys were placed with infected turkeys (71). However, limited transmission (8%) was observed when naïve turkeys were placed on fresh litter from turkeys excreting the trophozoites, suggesting that direct contact between birds may be needed for transmission of *C. anatis*. House flies have been implicated as a carrier based on polymerase chain reaction (PCR) detection using primers against the *C. anatis* mtDNA 16S gene, although it is uncertain whether the house flies were carrying live trophozoites (78).

Cochlosoma reproduces by longitudinal fission (30, 62, 89, 110). An infective pseudocyst was formed upon incubation on ice for 24 hours, but electron microscope studies did not demonstrate a true cyst wall (33).

Pathogenesis and Epidemiology

Natural and Experimental Hosts

Naturally occurring *C. anatis* infections have been described in the turkey, duck, goose, and coot (11, 19, 89, 110). *Cochlosoma* spp. infections have been reported in a wide variety of wild birds including the eastern robin, American magpie, woodcock, waxbill, and a variety of finches (38, 89, 91, 110). Experimental *C. anatis* infections have been produced in adult Bobwhite quail (67% infection) and 8-week-old chickens (12.5% infection) (71). These reports suggest that all poultry are susceptible to *C. anatis* infections and wild birds may serve as a reservoir.

Site of Infection

In the duck, *C. anatis* was found mainly in the lower ileum, cecum, and colon (62, 110, 113) and also seen in the jejunum at 25 days PI of a duckling experimentally inoculated at 1 day old (11). In the goose, *C. anatis* was found primarily in the colon and also in the cecum (89). In turkeys, trophozoites have been observed in the duodenum, jejunum, and ileum of all birds and also in the cecum and colon of some birds (19, 71, 81).

Clinical Signs and Pathogenicity

Kotlan noted that the intestinal wall of *C. anatis*-infected ducks was swollen and catarrhal at the point where a mass of the flagellates was attached and the intestinal contents were mixed with blood. Kimura was unable to attach any pathogenic significance to *C. anatis* in ducks, but noted inflamed intestinal tracts that he ascribed to a bacterial infection rather than to this parasite (62). Travis did not observe pathogenic changes associated with *C. anatis* infections in the birds he studied (110). Bollinger suspected *C. anatis* as the causative agent of severe runting and mortality associated with enteritis in inoculated ducklings (11), but could not make a definitive association because of a low number of *Trichomonas* and *Spironucleus* (Hexamita) flagellates and *Campylobacter jejuni* found in the inoculum used in the study. In addition, many of the ducklings also died of secondary Gram-negative bacterial septicemia (11). The authors, in a separate experiment, inoculated ducklings with a pure culture of *C. anatis*. They observed an increase in intestinal villus length and altered mucosal enzyme concentrations, but no effect on weight gain (12).

Finch aviaries have reported clinical signs including debility, dehydration, and significant mortality as the result of *C. anatis* infections (73, 91). However, Filippich reported that various species of apparently healthy finches sampled in pet shops were infected with *C. anatis*, suggesting that the parasite is not always pathogenic in finches (38).

The pathogenicity of *C. anatis* in the turkey is also unclear. McNeil and Hinshaw reported that *C. anatis* was always found in association with *Spironucleus* (Hexamita) or in combinations with *Spironucleus* and *Salmonella* (81). Experimental *C. anatis* infections in turkeys caused little or no microscopic change in the intestinal tract, suggesting that *C. anatis* alone did not cause clinical signs (14, 15). However, Campbell reported a case of *C. anatis* infection in turkeys in which affected turkeys, 2–10 weeks of age, had severe catarrhal enteritis (16). Various workers identified *C. anatis* as the likely etiologic agent in a series of cases of diarrhea and enteritis in turkeys with depressed body weights (19). A combination of *C. anatis* and turkey coronavirus was more pathogenic than *C. anatis* or coronavirus alone (106). All these experimental findings with *C. anatis* suggest that this parasite can cause limited pathology in turkeys but can worsen disease signs associated with other pathogens.

The exact role of the ventral adhesive disc in the pathogenicity of *Cochlosoma* is still unknown. Multiple scanning and transmission electron microscopy images revealed a clear indentation in the mucosal brush border of the hosts with the same size and shape as the ventral adhesive disk which suggests that the ventral disc serves as the attachment to the intestinal mucosa (19, 89, 113). It appears likely that this attachment to the microvillous border plays some role in pathogenicity.

Prevention and Control

Treatment

Historically, *C. anatis* infections were treated successfully with members of the nitroimidazole family and with roxarsone (11, 14, 15, 38, 82, 91). However, these products are no longer approved for use in poultry.

Prevention

Contamination is most likely introduced to poultry farms by wild birds or small rodents. Thus, biosecurity is key to prevention of infections. Because *C. anatis* survives only a short time in the environment, any residual parasites die out upon depopulation of the farm (15, 71). Disinfection with phenolic or quaternary ammonium compounds or formalin is highly effective (14).

Haemoproteus Infections

Haemosproteus (Apicomplexa) is a genus of blood parasites transmitted by biting diptera of the families Hippoboscidae and Ceratopogonidae (66). About 140 species of *Haemoproteus* have been reported in birds, with most occurring in wild waterfowl, raptors, and passerines (66, 67). Species found in domestic poultry include *H. meleagridis* in domestic and wild turkeys (46), *H. columbae and H. saccharovi* in pigeons and doves, and *H. nettionis* in waterfowl (72). Intermediate hosts include the hippoboscid *Pseudolynchia canariensis* for *H. columbae* and the ceratopogonid *Culicoides* for *H. nettionis* (64). Hosts for *H. meleagridis* include *C. edeni*, *C. hinmani*, *C. arboricoli*, *C. knowltoni*, and *C. haemoproteus* (5, 6). Infections occur throughout tropical and temperate areas wherever insect hosts and avian hosts coexist.

Etiology

Infections are characterized by schizogony (merogony) in visceral endothelial cells, gametocyte development in circulating erythrocytes, and the presence of pigment in granules in infected erythrocytes. Zygotes, ookinetes, and oocysts development occur in the insect hosts, with sporozoites infecting the salivary glands (42). Atkinson (6) reported at least 2 generations of schizogony. The first-generation schizonts mature 5–8 days PI and second-generation megaloschizonts develop after 8–17 days, yielding spherical merozoites that mature into erythrocytic gametocytes (Figure 28.24).

Pathogenesis and Pathology

Signs of *H. meleagridis* in turkeys include severe lameness, diarrhea, depression, emaciation, and anorexia (6). Anemia and enlarged livers are sometimes seen. Wild turkeys had pathology associated with megaloschizonts in skeletal and cardiac muscle (7). Pigeons infected with *H. saccharovi* had enlarged gizzards. Muscovy ducks (*Carina moschata*) infected with *H. nettionis* suffered lameness, dyspnea, and death, with hemorrhage on the heart, edematous lungs and swollen livers, spleens, and kidneys (59).

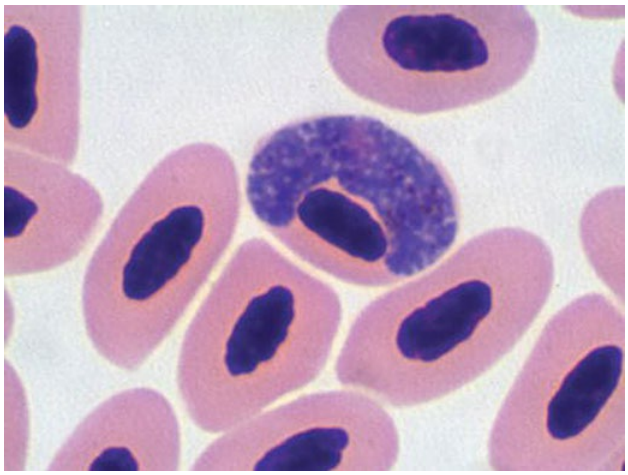


Figure 28.24 *Haemoproteus columbae*. Pigeon blood, Giemsa stain.

Diagnosis

Microscopic examination of stained blood smears is key to diagnosis of *Haemoproteus* infection. Restriction enzyme tests and PCR assays have been developed that allow differentiation of *Haemoproteus* spp., *Plasmodium* spp., and *Leucocytozoon* spp. (8, 48).

Treatment and Control

Control of insect vectors may be useful in local situations (61). However, complete life cycles are not known for most species, precluding specific control recommendations. No drugs are approved for commercial use.

Leucocytozoonosis

Leucocytozoon are hemosporidian parasites belonging to the Apicomplexa phylum and infect both wild and domestic avian species (66, 69). These parasites have a 2-host life cycle, involving birds and blackflies (*Simuliidae*) or biting midges (*Culicoides*). Sporogony occurs in the insect, but schizogony (tissue phase) and gametogony (blood phase) occur in the vertebrate host. Approximately 60 *Leucocytozoon* species have been described in birds (50, 66). Outbreaks of leucocytozoonosis are sporadic in North America (1), but are relatively common in the open type poultry houses of Southern and Eastern Asia (115), the Philippines, Indonesia, and Eastern Africa (21).

Leucocytozoon simondi Mathis and Leger 1910

Leucocytozoon simondi, synonymous with *L. anatis* and *L. anseris*, infects only ducks and geese. This parasite has been identified in 27 species of ducks and geese in the United States, Canada, Europe, and Vietnam (50, 93). Infection rates are high, with 14%–20% of ducks and geese along the northeastern seaboard of North America carrying infections. Eighty percent of geese at Seney Wildlife Refuge in Michigan had some parasitemia in 1963 just prior to the egg-laying season (9, 10, 49). The bloodsucking flies *Simulium venustum*, *S. croxtoni*, *S. euradminiculum*, and *S. rugglesi* serve as intermediate hosts for *L. simondi* in ducks.

Etiology. Sporogony occurs in the insect host upon ingestion of blood containing gametocytes and may be completed in 3–4 days. In the stomach, gametocytes differentiate into macrogametocytes (female) or microgametocytes (male) (32). Gamonts may be differentiated with a Romanowsky stain based on the dark blue staining cytoplasm of the macrogamete with its red nucleus, and the very pale blue staining cytoplasm of the microgamont with its pale pink nucleus (68). Fertilization of the macrogametocytes form the ookinetes which invade the intestinal cells and mature into oocysts. Sporozoites produced in the oocysts migrate to the salivary glands where they remain infective up to 18 days after the last blood meal (32).

Sporozoites enter the bird host when it is bitten by the infected fly and migrate to hepatocytes where they initiate schizogony. Merozoites released from hepatic schizonts either enter parenchymal cells of the liver and initiate another schizogonic cycle or enter erythrocytes or erythroblasts to develop into gametocytes. Syncytia are phagocytized by macrophages or reticuloendothelial cells throughout the body and develop there into megaloschizonts up to 400 μm in size. The megaloschizonts release merozoites into the blood that go on to form gametocytes in lymphocytes and other leukocytes (32). Kocan (64) described an antierythrocyte factor in sera from acutely infected ducks, which agglutinated and hemolyzed normal untreated erythrocytes as well as infected cells. This factor was believed to be a product of the parasite, and its action may account for the osmotic fragility of erythrocytes and anemia associated with *L. simondi* infections (74).

The gametocytes of *L. simondi* found in the blood average 14.5 × 5.5 μm and usually inhabit elongate, spindle-shaped host cells averaging about 48 μm in length. The parasite lies beside the nucleus of the host cell. Elongate gametocytes probably develop exclusively in leukocytes, predominantly lymphocytes and monocytes, whereas mature round gametocytes are found in erythrocytes. According to Allan and Mahrt (4), each *Leucocytozoon* species enters gametogony in only 1 type of host cell; therefore, the presence of 2 morphologic types in the same bird suggests a concurrent infection with 2 species. Desser et al. (20) observed infections in some areas of northern Michigan that were characterized by the presence of both hepatic schizonts and round gametocytes, which he attributed to different strains of *L. simondi*.

Clinical Signs and Pathogenesis. Clinical signs associated with *L. simondi* include inappetence, weakness, listlessness, dyspnea, and sometimes death within 24 hours caused by the pathologic effects of the organism which include: anemia, leukocytosis, splenomegaly, and degeneration and hypertrophy in the liver and heart. About 60% of fatalities occur 11–19 days postexposure. However, subclinical infections are common.

Leucocytozoon smithi Laveran and Lucet 1905

Leucocytozoon smithi infects both wild and domestic turkeys and has been reported in North America and Europe (17, 36, 104, 105). Economically significant outbreaks of *L. smithi*-infection in turkeys are rare, because most commercial turkeys are reared indoors and away

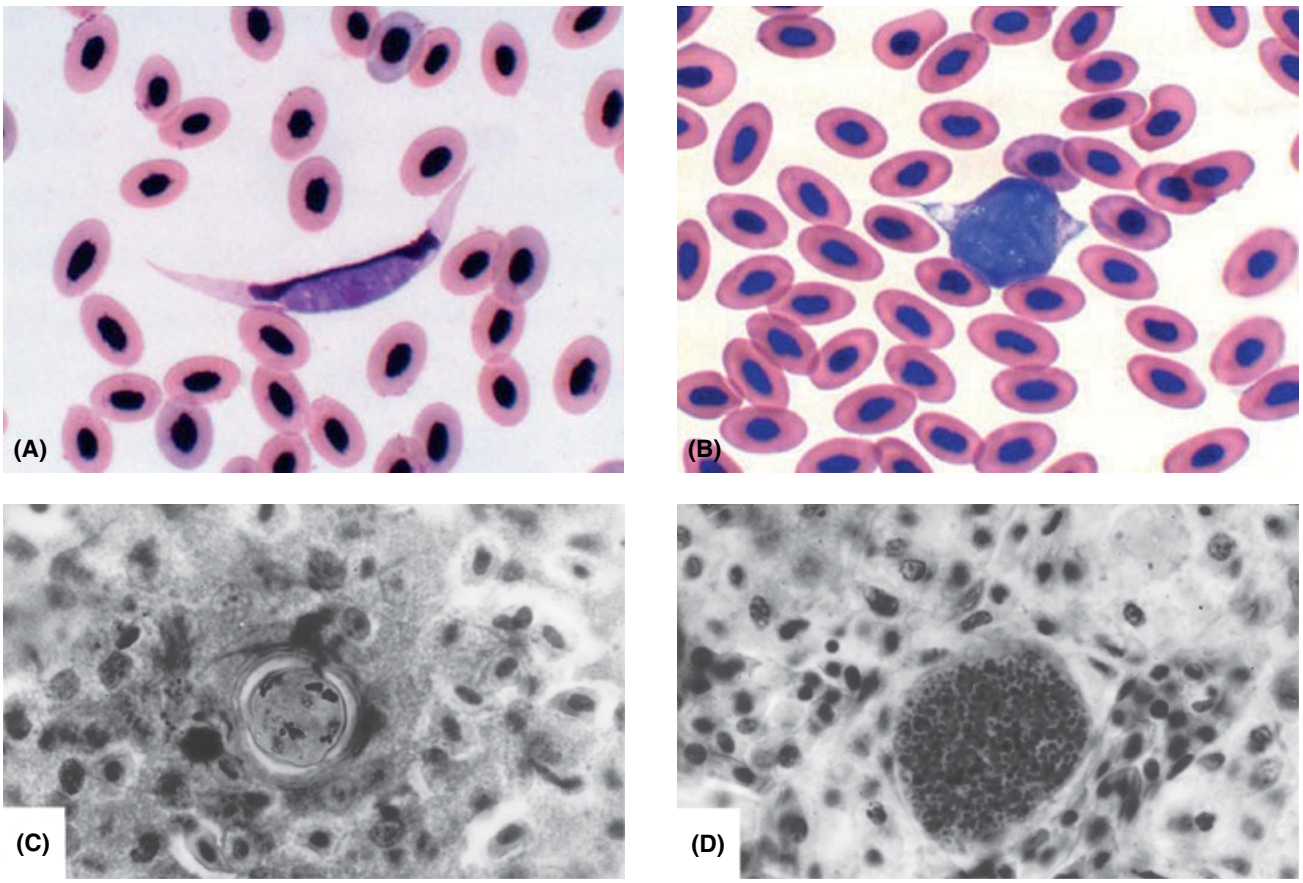


Figure 28.25 (A) *Leucocytozoon simondi*. Duck blood, Giemsa stain. Reproduced with permission of the American Association of Avian Pathologists. (B) *L. smithi*. Turkey blood, Wright-Giemsa. (C) *L. smithi*. Megaloschizont in turkey liver, ×1000. (D) *L. smithi*. Megaloschizont in turkey liver, day 10. (18)

from regions where bloodsucking flies and midges are abundant (1). *Simulium occidentale*, *S. aureum*, *S. meridionale*, *S. nigroparvum*, and *S. slossonae* have been listed as vectors for *L. smithi* (34, 63). *L. smithi* may be observed in the blood as rounded gametocytes that later become elongate, averaging 20–22 µm in length. They inhabit elongate cells averaging 45 × 14 µm, with pale cytoplasmic "horns" extending out beyond the enclosed parasite. Gamonts are found only in leukocytes. The staining characteristics of the gamonts with a Romanowsky stain are similar to those of *L. simondi* (68) (Figure 28.25). Both schizonts and megaloschizonts were observed and illustrated by Siccardi et al. (98).

Clinical signs include anorexia, excessive thirst, depression, somnolence, and sometimes muscular incoordination. Death may occur suddenly during the acute stage or when turkeys are subject to stress (72, 114). Johnson et al. (56) reported that death results from obstruction of the circulatory system by large numbers of parasites. Domestic hens infected with *Leucocytozoon* had decreased egg production, egg weight, and hatchability and higher mortality than uninfected hens (57). Males showed reduced mating activity (68). Recovered birds may harbor the parasite in their blood for more than 1 year and serve as a reservoir (21).

Leucocytozoon caulleryi Mathis and Leger 1909

The domestic chicken is the only confirmed host for *L. caulleryi* with the biting midge (*Culicoides arakawa*, *C. circumscriptus*, and *C. odibilis*) as its intermediate host (34). Infections have been found in southeast Asia, including Japan and South Korea (2, 83). Reports of leucocytozoonosis in South Carolina were suggested to be caused by *L. caulleryi*; however, subsequent research has suggested that these outbreaks were the result of a new, region-specific species, *Leucocytozoon andrewski* (2, 3, 34, 87).

Early schizonts occur in the lung, spleen, and thymus with megaloschizonts visible in numerous tissues, including the liver, spleen, kidneys, pancreas, heart, lungs, proventriculus, ventriculus, intestines, and brain (44, 50). Extensive hemorrhage occurs when merozoites are released from megaloschizonts. Mature gamonts are round and occupy round host cells, erythrocytes, and

leukocytes about 20 μm in diameter. The nucleus of the host cell reportedly disappears after infection, a characteristic that differs from other species with round gametocytes. Macrogametes (12–15 μm) stain more darkly than microgamonts with Romanowsky stain (10–15 μm) (68).

Infections of *L. caulleryi* in chickens are characterized by hemorrhage in the peritoneal cavity, perirenal hemorrhage, and subdural hemorrhage (44). In infected laying hens, the uterus is edematous on gross examination. Schizont development, granuloma formation, and inflammation of the uterus are noted on histopathology (86). Serious infections may cause death in growing chicks and reduced egg production in hens (83).

Leucocytozoon sabrazesi Mathis and Leger 1910

Leucocytozoon sabrazesi has been found in domestic chickens in southeast Asia, causing anemia, thickened oral discharge, and paralysis of the legs. A survey of southern China showed that chickens were infected with *L. sabrazesi* year-round with infection rates ranging from 0% to 80% (117). Megaloschizont formation has not been reported for this parasite. Zhao et al. (116) using antibodies specific for red blood cells and various leukocytes showed that gametocytes develop exclusively within thrombocytes. *Simulium* blackflies are believed to serve as the insect host.

Leucocytozoon schoutedeni Rodham, Pons, Vandenbranden, and Bequaert 1913

Leucocytozoon schoutedeni was reported only in east Africa, with 50% of chickens harboring infection (21). Gametocytes are round (11–13, μm) and found in round host cells (18 μm) in which the nucleus surrounds the parasite which is about one-half of the host cell's length. Staining characteristics of the gametocytes have not been reported. The blackfly genus *Simulium* serves as the invertebrate host.

Diagnosis

Leucocytozoon infections are diagnosed by microscopic observations of gametocytes in a blood smear, schizonts in tissue sections, or PCR-based DNA detection (48, 102). A variety of serologic tests have been developed for detecting antibodies to *L. caulleryi*, including the agar gel precipitation test, indirect fluorescent antibody (IFA), enzyme-linked immunosorbent assay (ELISA), and latex agglutination test (52, 54).

Treatment and Control

No drugs are approved for treatment of leucocytozoonosis. Experimentally, pyrimethamine (1 ppm) combined with sulfadimethoxine (10 ppm) was partially effective against *L. caulleryi*. Clopidol in feed effectively controlled *L. smithi* in turkeys according to Siccardi et al. (98). Tests of primaquine, ketotifen, clomipramine hydrochloride, desipramine hydrochloride, sulfaquinoxaline, and pyrimethamine, were largely ineffective against *L. sabrazesi* and *L. caulleryi* infections. Only primaquine reduced blood gametocytes of *L. sabrazesi*. Experimental vaccination against *L. caulleryi* using an oil-adjuvanted recombinant R7 vaccine from second-generation schizonts showed promising results in laboratory and field trials in chickens (53, 54). Control requires elimination of the insect host from the environment of the vertebrate host.

Plasmodium spp. (Avian Malaria)

Avian malaria, transmitted by mosquitoes, is caused by the parasites of the *Plasmodium* (phylum Apicomplexa) genus. Unlike mammalian *Plasmodium* species that only infect erythrocytes, exoerythrocytic stages found in avian malaria can result in extensive damage to many tissues and organs. Outbreaks of avian malaria are found mostly in Asia, Africa, and South America.

Etiology

Although domestic fowl can be infected experimentally by several species of *Plasmodium*, only a few appear to be natural hosts for these parasites: *P. gallinaceum* occurs in jungle fowl and domestic hens; *P. juxtanucleare* parasitizes domestic hens and turkeys; *P. durae*, *P. griffithsi*, *P. hermani*, *P. kempi*, and *P. lophura* occur in turkeys (40). Other species found in birds can infect domestic fowl or have been experimentally transmitted to them, including *P. relictum*, *P. elongatum*, *P. cathemerium*, *P. circumflexum*, *P. lophura*, and *P. fallax* (30, 39, 61).

Life Cycle

The life cycle of avian malaria is similar for all species of *Plasmodium*. Upon a blood meal by an infected mosquito, Sporozoites are inoculated into an avian host by the feeding behavior of mosquitoes and invade cells of the reticuloendothelial system. Development progresses through 2 generations of exoerythrocytic schizogony. Merozoites produced by the second generation schizonts invade erythrocytes resulting in schizogony (with released merozoites infecting other cells or erythrocytes) or gametogony in other cells. An interchange of parasites between blood and reticuloendothelial tissues may occur, resulting in secondary exoerythrocytic schizonts (phanerozoites), especially in spleen, kidney, and liver endothelial cells. Gametocytes are taken up by the mosquito when it feeds on infected birds, after which gamete

formation, oocyst development, and sporogony occur in the infected mosquito (95). Avian plasmodia develop in culicine mosquitoes, predominantly the genera *Culex* and *Aedes*.

Pathology and Pathogenesis

In avian hosts, the pathologic effects of malaria range from no apparent signs to severe anemia and death. *P. gallinaceum*, *P. juxtanucleare*, *and P. durae* are the most pathogenic for domestic fowl and may cause 90% mortality. Severe anemia and generalized hypoxia may occur in acute *P. gallinaceum* and *P. lophurae* malaria (61). *P. gallinaceum* and *P. durae* exoerythrocytic schizonts in the brain may block capillaries resulting in death caused by central nervous system dysfunction (41, 51).

Diagnosis

The diagnosis of *Plasmodium* infection requires microscopic examination of stained blood smears. A PCR assay has been developed for *P. gallinaceum* (35, 88).

Treatment and Control

The life cycle of the malarial parasite must be broken by the eradication of mosquitoes or by isolation of the flock from the intermediate host by suitable housing. There is no approved drug therapy for avian malaria. Experimentally, antimalarial drugs are effective (45, 101, 107). In turkeys, *P. durae* infections were successfully treated with a mixture of sulfachloropyrazine and sulfamonomethoxine, and treatment with halofuginone was partially effective (51).

Sarcocystosis

Avian sarcocystosis, caused by apicomplexan protozoa of the genus *Sarcocystis* Lankester 1882, is found throughout the world in wild birds, but is rare in domestic poultry (70). Contracting sarcocystosis from birds does not appear to be a public health hazard, because the parasites are killed by cooking and storage at subfreezing temperatures. Dubey et al. (29) has published an extensive overview of the history and current knowledge of toxoplasmosis in animals and humans.

Etiology

Sarcocystis have an obligatory 2-host life cycle (often carnivore–herbivore). When muscle tissues are eaten by a carnivore-host, cystozoites are released that penetrate the intestinal wall and develop into macrogametocytes and microgametocytes in subepithelial tissues. Oocysts (containing 2 sporocysts, each with 4 sporozoites) are produced and shed in feces as fully sporulated sporocysts. Sporozoites are released when sporocysts are ingested by the intermediate host (usually an herbivore) and invade the mucosa of the intestine. Schizogony (merogony) occurs in endothelial cells of various organs. After several asexual generations, the merozoites develop into young cyst stages, containing metrocytes and later cystozoites, that mature into the third-generation merонts (sarcocysts) in myocardial, skeletal, and smooth muscle tissues (75, 100).

Chickens are hosts for *Sarcocystis horvathi* (*S. gallinarum*, *S. horvathi*), and ducks are hosts for *S. rileyi* (*Balbiani rileyi*, *S. anatina*) (70). Levine (70) lists the chicken as the intermediate host and the dog as the definitive host for *S. horvathi*, although the life cycle is not completely defined.

Pathogenicity/Lesions

Sarcocystosis is recognized by the presence of elongated cysts (sarcocysts) running lengthwise in the musculature of the breast, thigh, neck, or esophagus (Figure 28.26) (97). Pathogenicity in birds is variable, with isolated cases reportedly causing debility and death in wild turkeys and distinct neurologic signs in backyard chickens (26, 85, 108).

Diagnosis

Diagnosis is based on the identification of sarcocysts or cystozoites in tissues. Large sarcocysts are seen easily in gross specimens; smaller cysts and cystozoites can be identified by histologic examination of muscle tissue.



Figure 28.26 Sarcocytosis in wild mallard, a severe, naturally occurring infection. (Photo by J. Runningen, Field Guide to Wildlife Diseases, US Department of Interior Fish and Wildlife Service, 1987)

Sarcocystis schizonts and merozoites can be differentiated from other systemic protozoal infections (*Toxoplasma* and *Neospora*) by immunohistochemistry (76, 85). Molecular techniques using PCR amplification and restriction endonuclease digestion are also being used in the identification of *Sarcocystis* species (27). In the definitive host, infections may be diagnosed by identification of sporocysts in feces.

Transmission, Carriers, and Vectors

Chemotherapy of avian sarcocystosis is not practical, placing the burden of control on prevention by breaking the infection cycle. *Sarcocystosis* appears to be most prevalent in hosts that frequently drink from shallow or stagnant water (puddling ducks, cattle, sheep, or swine) (103). Sporocyst-contaminated food is the common source of infection for the intermediate host (birds); infection in the carnivorous, definitive host (mammal) results from ingestion of sarcocyst-infected tissues of the intermediate hosts. Modern production systems prevent infections by preventing contact of poultry with oocyst-contaminated excreta of the definitive mammal host.

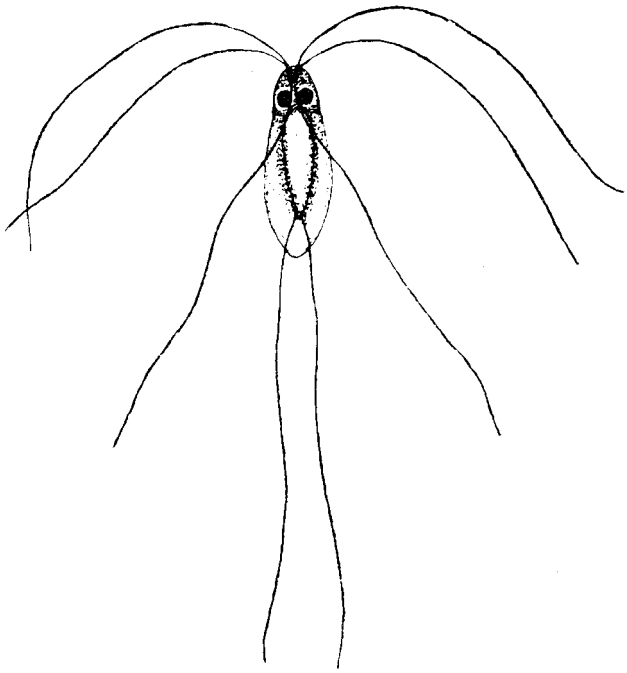


Figure 28.27 *Spironucleus meleagridis* from the intestine of a turkey. Reproduced with permission of the American Association of Avian Pathologists. (25)

Spironucleus meleagridis (Hexamitiasis)

Etiology and Distribution

Hexamitiasis, or infectious catarrhal enteritis, of poults is caused by the protozoan *Spironucleus meleagridis*. There is apparently no well-known common name for this parasite other than the original generic name *Hexamita*. There is no good measure of economic losses from this parasite. However, the United States Department of Agriculture (USDA) estimated that an annual loss of $667,000 occurred from hexamitiasis in turkeys from 1942 to 1951 (111). Cases of hexamitiasis are encountered sporadically in diagnostic laboratories in the United States. The disease has been reported from several areas of the United States, Canada, Scotland, England, and Germany. The organism has also been found in pheasants, quail, chukar partridges, and peafowl, which may be a source of infection for range-reared turkeys. The 8 prominent flagella include 4 anterior, 2 anterolateral, and 2 posterior. The 4 anterior flagella are recurved along the body (Figure 28.27). McNeil et al. (80) who named the species, described it as being 6–12.4 × 2–5 μm in size with binucleate large endosomes.

Pathology

Affected poults do not show specific signs, but a watery diarrhea occurs that may become yellowish later in the course of the disease. The poults at first are nervous and active but later tend to become listless and huddled. Convulsions and coma may occur as the terminal stage is approached.

Lesions include catarrhal inflammation and atony resulting in distention, especially in the upper small intestine. Intestinal contents are watery and large numbers of *S. meleagridis* may be seen in the crypts upon microscopic examination. A yellowish discoloration of the liver surface was described in an outbreak in Germany.

Diagnosis

The presence of watery diarrhea and the microscopic demonstration of flagellated *S. meleagridis* in fresh smears of duodenal contents are sufficient to establish the diagnosis. Survivors may become carriers; thus, the parasites may be seen without any signs of infection. These parasites are easily distinguished from other protozoa by their rapid, darting movement. They are small in comparison with other flagellates.

Control and Treatment

There is no effective treatment, although butynorate (0.0375%) and chlortetracycline (0.0055%) were approved for use at one time. There is no vaccine for this parasite. The removal of carrier birds, the separation of older stock from poults, and the exclusion of other avian host species from the area of the poult flock are thought to

minimize transmission. Attention to principles of good management is considered important in reducing losses to this and other parasitic diseases.

Toxoplasmosis

Toxoplasmosis is a parasitic disorder of mammals, birds, and reptiles affecting primarily the central nervous system, but sometimes the reproductive system, skeletal muscles, and visceral organs as well. A single species, *Toxoplasma gondii* (Apicomoplexa), is the cause of toxoplasmosis in all hosts, with the majority of infections being inapparent or latent. Overt toxoplasmosis in humans may result at times of stress or immunosuppression (25, 66). The literature presents extensive overviews of history and current knowledge of toxoplasmosis (22, 28, 37, 50, 58, 109).

Toxoplasmosis is uncommon in commercial chickens and is of little significance to the health of birds (23, 28, 60). Only a few cases of the disease have been described in chickens and turkeys (22, 43, 92, 99), but 1 study reported a high prevalence of *T. gondii* in backyard (up to 100%) and free-range organic (30%–50%) chickens (28). Although the consumption of any raw contaminated meat product, including uncooked poultry or eggs, is a potential source of human infection, commercial poultry products have not been implicated as a significant risk factor (28).

Etiology

The life cycle of *T. gondii* is divided between the definitive host (Felidae) and an intermediate host (non-Felidae) (24, 58). Schizogonic and gametogenic developmental cycles only occur in the intestinal epithelium of some members of the cat family (domestic cats, ocelots, pumas, jaguarundi, bobcats, and Asian leopards). Oocysts are shed into the environment with feline feces. Birds, cats, and other nonfelines can become infected by ingestion of these oocysts or tachyzoites and bradyzoites from infected host tissue. Once ingested, tachyzoites multiply by endodyogeny within parasitophorous vacuoles of many cell types and eventually develop into tissue cysts or bradyzoites that are walled off from the host immunity system (22, 96). Cysts may persist for the life of the host or, if immunity wanes, bradyzoites may be released and a proliferation of tachyzoites renewed. The tissue cycle may reverse again with cysts forming from tachyzoites (55, 58, 109).

Diagnosis

As reviewed by Montoya (84), *T. gondii* infections can be diagnosed by serological tests, PCR techniques, histological methods, or isolation of the organism.

Treatment, Prevention, and Control

Chemotherapy has not been used to control avian toxoplasmosis. Prevention of avian toxoplasmosis requires management practices that eliminate the source of *T. gondii* infective tachyzoites and oocysts by preventing exposure to rodents, coprophagous arthropods, earthworms, and cats (96, 112).

Trichomoniasis

Introduction

Trichomoniasis in birds, affecting the upper digestive tract, is caused by the flagellated protozoan *Trichomonas gallinae* (Figure 28.28A,B). In pigeons, it causes a condition

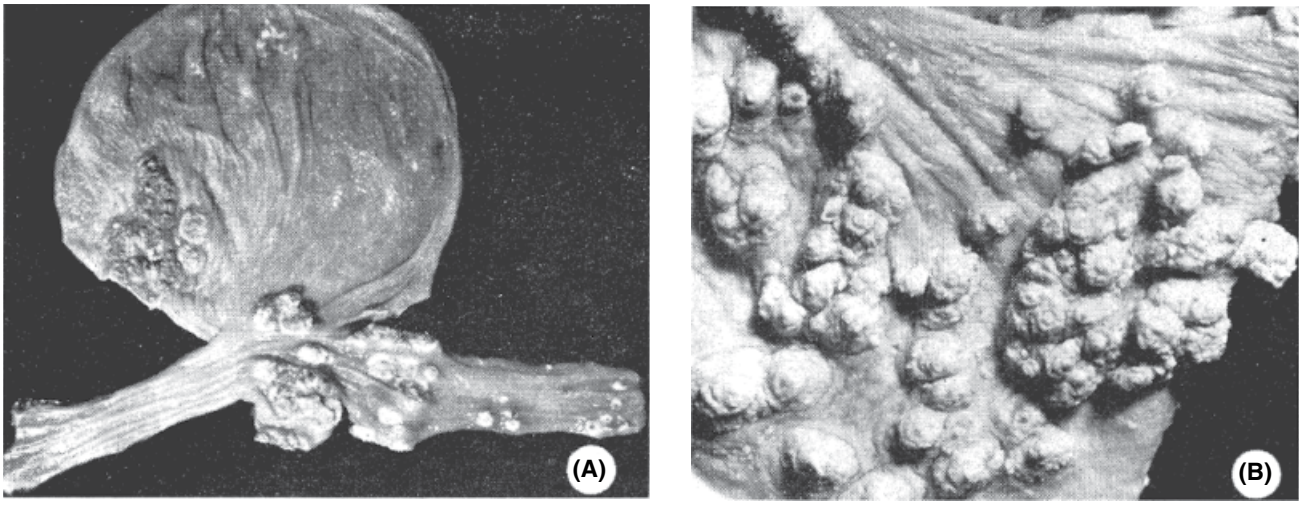


Figure 28.28 (A) Necrotic ulceration of the esophagus and crop seen in trichomoniasis. (B) Necrotic ulcers characteristic of trichomoniasis of upper digestive tract. Note pyramidal shape of tissue. (W.R. Hinshaw and A.S. Rosenwald)

known as canker. Turkeys, chickens, and a wide variety of wild birds are parasitized with varying degrees of pathogenicity (68).

Description

These intestinal flagellates are rapidly moving, pear-shaped protozoa that range in size from 5 to 9 μm in length and from 2 to 9 μm in width (Figure 28.29). There are typically 4 free flagella arising from a basal granule at the anterior pole of the organism. A slender axostyle usually extends well beyond the posterior end of the body. An undulating membrane originates at the anterior pole of the body and ends short of the posterior pole, with the enclosed flagellum not trailing free at the posterior end. The flagella and internal structures can be seen only with the aid of phase-contrast microscopy or special stains.

Incidence and Distribution

Squabs usually become infected with their first taste of "pigeon milk" from the crop of adults and usually remain carriers throughout life. With virulent strains, mortality may be as high as 50% before sufficient protective immunity develops. Pigeons are often blamed for transmission of trichomoniasis to turkeys and chickens. The economic impact of the disease in turkeys and chickens is difficult to assess, although infections are occasionally reported. When captive birds of prey such as falcons are allowed to feed on pigeons, infection may result in a condition known as frounce among falconers.

Life Cycle

Trichomonas gallinae reproduces by longitudinal binary fission. Cysts, sexual stages, or vectors are not known. The organism is transferred to squabs by infection of "pigeon milk" from adults. In chicken and turkey flocks, infection is spread by contamination of drinking water and perhaps feed.

Pathogenesis and Pathology

Nearly all pigeons are carriers of this organism. The virulence of *Trichomonas* varies widely, with some strains capable of causing mortality. At one time investigators considered trichomoniasis to be synonymous with blackhead disease. However, these investigators failed to consider that more than 1 parasite might produce lesions of similar appearance. Affected birds may cease to feed and become listless, ruffled in appearance, and emaciated before death. A greenish to yellowish fluid may be seen in the oral cavity and may drip from the beaks of infected birds.

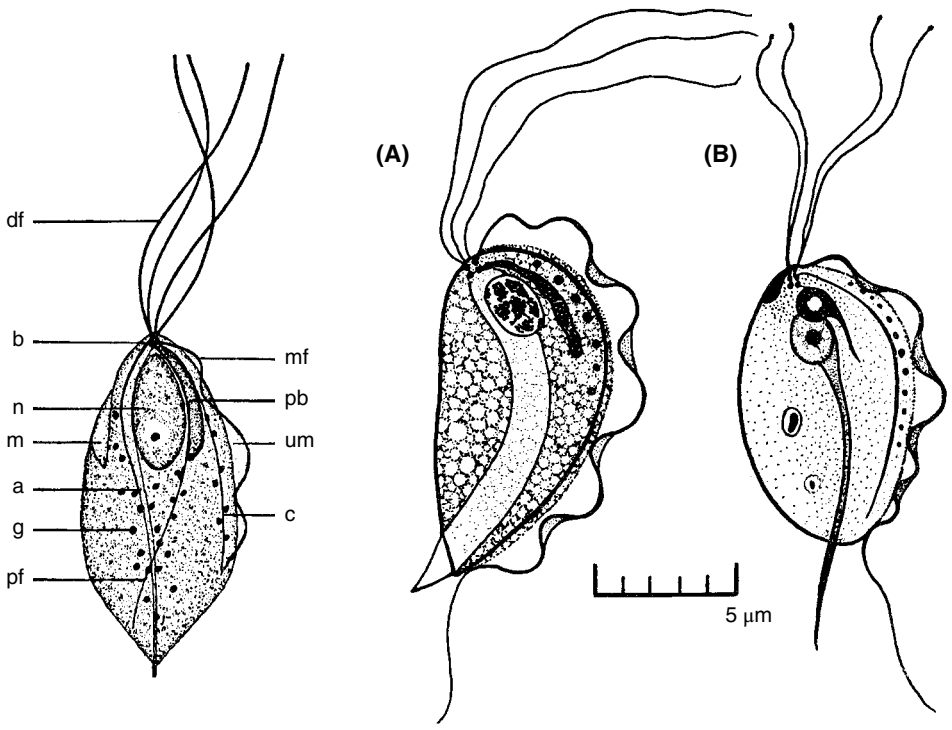


Figure 28.29 *Trichomonas gallinae*, semidiagrammatic (left): (a) axostyle, (af) anterior flagellum, (b) blepharoplast, (c) costa, (g) cytoplasmic granules, (m) mouth, (mf) marginal filament, (n) nucleus, (pb) parabasal body, (pf) parabasal fibril, (um) undulating membrane. (S Tabler) Two common trichomonads of the lower digestive tract of domestic birds (right), as specimens fixed in Schaudinn's fluid and stained with Heidenhain's hematoxylin may appear. (A) *Tritrichomonas eberthi*. (B) *Trichomonas gallinarum*. (E.E. Lund)

Gross

Trichomonas gallinae invade the mucosal surface of the buccal cavity, sinuses, pharynx, esophagus, and crop and occasionally the conjunctiva and proventriculus (Figure 28.28). The liver is frequently invaded, and occasionally other organs – but not the digestive tract below the proventriculus – are involved.

Lesions appear initially as small, circumscribed caseous areas on the surface of the oral mucosa, which may be surrounded by a thin zone of hyperemia. These may enlarge and become confluent. The build-up of caseous material may be sufficient to occlude the lumen of the esophagus partially or completely. These lesions eventually may penetrate tissue and extensively involve other regions of the head and neck, including the nasopharynx, orbits, and cervical soft tissues. In the liver, lesions appear on the surface and extend into the parenchyma as solid, white to yellow circular or spherical masses.

Histopathology

Pigeons infected with a virulent strain of *T. gallinae* had purulent inflammation with caseous necrosis as the predominant lesion (94). Trichomonads multiply in secretions and on the mucosal surface of the oropharynx. Ulceration of the mucosa with a massive inflammatory response, primarily heterophils, is well established by the fourth day of experimental infections. In the liver, focal necrotic abscesses occurred in all zones of lobules, with an inflammatory reaction characterized by mononuclear cells and heterophils. As liver lesions progressed, no intact hepatocytes remained in the center of foci; trichomonads were most numerous at the periphery.

Immunity

The relatively high incidence of infections in otherwise normal pigeons can be attributed to strain variations, acquired immunity, or both. Pigeons are immune to disease from virulent strains of trichomonads after recovery from sublethal trichomoniasis. Plasma from pigeons harboring any of 3 strains of *T. gallinae* could protect other pigeons against disease but not infection from a virulent strain.

Antigens of *T. gallinae* have been studied in regard to taxonomy with the conclusion that virulence and antigenic composition were related (31).

Diagnosis

Clinical signs and gross lesions are highly suggestive and may be confirmed by microscopic observation of organisms in direct wet smears from the mouth or crop. Histopathologic examination or cultivation of organisms in artificial media may help in cases in which the parasites are absent in fresh smears. Trichomoniasis must be differentiated from candidiasis and hypovitaminosis-A, which can produce somewhat similar lesions. History, cultivation for fungi, and histopathologic examination may prove useful in resolving problem diagnoses.

Several other species of flagellates that inhabit the avian gastrointestinal tract are frequently misidentified as *T. gallinae*. These other species of trichomonads and more distantly related flagellates have never been unequivocally demonstrated to be pathogenic for the avian host. Their recognition as harmless commensals will prevent unnecessary expenditure for therapeutic measures.

One trichomonad, *Tetratrichomonas gallinarum*, is a common inhabitant of the cecum of chickens and other gallinaceous birds. This trichomonad or a closely related species occasionally has been isolated from liver and blood. Although lesions have been ascribed to this organism, no confirmation of pathogenicity has come from experimental infection.

Other lower intestinal protozoa such as *Chilomastix gallinarum* (Figure 28.30), a cyst-forming flagellate with a large cytostomal cleft but no undulating membrane, and *Cochlosoma anatis*, with a ventral sucker covering

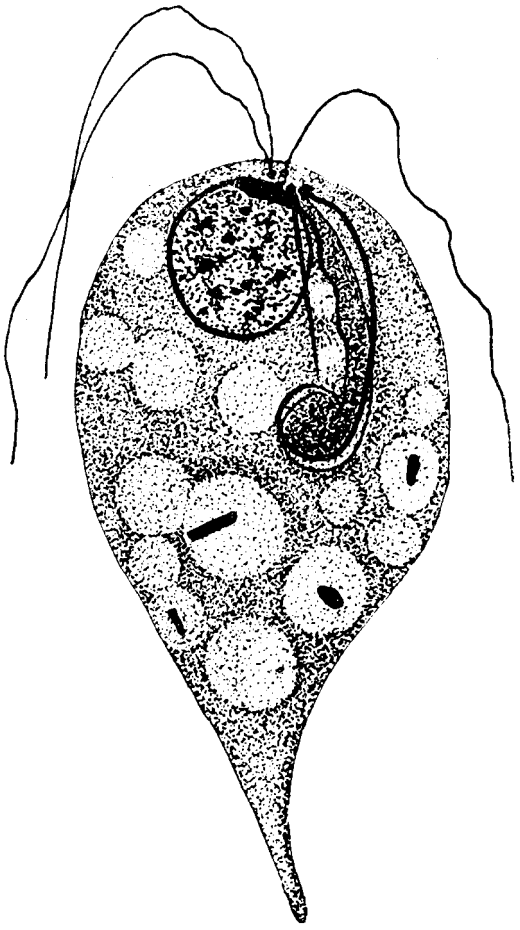


Figure 28.30 *Chilomastix gallinarum*, semidiagrammatic, illustrating details of morphology. (W.C. Boeck and M. Tanabe)

half the surface of the body, are apparently nonpathogenic. Although additional controlled experiments with flagellates found in the lower intestine are needed, for the present, they should not be considered important.

Prevention and Control

Because *T. gallinae* is transmitted from parent to squab in pigeons and by contamination of feed and water by oral fluids in the case of domestic fowl, sick birds should be removed from a flock. Drugs with activity against other related protozoa (*H. melelagridis*, *Entamoeba histolytica*, and *Giardia lamblia*) are active against trichomoniasis in pigeons or turkeys; however, none is approved for use in domestic birds. McLoughlin (79) found dimetridazole useful at a level of 0.05% in drinking water for pigeons. This drug is no longer available in the United States. There is no vaccine for this parasite.

Sporadic Protozoa in the Digestive Tract

Several species of the genera *Entamoeba* and *Endolimax* occur naturally in the ceca or feces of various domestic fowl or can be established experimentally. Apparently, none of these are pathogenic; they exist by feeding on intestinal contents.

The amebas have irregularly shaped trophozoites with a single nucleus with a more or less prominent endosome. They produce cysts containing 1, 4, or 8 nuclei. Phase-contrast microscopy or stained preparations is recommended for observing these organisms. A number of species have been reported (68, 77).

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Section VI

Noninfectious Diseases

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Nutritional Diseases

Kirk C. Klasing and Doug R. Korver

Summary

Agents and Disease. Of the approximately 36 nutrients required by poultry, the macroingredients (i.e., cereal grains and plant and animal proteins) used in commercial diets are typically supplemented with methionine, lysine, threonine, sodium chloride, calcium, phosphorus, zinc, manganese, copper, iron, selenium, and each of the essential vitamins. These added nutrients are most likely to cause deficiencies or toxicities caused by errors in manufacturing or formulation. Marginal deficiencies or toxicities often result in suboptimal production and not clear deficiency signs, but when severe, characteristic pathologies to specific organs and tissues develop. Deficiencies are most likely to be expressed in fast-turnover tissues, including feather follicles, skin, hematopoietic tissues, and the growth plate of bones. Some characteristic pathologies (e.g., tibial dyschondroplasia, chondrodystrophy, rickets, and anemia) can be caused by a deficiency of one of several different nutrients and differential diagnosis requires examination of nutrient levels in the feed or in the bird's tissues. Although large excesses of many nutrients are well tolerated, several nutrients (e.g., sodium, calcium, phosphorus, selenium, and vitamin A) become toxic at levels that are quite low relative to the bird's requirement and are the most likely to occur. From a human health perspective, levels of vitamins and trace-minerals in meat and eggs are highly dependent on dietary levels. Poultry can serve as buffers for high levels of nutrients found in foodstuffs, thereby reducing human exposure. However, some nutrients (e.g., selenium, iodine, copper, fluoride, and vitamin A) may accumulate in meat or eggs to levels that might adversely affect human health.

Diagnosis and Intervention. Prompt diagnosis and correction of toxicities is important for safeguarding the human food supply.

Introduction

Feed is the most expensive input in commercial production of poultry meat and eggs. Consequently, poultry nutrition has been an area of intensive research and optimization. Poultry require at least 36 dietary nutrients at appropriate concentrations and balance. Although chickens and turkey are omnivores, for economic reasons they are fed largely vegetarian diets supplemented with purified sources of nutrients to meet their nutritional requirements. Most poultry diets are based on soymeal as the primary protein source, and grains such as corn, sorghum, or wheat, as the primary energy source. To prevent nutrient deficiencies, grain-soy based diets are typically supplemented with concentrated sources of methionine, lysine, threonine, sodium chloride, calcium, phosphorus, zinc, manganese, copper, iron, selenium, and all of the essential vitamins. Nutrient deficiencies or toxicities most often result from errors in diet formulation or milling, and these supplemented nutrients are the most probable causes of nutritional problems. Severely deficient or toxic levels often are expressed as characteristic pathologies to specific organs and tissues. Marginal deficiencies or toxicities often result in suboptimal growth, egg production, and fertility, or resistance to infectious diseases but rarely result in clear deficiency signs. However, more severe errors in diet formulation or manufacturing cause diagnostically relevant changes.

Providing levels of nutrients that maximize productivity may exacerbate some developmental and metabolic diseases for genetically susceptible breeds of poultry. Nutrient restriction markedly reduces ascites (27), sudden death syndrome (57), and skeletal disorders (16). Chapter 30 should be consulted for information on these diseases. The following sections examine the effects of individual nutrient deficiencies or toxicities on poultry health.

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Water

Poultry can survive much longer without food than without water. Unlike larger farm animals, chickens and turkeys are most productive with access to a continuous water supply because they drink only small amounts at a time. An insufficient amount results in decreased growth, egg production, and resistance to heat stress, signs of dehydration including dark, dry shanks and contraction of the skin, dilated ureters with white urate crystals, and hemoconcentration. Hepatic histopathology reveals luminal dilation and attenuation of associated epithelium of distal segments of nephrons (67).

The quantity of water consumed is correlated directly with dietary salts and protein (29). Sodium or potassium bicarbonates cause similar increases in water intake of broiler chicks. Chlorine and phosphorus also increase water intake, but not as much as sodium or potassium, and calcium has little effect. The effect of protein is presumably caused by increased excretion of nitrogen and minerals such as phosphorus and sulfur that are constituents of protein. Excess water excretion often results in wet litter, which predisposes birds to footpad dermatitis.

Proteins and Amino Acids

Commercial diets usually are formulated using a "least cost" approach, and meeting the essential amino acid requirements greatly impacts the cost of the diet. For this reason, the limiting amino acids in the diet are typically supplied with very little margin of safety. The protein requirement represents the collective need for 10 absolutely essential amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine), 2 amino acids (cysteine and tyrosine) that can be synthesized from essential amino acid precursors, 2 amino acids that are essential for the young chick (glycine or serine and proline), plus additional amino acids to satisfy the amino nitrogen requirement for synthesis of nonessential amino acids, purines, pyrimidines, and other nitrogenous compounds.

Although poultry are omnivores, in commercial production they are fed plant-based diets. Grains such as corn, sorghum, and wheat are most deficient in lysine, whereas soybean meal is most deficient in methionine. It is often cost effective to supply these limiting amino acids in the form of pure amino acids, especially lysine, methionine, and threonine. Tryptophan, arginine, and isoleucine also may be supplied as synthetic amino acids when economically advantageous.

Amino Acid Deficiency

In contrast to the specific signs that may occur as a result of vitamin or mineral deficiencies, the effects of essential amino acid deficiencies are nonspecific: reduced growth, reduced feed consumption, lower meat yield, decreased egg production and egg size, and loss of body weight in adults. Unlike all other nutrients, a decrease in feed intake caused by a severe deficiency of a single amino acid occurs within hours of consumption of a deficient diet and is caused by a distortion in plasma and tissue amino acid levels. Unlike severe deficiencies, marginal amino acid deficiencies often result in increased food intake with concomitant reduction of body weight gain and lean tissue growth resulting in increased body fat. Some amino acids have additional effects. Threonine is found in high concentrations in the ileal endogenous protein losses, particularly in the mucins. Marginally low dietary threonine results in changes in morphology of the small intestine including decreased villus height, goblet cells, and mucous production (7, 46). Methionine deficiency may exacerbate choline or vitamin B_{12} deficiencies owing to its role in methyl group metabolism. Lysine deficiency causes impaired pigmentation of bronze turkey poults (19) and can result in stunting and retarded development in chicks (Figure 29.1). Arginine deficiency tends to cause the wing feathers to curl upward, giving the chick a distinct ruffled appearance. Several other amino acids have been reported to affect feather growth and structure (19).

Toxicity

When animals are provided with dietary protein in excess of their requirements, the surplus protein is catabolized, and the nitrogen released is converted to uric acid. An excess of protein may cause hyperuricemia and exacerbate articular gout or visceral gout in growing chickens. High protein diets also cause metabolic acidosis caused by the oxidation of excess methionine, lysine, arginine, and phosphorylated amino acids decreasing bone mineralization, eggshell thickness, and growth.

High levels of well-balanced protein are well tolerated, but excesses of individual amino acids caused by feed mixing errors are especially toxic. Acute toxicity of individual amino acids manifests as a severe decrease in food intake and growth rate. Methionine is the most toxic of the amino acids and also the amino acid most likely to be supplemented to a poultry diet. Methionine toxicity causes splenic hemosiderosis leading to the accumulation of heme Fe and darkening of the spleen (11). The safety margin for supplemented DL-methionine is very narrow, with toxicity occurring at about 2 to 3 times the requirement in growing chicks. Methionine



Figure 29.1 Lysine deficiency. Stunting and retarded development are apparent in this chick (right) fed a diet without sufficient lysine when compared with the normal control chick (left) fed adequate lysine. (D.E. Swayne)

is often supplemented as the analog DL-2-hydroxy-4-methylthiobutanoic acid (HMB), which is less toxic than L- or DL-methionine (3, 81). The relative order of toxicity of amino acids for growing chickens is methionine → phenylalanine → tryptophan → histidine → lysine → tyrosine → threonine → isoleucine → arginine → valine → leucine. Amino acid toxicity usually can be diagnosed by high levels of the toxic amino acid in the blood or the diet.

Carbohydrates

Starch is the primary source of metabolizable energy in practical poultry diets. Nonstarch polysaccharides (NSP) are not digested by the endogenous enzymes and may have either positive or negative effects on intestinal health depending on their chemical and physical properties. Some grains (e.g., wheat, barley, and rye), legumes, and distillers dried grains have NSP that increase the susceptibility of poultry to several important bacterial and parasitic pathogens. Grain NSP have been implicated in susceptibility to dysbacteriosis, *Clostridium perfringens*, and *Salmonella enteriditis* (44, 45, 72, 73, 86). This effect of NSP is due, at least in part, to increased viscosity of the digesta in the ileum and ceca, inducing epithelial inflammation and providing nutritional substrates that facilitate microbial overgrowth. In recent years, the diminishing use of antibiotics in the feed of poultry has exacerbated this problem (86) unless exogenous enzymes are added to break down the NSP. Because NSP increase the viscosity and moisture of feces, they are predisposing factor for footpad dermatitis (69).

Intestinal lactase activity is low in chickens; this limits the amount of lactose that can be hydrolyzed. Milk byproducts, such as whey, are excellent nutritionally but excessive levels in the diet cause growth depression and severe diarrhea.

Fats

Fats are important in the diet of poultry as concentrated sources of energy and sources of the essential fatty acids linoleic acid and alpha-linolenic acid. Linoleic acid deficiency in young chicks results in suboptimal growth and enlarged fatty livers, and in laying hens results in lowered egg production, egg size, and hatchability (76). Reduced concentrations of arachidonic acid and increased concentrations of eicosatrienoic acid in tissue and egg lipids are a characteristic sign of essential fatty acid deficiency (76).

Unsaturated fatty acids in the feed may undergo oxidative rancidity, with multiple effects: essential fatty acids are destroyed; aldehydes that are formed may react with free amino groups in proteins, reducing amino acid availability; and reactive oxygen intermediates generated during rancidification may destroy activities of vitamins A, D, and E and water-soluble vitamins such as biotin. The addition of synthetic antioxidants or plethoric levels of tocopherols to poultry feeds provides protection to essential fatty acids and other essential nutrients.

Vitamins

The amounts of various vitamins needed in poultry diets are very low, ranging from about 0.001% for pantothenic

acid to about 0.000,000,3% for vitamin B_{12}, yet moderate deficiencies are often more debilitating than those of protein, energy, or fat (28). The vitamins function as cofactors for enzymes, hormones (e.g., vitamins A,D), or antioxidants (previtamin A and vitamin E). Although the specific functions of the water-soluble vitamins in metabolism are biochemically distinct, the deficiency symptoms are often similar. Usually the growth rate of young birds is impaired and tissues that turn over rapidly are most affected, including feather follicles, the epithelial surfaces of skin, hematopoietic tissues, and the growth plate of bones. Common symptoms that arise from defects in these tissues are dyschondroplasia, chondrodystrophy, dermatitis, poor feathering, anemia, and increased susceptibility to infectious diseases. For example, chondrodystrophy ("perosis") occurs in young birds when the diet is deficient in choline, nicotinic acid, pyridoxine, biotin, folic acid, zinc, or manganese. Chondrodystrophy is an anatomic deformity of leg bones of young birds that is characterized by decreased linear bone growth, enlargement of the tibiometatarsal joint, and twisting or bending of the distal end of the tibia and proximal end of the metatarsus, with secondary varus or valgus deformation of the legs. When severe, the gastrocnemius tendon slips from its condyles and mobility is greatly reduced. It appears that chondrodystrophy occurs because the growth plate is an avascular tissue and chondrocyte growth and maturation depends on nutrient diffusion into the connective tissue matrix, which makes this region very susceptible to deficiencies of a variety of nutrients that have unrelated biochemical functions. Analysis of the diet may be the only way to determine whether a specific nutritional deficiency is responsible for the condition.

Modern poultry diets using grain and soymeal are deficient in most of the vitamins if they are not supplemented. In practice, poultry diets should be formulated to contain a large margin of safety for all of the vitamins to compensate for possible losses during feed processing, transportation, and storage, and for variations in feed composition and environmental conditions.

Birds with replete tissue stores of the fat-soluble vitamins A, D, and E can withstand long periods of depletion before deficiency symptoms manifest. Birds with adequate stores of most of the vitamins also can withstand feed restriction without deficiency symptoms because tissue catabolism liberates vitamins from muscle, fat, and other tissues. Thus, vitamin deficiencies are not usually important determinants of the clinical signs of feed restriction or starvation. The level of deposition of many of the vitamins into the egg directly reflects the amount in the diet. Marginal deficiencies of vitamins may not decrease egg production but are likely to influence embryonic development (29).

Excretion rates of vitamins A and D are limited and toxicity of these vitamins are more common than the other vitamins. Water-soluble vitamins are not stored to a large extent, excesses are excreted mostly in urine, and they are relatively nontoxic.

Vitamin A

From a nutritional perspective, vitamin A is the most challenging of the vitamins because it is deficient in many feedstuffs and is among the most likely to become toxic upon oversupplementation. Corn has moderately high levels of beta-carotene, which is converted to retinol, so diets that are high in corn are less likely to cause severe deficiencies compared with diets based on wheat or sorghum. Nevertheless, corn-based diets still require supplementation with additional vitamin A when fed to chicks or laying hens. Vitamin A is usually supplemented to poultry diets as retinyl-esters of acetate or palmitate at a level of 4,000 to 12,000 IU/kg.

With the exception of their role in vision, retinol and retinal must be converted to retinoic acid for their required function. Retinoic acid binds to specific nuclear receptors and induces or suppresses the expression of more than 500 genes that regulate cell replication, differentiation, and death. Retinoic acid is among the most important signaling molecules in ontogenesis. It regulates the expression of *Hox* genes along the axis of the early-stage embryo, the induction of digit formation, and the connection of the extraembryonic blood supply to the forming heart (91). Retinoic acid also regulates the commitment of differentiating stem cells along specific paths important in epithelial differentiation, bone modeling, spermatogenesis, and leukopoesis. For example, retinoic acid induces the differentiation of epithelial basal cells into the cubodial, columnar, and goblet cells characteristic of a soft, moist epithelia. When vitamin A is deficient, the basal cells differentiate into squamous cells (squamous metaplasia), which may become stratified and keratinized to form a hard, dry epithelia characteristic of the skin. Vitamin A deficiency can affect the epithelia of the esophagus, intestines, cloaca, respiratory tract, conjunctiva of the eye, ureters, cloacal bursa (bursa of Fabricius), and vaginal region of the oviduct.

Retinal, the aldehyde form of retinol, covalently binds to opsins in sensory cells of the retina and plays an essential role in the detection of light, especially in dim lighting.

Vitamin A Deficiency

Clinical Signs and Signalment. Vitamin A deficiency in the embryo often results in death during the first week of incubation. Those embryos that survive to term may be too weak to hatch or die shortly thereafter (77). A severe deficiency results in a grossly abnormal cardiovascular

system, characterized by an absence of vascular networks and by a ballooned, noncompartmentalized, randomly positioned heart without an inflow tract at the posterior site of the heart (29).

In growing chicks and poults, severely deficient birds display decreased growth, depression, inappetence, unthriftiness, increased mortality caused by infections, unsteady gait, and postural imbalance (29). In the absence of accompanying infectious diseases, deficiencies of vitamin A may cause epithelial damage in the oropharynx and esophagus without marked growth depression. Periorbital edema may occur (Figure 29.2A). In broilers, severe deficiencies result in hyperproliferation and decreased maturation of enterocytes, decreased mucin production, a decrease in the number of goblet cells, and blunting of villi with decreased expression of brush-border enzymes. Impaired growth rate of deficient broiler chicks appears to be secondary to diminished digestive function. Deficiencies of vitamin A are deleterious for development of the cloacal bursa and thymus. Infectious diseases may occur secondary to immunodeficiency and death from infection may occur before other clinical signs of vitamin A are evident (29).

Vitamin A can be stored in relatively large amounts as retinyl-fatty acid esters in the liver. Depending on storage levels, it usually takes 2–5 months for deficiency signs to develop in adult chickens or turkeys. The plasma vitamin A level tends to be maintained until storage pools are depleted and is not a reliable indicator of status until the deficiency is severe. Hepatic levels are a better indicator prior to the onset of deficiency symptoms. Deficient hens display weakness, weight loss, ruffled feathers, a sharp drop in egg production, increased incidence of blood spots in eggs, and increased susceptibility to infections, followed by swelling of the nictitating membrane and a watery discharge from the nostrils and eyes (30). As the deficiency continues, milky white, caseous material accumulates in the eyes, displacing the nictitating membrane, and eyelids may become stuck together. When chronic, infection of the eye results in necrosis and irreversible blindness that is unrelated to vision loss caused by depletion of retinal in the opsin pigments of the eye. An increased frequency of atretic ovarian follicles containing hemorrhages either throughout the follicle or between the theca interna and granulosa cell layer has been observed in hens exposed to vitamin A deficiency over a period of 5–8 months.

Pathology. Vitamin A-deficiency lesions first appear in the esophagus and pharynx and are confined largely to mucous glands and their ducts. The original epithelium is replaced by a keratinizing epithelium (i.e., squamous metaplasia) that blocks ducts of the mucous glands, causing them to become distended with secretions and necrotic materials. Small, white nodules may be found in the nasal passages, mouth, esophagus, and pharynx and may extend into the crop. Nodules range in size from microscopic lesions to 2 mm in diameter (Figure 29.2B). As the deficiency progresses, lesions enlarge, are raised above the surface of the mucous membrane, and have a depression in the center. Small ulcers surrounded by inflammatory products may appear at the site of these lesions. This condition resembles certain stages of fowlpox, and the 2 conditions can be differentiated only by microscopic examination. Bacterial and viral infections often occur because of breakdown of the mucous membrane (30).

Clinical signs and lesions of vitamin A deficiency of the respiratory tract are variable; it is difficult to differentiate this condition from infectious coryza, fowlpox, or infectious bronchitis. In vitamin A deficiency, thin diphtheritic membranes and nasal plugs usually are limited to the cleft palate and its adjacent epithelium. They may be removed easily without bleeding. Atrophy and degeneration of the respiratory mucous membrane and its glands occur. Later, the original epithelium is replaced by a stratified squamous keratinizing epithelium. In the early stages of vitamin A deficiency in chickens, turbinates are filled with seromucoid water-clear masses that may be forced out of the nodules and cleft palate by application of slight pressure. The vestibule becomes plugged and overflows into paranasal sinuses. Exudate also may fill sinuses and other nasal cavities, causing swelling of 1 or both sides of the face. Mucous membranes, cleared of inflammatory products, appear thin, rough, and dry (31).

Similar lesions frequently may be found in the trachea and bronchi. In early stages, these may be difficult to see. As the condition progresses, the mucous membrane is covered with a dry, dull, fine film that is slightly uneven, whereas a normal membrane is even and moist. In some cases, small nodule-like particles may be found in or beneath the mucous membrane in the upper part of the trachea.

Chronic vitamin A deficiency causes damage to the kidney tubules, which leads to azotemia and visceral urate deposits (e.g., "visceral gout") in severe cases.

Histopathology. An early histologic lesion of vitamin A deficiency is atrophy and deciliation of columnar-ciliated epithelium of the respiratory tract. Nuclei often present with marked karyorrhexis. A pseudomembrane formed by the atrophying and degenerating ciliated cells may hang as tufts on the basement membrane; later these are sloughed. During this process, new cylindric or polygonal cells may be formed singly or in pairs and appear as islands beneath the epithelium. These new cells proliferate, and their nuclei enlarge, containing less chromatin as they develop. Cell boundaries are less clearly defined; finally, the columnar ciliated epithelial lining of nasal cavities and communicating sinuses,

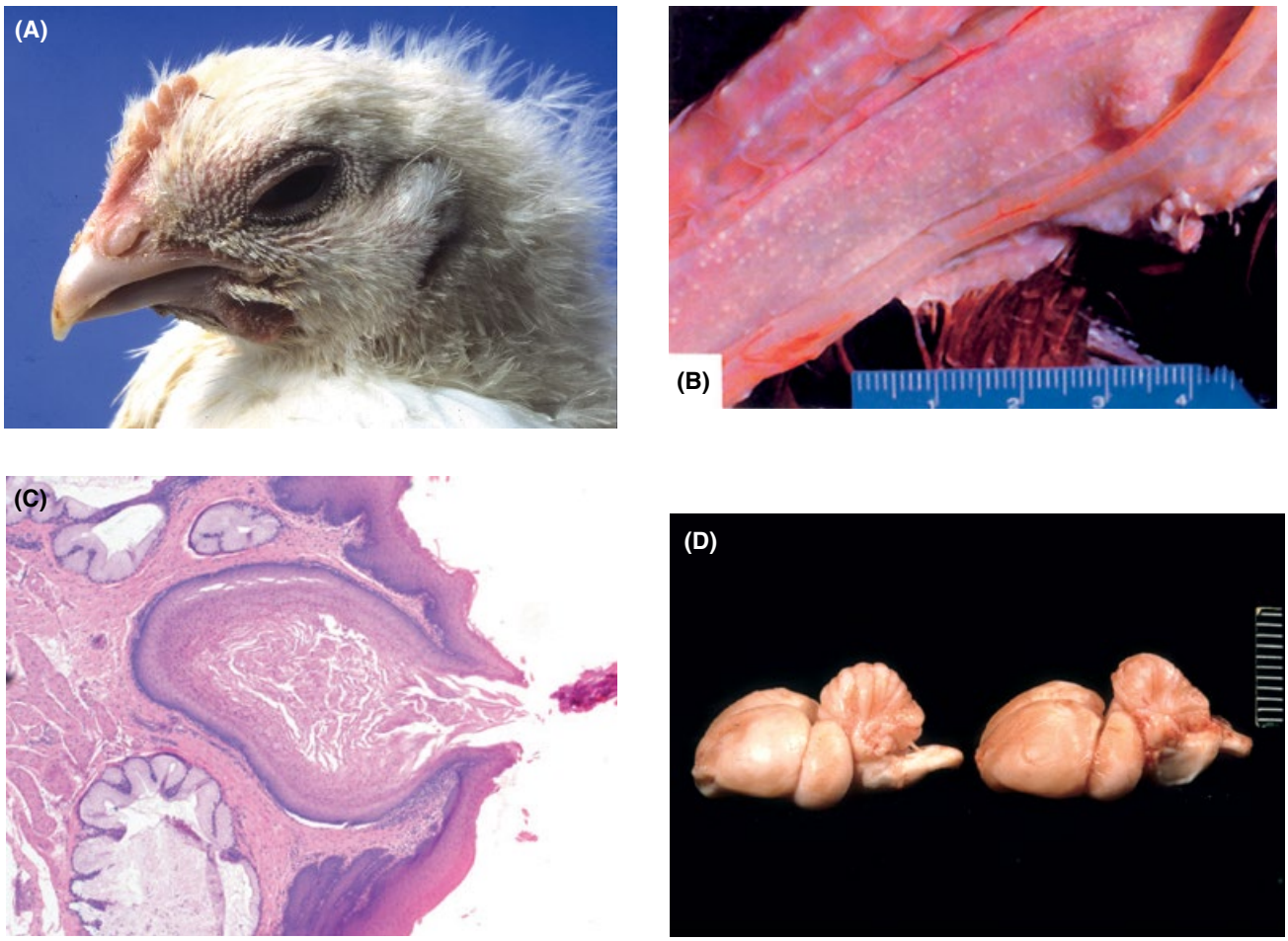


Figure 29.2 Vitamin A deficiency. (A) Periorbital edema and lack of pigmentation. (D.E. Swayne) (B) Distended, impacted mucosal glands resembling pustules in the esophagus. (H.J. Barnes) (C) Squamous metaplasia has replaced all but a few focal areas of normal mucosa in the base of this esophageal gland. Distention has resulted from occlusion of opening and accumulation of keratin and cellular debris in the lumen. Inflammation resulting in formation of a pustule will occur if contents contact surrounding tissues. (D.E. Swayne) (D) Normal brain with prominent cerebellar folia (left) and vitamin A-deficient brain with flattened cerebellar folia (right). (D.E. Swayne)

trachea, bronchi, and submucous glands are transformed into a stratified squamous keratinizing epithelium. Lesions in glands of the tongue, palate, and esophagus (Figure 29.2C) are similar to those of the respiratory tract (29, 30).

Vitamin A deficiency in young chicks and ducks causes marked retardation and suppression of endochondral bone growth. The proliferating zone is reduced. Hypertrophied cells accumulate, surrounded by uncalcified matrix. Vascular invasion of the epiphyseal cartilage is reduced and exhibits irregular patterns such as branching. The number of endosteal and periosteal osteoblasts is decreased, leading to impaired bone growth and thinning of bone cortex. Bone remodeling is inhibited. Disproportionate growth of brain and spinal cord relative to that of the axial skeleton appears to cause compression of brain tissue (Figure 29.2D). Increased cerebrospinal fluid pressure is one of the earliest signs of vitamin A deficiency (30).

Treatment of Deficiency. Poultry found to be severely deficient in vitamin A should be given a stabilized vitamin A preparation at a level of approximately 10,000 IU vitamin A/kg of ration. It also may be provided in the water or via injection. Absorption of vitamin A is rapid; therefore, chickens or turkeys not in advanced stages of deficiency should respond promptly, except for blindness caused by xerophthalmia, which is often permanent.

Toxicity

Mistakes in formulation of vitamin premixes can result in toxicity at vitamin A levels between 35,000 and 60,000 IU/kg. In broilers, signs of toxicity include slow growth, an unsteady gait, reluctance to walk, reduced bone mineralization and a higher incidence of tibial dyschondroplasia as indicated by widened epiphyseal growth plates with irregular tunneling by blood vessels. Severe toxicity results in anorexia, conjunctivitis,

adhesions of the eyelids, encrustations around the mouth, and thinning of the frontal bones of the skull with thickened osteoid seams (35, 71). Reduced bone growth and mineralization is caused by reduced osteoblastic cell activity and inhibited expression of calcium binding protein excretion (20). In broiler breeders, doses of 45,000 IU/kg and above significantly decreased egg weight, yolk color, eggshell thickness and strength, and reproductive performance (88).

Signs of hypervitaminosis A in Leghorn chicks differ from those of broiler chicks administered similar levels of vitamin A (71). The epiphyseal growth plates in tibiae of Leghorn chicks are normal in width but contain a narrower proliferative or maturation zone and a wider hypertrophic zone. Leghorns have normal osteoid seams in the skull and parathyroid morphology, whereas parathyroid hyperplasia occurs in broiler chicks.

Vitamin D

Vitamin D_3 (cholecalciferol) can be synthesized from 7-dehydrocholesterol in the skin under the influence of ultraviolet light. Although this synthesis can reduce the dietary requirement for vitamin D, it is not sufficient to satisfy requirements of poultry raised in conventional confinement housing. Poultry diets commonly are supplemented with vitamin D_3; however, 25-(OH)D_3 or 1-(OH)-25 D_3 are also sometimes used because they have higher bioavailability and can reduce the incidence of tibial dyschondroplasia and osteomyelitis in broilers and egg quality in older hens (21, 68, 78). The plant source of vitamin D activity, ergocalciferol (vitamin D_2), is not efficiently used by poultry and should not be used as a supplement.

Vitamin D acts as a hormone to regulate calcium and phosphorus metabolism in poultry and is critical for the formation and maintenance of a normal skeleton and for strong eggshells. The metabolically active form of vitamin D is 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$). The first hydroxylation occurs in the liver to give 25-(OH)D_3, which is the primary storage and circulating form of vitamin D. The second hydroxylation occurs in the kidneys and is tightly regulated by calcium status, being activated by parathyroid hormone or low blood calcium or phosphate. Many other hydroxylation products of 25(OH)D_3 have been identified but their functions have not been fully characterized. $1,25(OH)_2D_3$ functions in the regulation of calcium metabolism by stimulating the intestinal absorption of calcium, influencing osteoblast and osteoclast activity, and increasing renal tubular reabsorption of calcium in response to metabolic demands for calcium. $1,25(OH)_2D_3$ also influences cellular differentiation in the immune system, skin, and growth plate of the bone. $1,25(OH)_2D_3$ traverses the cellular membranes and binds to receptors in the nucleus. Receptors are present in almost all tissues of the body and more than 100 genes are known to be regulated by $1,25(OH)_2D_3$, including calbindin, which is important in calcium absorption and egg shell mineralization, and osteocalcin, which is important in bone mineralization. The opening of calcium channels in the cell membrane is an important nongenomic action of $1,25(OH)_2D_3$, which initiates a variety of immediate, nongenomic regulatory actions in cells (29).

Vitamin D Deficiency

Clinical Signs and Signalment. Hatchability is reduced markedly by vitamin D deficiency mainly caused by increased embryonic mortality late in incubation. Chicks and poults that do not hatch have a high incidence of chondrodystrophy in which the upper or lower mandible is shortened or deformed. The first signs of vitamin D deficiency in growing chicks or poults are slower growth and an awkward gait. As the deficiency advances, rickets becomes evident as severe fragility and bending of long bones caused by poor mineralization. Beaks and claws become soft and pliable, feathering is poor, and birds walk with obvious effort and take a few unsteady steps before squatting on their hocks, which they rest upon while swaying slightly from side to side. A moderate deficiency of vitamin D results in an increased incidence of tibial dyschondroplasia especially when calcium or phosphorus levels are not optimal. Many of the signs of a vitamin D deficiency may be similar to calcium deficiency, and analysis of levels of these two nutrients in the diet, or 25-(OH)D_3 in blood plasma, will confirm the cause (29).

In confined laying hens, signs of deficiency begin to occur as soon as 2 weeks after they are deprived of vitamin D. The first sign is a marked increase in the number of thin-shelled and soft-shelled eggs, followed soon after by a marked decrease in egg production. Biochemical indicators include a rapid decrease in the concentrations of 25-(OH)D_3 in the blood, followed by a decrease in blood calcium concentration. Egg production and eggshell strength may vary in a cyclic manner. Several cycles of decreased egg production and shell strength may each be followed by periods of relatively normal production and eggshell strength. Individual hens may show temporary loss of the use of the legs, with recovery after laying an egg that is usually shell-less. During periods of extreme leg weakness, hens show a characteristic posture that has been described as a "penguin-type squat." Later, beak, claws, and keel become very soft and pliable. The sternum usually is bent, and ribs lose their normal rigidity and turn inward at the junction of the sternal and vertebral portions, producing a characteristic inward curve of the ribs along the sides of the thorax (29, 30).

Pathology. In laying and breeding chicken and turkey hens deficient in vitamin D, characteristic changes observed on necropsy are confined to bones and parathyroid glands. The latter become enlarged from hypertrophy and hyperplasia. Bones are soft and break easily. Well-defined knobs are present on the inner surface of the ribs at the costochondral junction (rachitic rosary) (Figure 29.3A). Many ribs show evidence of pathologic fracture in this region. In chronic vitamin D deficiency, marked skeletal distortions become apparent. The spinal column may bend downward in the sacral and coccygeal region; the sternum usually shows a lateral bend and an acute dent near the middle of the breast. These changes reduce the size of the thorax with consequent crowding of vital organs. The beak may be soft and pliable (Figure 29.3B).

The most characteristic internal signs of vitamin D deficiency in chicks and poults are a bending of the ribs at their juncture with the spinal column and a bending of the ribs downward and posteriorly (Figure 29.3A). Poor calcification can be observed at the epiphysis of the tibia or femur (Figure 29.4). Bones of vitamin D-deficient chicks have reduced calcium content with an increased proportion of osteoid, and a greater proportion of bone mineral is present as a low-density amorphous form of calcium phosphate. The epiphyseal plate of long bones becomes wide and degenerative caused by the failure of cartilage-producing cells to hypertrophy and mature, leading to their accumulation rather than replacement by hydroxyapatite. As the deficiency progresses, the columns of chondrocytes in the degenerating hypertrophic zone of the epiphyseal plate become shortened and thickened, and exhibit an irregular pattern of invasion by metaphyseal blood vessels. Irregular patterns of cartilage and bone development occur in the primary and secondary spongiosa. Porosity of cortical bone increases caused by resorption of bone in Haversian canals. Increased osteoclast resorbing activity decreases trabecular bone volume and contributes to diminished mechanical strength of long bones. When severe, the loss of strength leads to fractures (Figure 29.4) (17, 29, 33, 41).

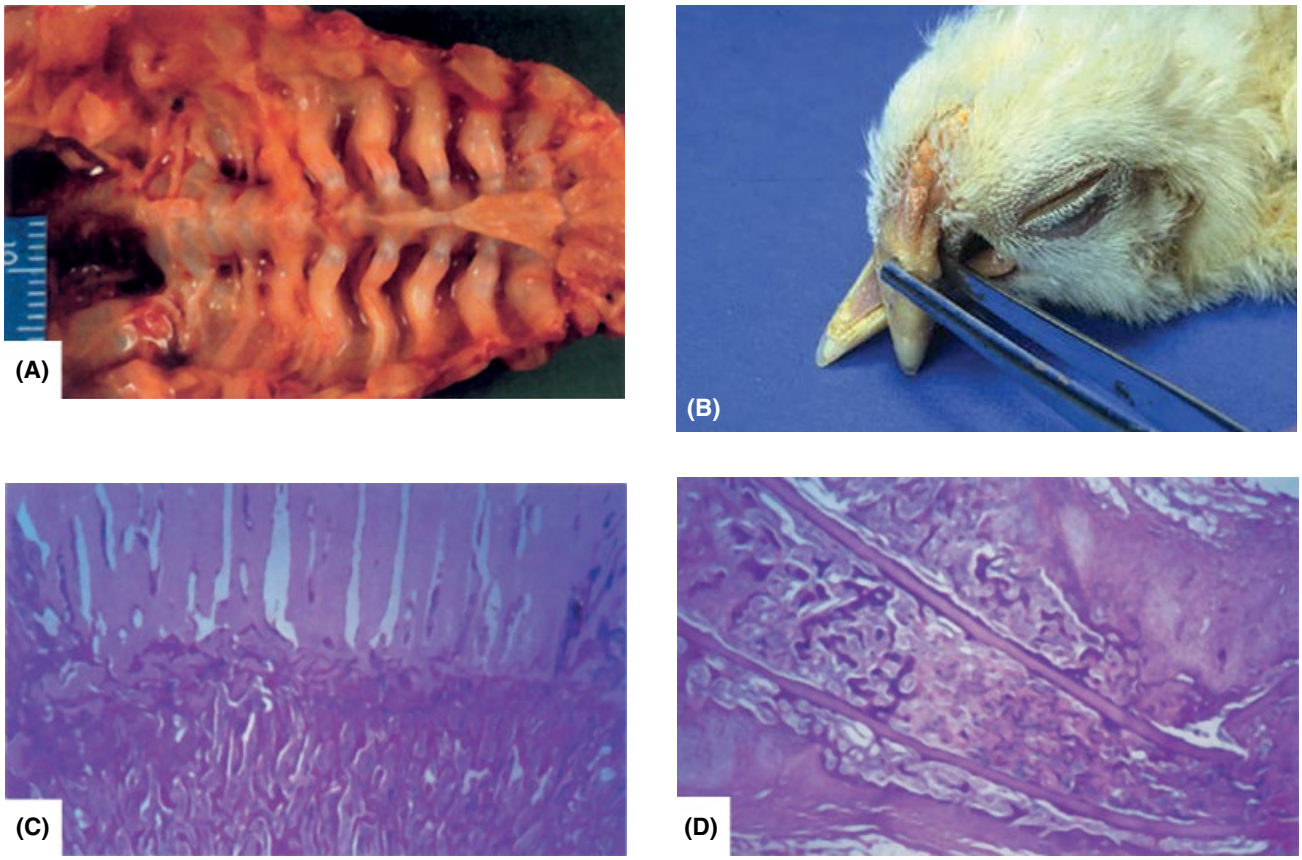


Figure 29.3 Rickets. (A) Soft, thick ribs form a flattened thorax in this severely affected 8-day-old broiler chicken. Vertebrae are also short and thick. In less affected birds, enlargement at junctions of ribs with vertebrae and sternum, folding of sterna portions of caudal ribs resulting in a flat, broad thorax, and occasionally pathologic rib fractures may be seen. (L. Munger) (B) Beak of affected chicken is soft and easily bent. (D.E. Swayne) (C) Field or infectious rickets in turkeys occurs secondarily to intestinal disease. In this affected poult, there is excess, hypertrophic cartilage that is poorly vascularized because of a compression-induced fold fracture involving trabeculae at the physical–metaphyseal junction. (H.J. Barnes) (D) Osteopenia ("caged layer fatigue"). Pathologic fracture of rib with imperfect callous formation. There is minimal mineral being deposited at the fracture site. (H.J. Barnes)



Figure 29.4 Effects of nutrient deficiencies on tibiotarsal bones of broiler chickens. (D.E. Swayne) (A) Control fed an adequate diet. (B) Phosphorus deficiency; prominent wide zone of hypertrophy. (C) Calcium/phosphorus deficiency; widened zone of proliferation. (D) Lysine deficiency; hypoplasia.

The histopathology of rickets differs significantly depending on the nutrient deficiency causing the disease (17, 33, 41). Refer to the section on calcium and phosphorus for further information on this topic.

Treatment of Deficiency. Feeding a single massive dose of 15,000 IU vitamin D_3 cured rachitic chicks more promptly than when generous levels of the vitamin were added to feed (22). In giving massive doses to rachitic chicks, it should be remembered that excess vitamin D can be toxic. The dose should be scaled to the degree of deficiency, and excessive amounts of vitamin D should not be added to feed.

Toxicitiy

The relative toxicity of vitamin D and its metabolites follows the same pattern as their bioactivity: $D_2 < D_3 < 25\text{-}(OH)D_3 < 1\text{-}(OH)D_3 < 1,25(OH)_2D_3$ (63). Although vitamin D_3 and 25-$(OH)D_3$ have little metabolic activity themselves, their affinity for the vitamin D transport protein causes the displacement of vitamin $1,25(OH)_2D_3$, which is then free to activate calcium mobilization. Elevated rates of calcium absorption and mobilization from the bone causes elevated levels of calcium in body fluids, resulting in soft-tissue calcification, cellular degeneration, and inflammation. Vitamin D toxicity is exacerbated by high levels of dietary calcium or

phosphorus, especially in the growing chick. In broiler chicks, pathology can be detected at 30,000 IU/kg diet of vitamin D_3 when fed throughout the growth period. Clinical signs include anorexia, diarrhea, weight loss, dehydration, bone demineralization, weakness, and difficult movement (32). Lesions include coagulative necrosis and metastatic calcification of the renal tubular epithelium, atrophy of the parathyroid gland associated with the proliferation of connective tissues, calcium deposits in basal areas of the aortic valve and epithelial calcification in blood vessel walls that in the brain cause vacuolization and necrosis (8, 32). High levels of vitamin D_3 (80,000 IU /kg diet) cause cardiac arrhythmia and a 2.5-fold increase in the incidence of sudden death syndrome that is associated with changes in cardiomyocytes and apoptotis in the His–Purkinje conductive system (48). In broilers, 25-(OH)D_3 is toxic at 690 mg/kg diet (85) and 1-(OH)D_3 is toxic at around 15 μg/kg (54).

Hens are generally more resistant to vitamin D toxicity than growing chicks, but toxic levels can be transferred to the egg, causing excessive mobilization of eggshell calcium and late embryonic death. Very high levels of vitamin D_3 (4 million IU/kg diet) rapidly induce renal damage from dystrophic calcification of kidney tubules. Calcification may be less often observed in the aorta and other arteries. A moderate excess of vitamin D has been reported to increase the incidence of eggshell pimpling (18) caused by excessive localized calcareous deposits on and within the eggshell. In laying hens, 25-(OH)D_3 becomes toxic at around 825 μg/kg feed (74).

Vitamin E

The term vitamin E refers to 2 groups of compounds that have antioxidant activity in cellular membranes: the tocopherols and the tocotrienols. Vitamin E is usually supplemented to poultry diets as ∝-tocopherol. In the feed, vitamin E is a very effective antioxidant; it protects unsaturated fatty acids, including the essential fatty acids, as well as many vitamins and xanthophylls. Diets that contain high levels of unstabilized polyunsaturated fatty acids become depleted of vitamin E and are most likely to cause deficiencies. Vitamin E has a low level of toxicity for poultry, and problems with excess levels are often caused by interference with vitamin A absorption and metabolism (89).

Vitamin E integrates into cellular membranes and protects against oxidative damage that lead to cell death. Cell membranes also are defended against oxidative damage by the selenium-dependent enzyme glutathione peroxidase. Thus, vitamin E and selenium have interactive effects on cell viability.

Deficiency of Vitamin E

Moderate vitamin E deficiencies do not markedly impact growth rates but do negatively impact a variety of reproductive and immunological processes. Reproductive effects of deficiency include decreased fertility of males and egg production in hens (10). With severe vitamin E deficiency, oxidative damage may cause: encephalomalacia, exudative diathesis, and nutritional myopathy (muscular dystrophy) in chicks; enlarged hocks and dystrophy of the ventricular musculature in turkeys; and nutritional myopathy in ducks. Vitamin E deficiency is exacerbated by low levels of dietary selenium, and vice versa (23, 47). Exudative diathesis in chicks and myopathies of the ventriculus and heart in young poults may not occur during vitamin E deficiency when dietary selenium is adequate. Meat and eggs from vitamin E-deficient birds have increased susceptibility to oxidative rancidity during processing and storage.

Hatchability of eggs from vitamin E-deficient hens is reduced markedly with embryonic mortality occurring as early as the fourth day of incubation, depending on the severity of the deficiency. Symptoms in the vitamin E-deficient embryo include cloudy spots in the eyes, blindness, abnormal vascular system, hemorrhages, and stunting. Turkey embryos may have bilateral cataracts that can cause blindness. Testicular degeneration occurs in males deprived of vitamin E for prolonged periods (29, 30).

Because of the similarities of vitamin E and selenium deficiency syndromes, measurement of their concentrations in blood plasma will reveal if either or both are deficient (23). Measuring vitamin E levels in feed may not be indicative of levels consumed by birds because of the rapid loss during storage.

Clinical Signs, Signalment, and Pathology. **Encephalomalacia in Chicks**. Encephalomalacia is a nervous syndrome characterized by ataxia or paresis (Figure 29.5A), backward or downward retractions of the head (sometimes with lateral twisting), forced movements, decreasing coordination, rapid contraction and relaxation of the legs, and finally complete prostration and death. Low levels of dietary selenium or high levels of dietary long-chain polyunsaturated fatty acids, especially linoleic acid, increase the severity of encephalomalacia (29).

The cerebellum, striatal hemispheres, medulla oblongata, and mesencephalon are affected most commonly (31). In chicks killed soon after the appearance of signs of encephalomalacia, the cerebellum is softened and swollen, and the meninges are edematous (Figure 29.5B). Minute hemorrhages are often visible on the surface of the cerebellum. The convolutions are flattened. As much as four-fifths of the cerebellum may be affected, or lesions may be so small they cannot be recognized grossly. A day or 2 after signs of encephalomalacia appear, necrotic areas present a green-yellow opaque appearance. One or 2 days later, the cerebellum may become pale and shrunken (Figure 29.5C). In the corpus

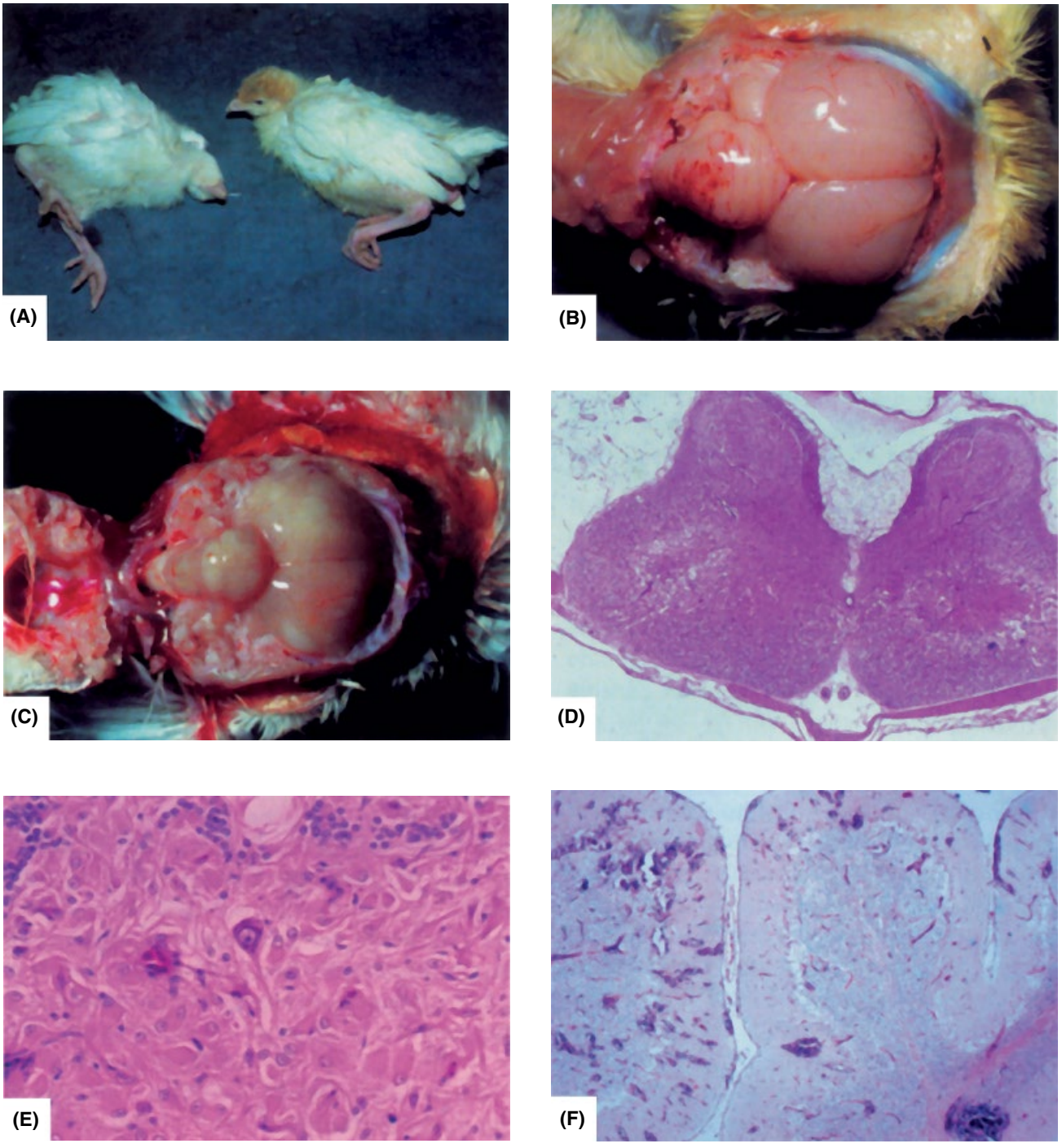


Figure 29.5 Nutritional encephalomalacia (vitamin E deficiency). (A) Paresis in 1 poult and another with pronounced neurologic signs. Whereas either clinical manifestation can be seen in turkeys, the latter is seen in chickens ("crazy chick disease"). (H.J. Barnes) (B) Birds with neurologic signs have cerebellar swelling, edema, hemorrhage, and attenuation of folia. Coning of the swollen cerebellum into the foramen magnum is often seen. Lesions in the cerebrum also may occur but are not common. (H.J. Barnes) (C) This bird with chronic nutritional encephalomalacia survived 3 days after onset of signs. Affected areas are now pale and shrunken. (H.J. Barnes) (D) Severe malacia of cerebellum. Variable portions of affected outer folia are sharply separated from inner normal tissue. There is congestion and hemorrhage. At higher magnification characteristic fibrin thrombi in small vessels would be seen. Inflammatory cells are minimal to absent. (H.J. Barnes) (E) Increased swollen astrocytes replace much of the normal cerebellar architecture in this bird with chronic encephalomalacia. Only isolated parts of the granular layer and individual Purkinje cells remain. (H.J. Barnes) (F) Poults with paresis usually do not have brain lesions but have bilateral poliomyelomalacia, as seen here. (H.J. Barnes)

striatum, necrotic tissue is frequently pale, swollen, and wet, and in early stages becomes sharply delineated from remaining normal tissue. The greater portion of both hemispheres may be destroyed. In other cases, lesions are apparent only on microscopic examination. Medullary lesions are not so readily noted in a macroscopic examination.

Histologically, lesions include circulatory disturbances (ischemic necrosis), demyelination, and neuronal degeneration (Figure 29.5D,E). Meningeal, cerebellar, and cerebral vessels are markedly hyperemic, and a severe edema usually develops. Capillary thrombosis often results in necrosis of varying extent. In the normal chick cerebellum, myelinated tracts exhibit a strongly positive reaction with Luxol fast blue, whereas in affected chicks, the staining reaction is markedly diminished, diffusely or locally accentuated (31). Degenerative neuronal changes occur everywhere but are most prominent in Purkinje cells and in large motor nuclei. Ischemic cell change is most frequently encountered. Cells are shrunken and intensely hyperchromatic, and the nucleus is typically triangular. Peripheral chromatolysis with the Nissl substance packed along the periphery of the cell nucleus also is common.

Signs of encephalomalacia in turkey poults are similar to those observed in chicks. Poults with paresis usually do not have brain lesions but have poliomyelomalacia (Figure 29.5F).

Exudative Diathesis. Exudative diathesis is an edema of subcutaneous tissues (Figure 29.6) associated with abnormal permeability of capillary walls. In severe cases, chicks stand with their legs far apart as a result of accumulation of fluid under the ventral skin. This green-blue viscous fluid is seen easily through the skin and usually contains some blood components from slight hemorrhages that appear throughout the breast and leg musculature and in the intestinal walls. Edematous subcutaneous tissue contains hyaline vascular lesions and hemorrhages. In laying hens, the thigh muscles are more susceptible to deficiency lesions than the breast muscles and show degenerative muscle fibers including calcium deposits, vascular lesions, and hemorrhages. Distention of the pericardium and sudden death have been noted (31).



Figure 29.6 Exudative diathesis in chicks. (M.L. Scott)

Vitamin E deficiency in the presence of adequate dietary selenium does not result in severe signs of exudative diathesis. The combined deficiency appears to be because of their complementary antioxidant functions and their interactions on the expression of selenoprotein genes (29).

Nutritional Myopathy (Muscular Dystrophy). When vitamin E deficiency is accompanied by a sulfur amino acid deficiency, chicks show signs of nutritional myopathy, particularly of the breast muscle, at about 4 weeks of age. The condition is characterized by light-colored streaks of easily distinguished affected bundles of muscle fibers in the breast (Figure 29.7A). A similar dystrophy occurs throughout all skeletal muscles of the body in vitamin E-deficient ducks.

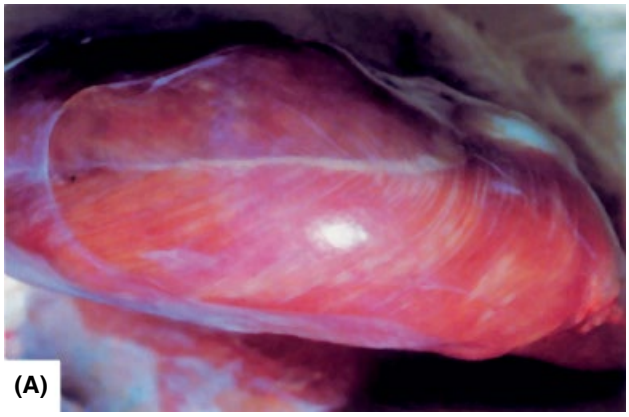
The initial histologic change is hyaline degeneration. Mitochondria undergo swelling, coalesce, and form intracytoplasmic globules. Later, muscle fibers are disrupted transversely. Extravasation separates groups of muscle fibers and individual fibers. The transuded plasma usually contains erythrocytes and heterophilic leukocytes. In more chronic conditions, reparative processes dominate the picture. There is a pronounced proliferation of cell nuclei and also fibroplasia, leaving a scar in the degenerated muscle.

Vitamin E and selenium deficiency in chickens and especially in turkeys may result in an extreme myopathy of the ventriculus (Figure 29.7B) and heart muscles.

Treatment of Deficiency. If not too far advanced, exudative diathesis and nutritional myopathy in chicks are readily reversed by the administration of vitamin E and selenium by injection, by oral dosing, or in feed. Encephalomalacia may or may not respond to treatment with vitamin E, depending on the extent of damage to the cerebellum.

Vitamin K

Vitamin K is required as a cofactor in the post-translational carboxylation of glutamic acid in prothrombin, osteocalcin, and several other calcium-binding proteins. In the absence of vitamin K, an abnormal prothrombin lacking gamma-carboxyglutamic acid is secreted into the blood by the liver. Because prothrombin is an important part of the blood-clotting mechanism, deficiency of vitamin K results in markedly prolonged blood-clotting time. An affected chick or poult may bleed to death from



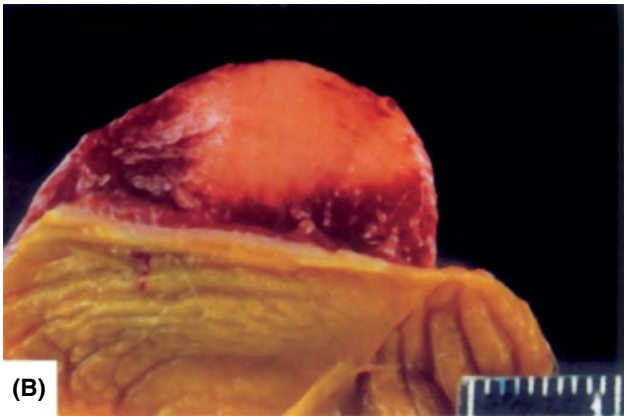


Figure 29.7 Nutritional myopathy. (A) Degeneration of muscle fibers can result from inadequate vitamin E and/or selenium. These are seen as pale, often fusiform, linear streaks in skeletal muscle. Fibrosis, intramuscular fat deposition, and other myopathies can produce similar changes. (H.J. Barnes) (B) Ventricular myopathy. Deficiency of vitamin E and/or selenium can produce myopathic changes in smooth muscle as well as cardiac and skeletal muscle. Lesions are seen as extensive, pale areas in ventriculus musculature. Turkeys are commonly affected. (L. Munger)

a slight bruise or other injury. Vitamin K deficiency is relatively rare and is most likely to occur following the administration of antibiotics, such as sulfaquinoxaline, or by consumption of warfarin-related rodenticides (75).

Clinical Signs, Signalment, and Pathology of Deficiency

In growing chicks, signs of vitamin K deficiency occur as early as 2 weeks; hemorrhages appear externally at areas that receive abrasions such as the feet and wings. Internally, petechial hemorrhages in the liver and erosion of the kaolin lining of the gizzard may occur. Chicks show an anemia that may result partly from loss of blood but also from development of a hypoplastic bone marrow. Although blood-clotting time is a fairly good measure of vitamin K deficiency, a more accurate one is obtained by determining prothrombin time.

Inadequate vitamin K in breeder diets causes increased embryo mortality late in incubation. Dead embryos appear hemorrhagic (29).

Treatment of Deficiency

Within 4–6 hours after vitamin K is administered to deficient chicks, blood clots normally, but recovery from anemia or disappearance of hemorrhages cannot be expected to take place promptly.

Thiamin (Vitamin B_1)

The active form of thiamin is thiamin pyrophosphate, which is an important cofactor in decarboxylation, dehydrogenase, and transketolase reactions. These enzymes are critical in the metabolism of carbohydrates, lipids, and branched-chain amino acids. Deficiency of thiamin leads to extreme anorexia, polyneuritis, and death. Adequacy can be tested by the erythrocyte transketolase activation coefficient assay or thiamin concentrations in blood.

Excess thiamin that is not needed by tissues is excreted in the urine and no appreciable storage pools are known. Excess thiamin is well tolerated, even at very high levels, but may have an analgesic effect.

Clinical Signs, Signalment, and Pathology of Deficiency

Anorexia is usually the first sign of deficiency and can quickly become severe, resulting in weight loss and leg weakness. Polyneuritis is observed in mature chickens approximately 3 weeks after they are placed on a thiamin-deficient diet. In young chicks, it may appear before 2 weeks of age. Onset is sudden in young chicks but more gradual in mature birds. Adult chickens often show a blue comb. As the deficiency progresses, paralysis of muscles occurs, beginning with the flexors of the toes and progressing upward, affecting the extensor muscles of legs, wings, and neck. The chicken characteristically sits on its flexed legs and draws back the head in a "stargazing" position (Figure 29.8). Retraction of the head is caused by paralysis of the anterior muscles of the neck. The chicken soon loses the ability to stand or sit upright, and it topples to the floor, where it may lie with the head still retracted.

The body temperature may drop to as low as 35.6°C. A progressive decrease in respiration rate occurs. Adrenal glands hypertrophy more markedly in females than males. Apparently, the degree of hypertrophy determines the degree of edema, which occurs chiefly in the skin. The epinephrine content of the adrenal gland increases as the organ hypertrophies. Atrophy of genital organs is more pronounced in males than females. The heart shows a slight degree of atrophy; the right side may be dilated, with the auricle more frequently affected than the ventricle. Atrophy of the stomach and intestinal walls may be sufficiently severe to be easily noted.

Crypts of Lieberkühn in the duodenum of deficient chicks become dilated (Figure 29.9). Mitosis of epithelial cells in the crypts decreases markedly. In advanced stages of deficiency the mucosal lining disappears, leaving a connective tissue framework. Necrotic cells and cell debris accumulate in the enlarged crypts. Exocrine cells of the pancreas show cytoplasmic vacuolation with the formation of hyaline bodies (29).

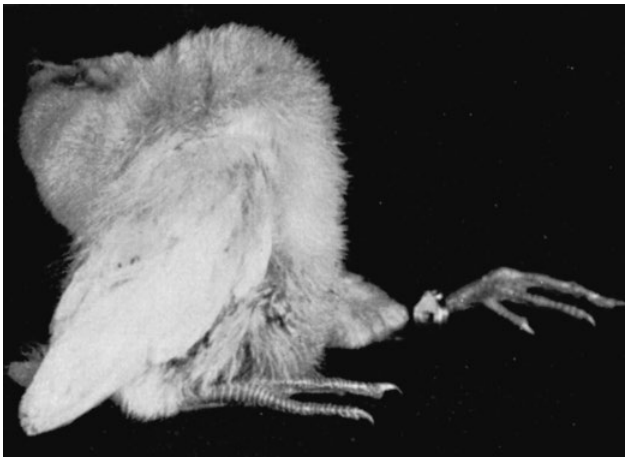


Figure 29.8 Typical stargazing pose displayed by chick suffering from thiamin deficiency. (M.L. Scott)

Treatment of Deficiency

Chickens suffering from thiamin deficiency respond in a matter of a few hours to oral administration of the vitamin. Because thiamin deficiency causes extreme anorexia, supplementing feed with the vitamin is not a reliable treatment until after chickens have mostly recovered.

Riboflavin (Vitamin B_2)

Riboflavin is a cofactor in more than 50 enzymes, many of which are vitally associated with oxidation-reduction reactions involved in cell respiration.

Clinical Signs, Signalment, and Pathology of Deficiency

When chicks are fed a diet deficient in riboflavin, they grow very slowly and become weak and emaciated, and diarrhea develops between the first and second weeks (29). Appetite is not affected as much as with a thiamin deficiency. Chicks may not walk except when forced to, and then they frequently walk on their hocks with the

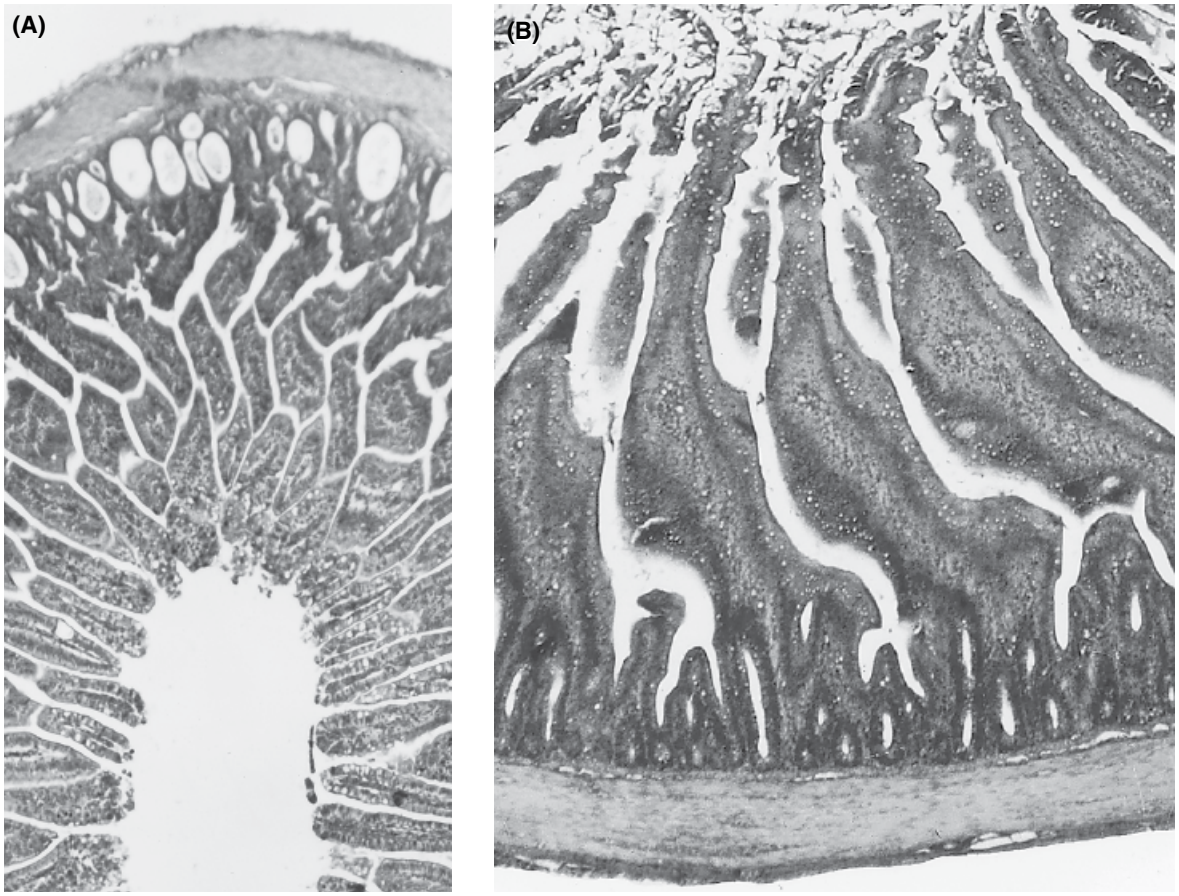


Figure 29.9 Duodenum from thiamin-deficient chick, with severe dilation of crypts of Lieberkühn (A). Control (B). (M.L. Scott)



Figure 29.10 Curled-toe paralysis (riboflavin deficiency). Typical signs include poor growth, reluctance to stand or walk, sitting on hocks, and toes curled inward. (D.E. Swayne)

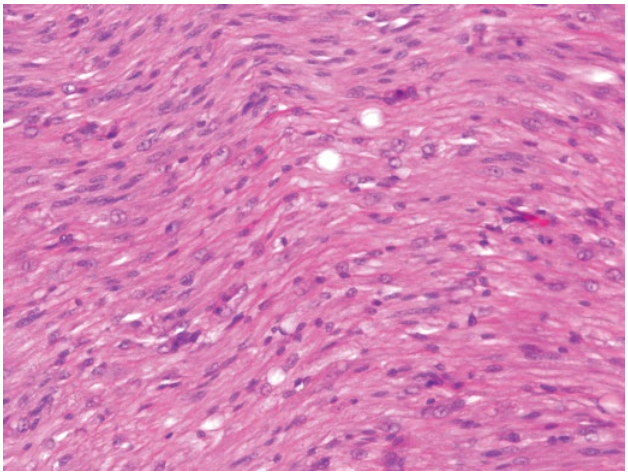


Figure 29.11 Curled-toe paralysis. Peripheral neuropathy characterized by axonal swelling and degeneration, Schwann cell activation and proliferation, and myelin degeneration. (D.E. Swayne)

aid of their wings. Leg paralysis may be more prevalent than curled-toe paralysis. Toes curl inward when both walking and resting (Figure 29.10). Chicks are usually found in a resting position with drooping wings. Leg muscles are atrophied and flabby, and the skin is dry and harsh. Young chicks in advanced stages of deficiency do not move around but lie with their legs sprawled out.

Riboflavin deficiency in young turkeys is characterized by poor growth, poor feathering, leg paralysis, and encrustations in the corners of the mouth and on the eyelids. Severe dermatitis and edematous swelling may occur on the feet and shanks.

In severe cases of riboflavin deficiency, chicks show marked swelling and softening of sciatic, cervical, and lumbar spinal nerves (29). Sciatic nerves usually undergo the most pronounced changes, sometimes reaching a diameter 4–5 times normal size. Histologically, affected nerves show degenerative changes in myelin sheaths of the main peripheral nerve trunks (Figure 29.11). This may be accompanied by axis cylinder swelling and fragmentation. Schwann cell proliferation, myelin changes, gliosis, and chromatolysis occur in the spinal cord. Fine structural examination of the sciatic nerve reveals that redundant folds and loops of myelin form symmetric or asymmetric expansions of the sheath resulting in segmental demyelination. In broiler chicks with riboflavin deficiency, ventral and dorsal spinal nerve roots, distal intramuscular nerves, and subcutaneous nerves are much less affected. In cases of curled-toe paralysis, degeneration of the neuromuscular end plate and muscle tissues is often found. Riboflavin is probably also essential for myelin metabolism of the main peripheral nerve trunks. No gross dystrophy develops, although muscle fibers are in some cases completely degenerated. The sciatic nerve exhibits myelin degeneration in 1 or more branches. Similar changes are apparent in the brachial nerve trunks. Chicks fed riboflavin-deficient diets develop pancreatic and duodenal lesions as described for thiamin deficiency in addition to the more classic nervous signs.

A deficiency of riboflavin in the diet of hens results in decreased egg production, increased embryonic mortality, and an increase in size and fat content of the liver. Hatchability of eggs decreases within 2 weeks after hens are fed a riboflavin-deficient diet but improves to near normal levels within 7 days after adequate amounts of riboflavin are added to the diet. Embryos that fail to hatch from eggs of hens fed diets low in this vitamin are dwarfed with a high incidence of edema, degeneration of Wolffian bodies, and defective down (64). The down is referred to as "clubbed" and results from failure of the down feathers to rupture the sheaths, causing feathers to coil in a characteristic way. The major metabolic consequence of embryonic riboflavin deficiency appears to be a severe impairment of fatty acid oxidation.

Treatment of Deficiency

Two 100 µg doses of riboflavin should be sufficient for treatment of riboflavin-deficient chicks or poults, followed by incorporation of an adequate level in the ration. When the curled-toe deformity is of long standing, however, irreparable damage has occurred and administration of riboflavin no longer cures the condition.

Pantothenic Acid

Pantothenic acid is a component of coenzyme A, which is involved in the formation of citric acid in the Krebs cycle, synthesis and oxidation of fatty acids, oxidation of keto acids resulting from deamination of amino acids, acetylation of choline, and many other reactions.

Clinical Signs, Signalment, and Pathology of Deficiency

Signs of pantothenic acid deficiency in chicks are difficult to differentiate from those of a biotin deficiency; deficiencies of either result in dermatosis, ruffled and broken feathers, chondrodystrophy, poor growth, and mortality (29). Chicks are emaciated and crusty, scab-like lesions appear in corners of the mouth. Eyelid margins are granular and small scabs develop on them. Eyelids are frequently stuck together by a viscous exudate and vision is restricted. There is slow sloughing of the keratinizing epithelium of the skin. Outer layers of skin between the toes and on bottoms of the feet sometimes peel off; small cracks and fissures appear at these points. These cracks and fissures enlarge and deepen, so chicks move about very little. In some cases, skin layers of the feet of deficient chicks cornify, and wart-like protuberances develop on the balls of the feet.

Necropsy shows the presence of a pasty substance in the mouth and an opaque gray-white exudate in the proventriculus. The liver is hypertrophied and may vary in color from a faint to dirty yellow. The spleen is atrophied slightly and the kidneys are somewhat enlarged. Nerves and myelinated fibers of the spinal cord show myelin degeneration. These degenerating fibers occur in all segments of the cord down to the lumbar region.

Embryos from pantothenic acid-deficient hens have high mortality and show hemorrhages and severe edema. The peak day of embryonic mortality depends on the degree of pantothenic acid deficiency and borderline deficiencies produce extremely weak chicks that fail to survive.

Dietary pantothenic acid deficiency in chicks produces duodenal and pancreatic lesions as described under thiamin deficiency (but of lesser extent), dermatosis, and severe ataxia progressing to inability to stand. In addition, there is pronounced lymphocytic necrosis and lymphoid depletion in the cloacal bursa (bursa of Fabricius), thymus, and spleen.

Treatment of Deficiency

Pantothenic acid deficiency appears to be completely reversible, if not too far advanced, by oral treatment or injection with the vitamin followed by restoration of an adequate level in the diet.

Nicotinic Acid (Niacin)

Nicotinic acid is the vitamin component in 2 important coenzymes, nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), that are required for more than 200 reactions involved in the metabolism of fatty acids, carbohydrates, amino acids, and nucleic acids. Niacin can be synthesized from tryptophan, though this conversion is not efficient, especially in turkeys and ducks. Modern diets rarely have sufficiently excess tryptophan to provide adequate niacin and must be supplemented. Excessive supplementation should be avoided because levels above 0.75% dietary niacin cause decreased bone thickness dimensions and bone strength (29).

Clinical Signs, Signalment, and Pathology of Deficiency

Severely deficient chicks show anorexia, slow growth, inflammation of the oral cavity, dermatitis, poor feathering, an enlargement of the hock joints (chondrodystrophy), and bowing of the legs (perosis). The perosis caused by a niacin deficiency is not as severe as that caused by a manganese deficiency and the Achilles tendon rarely slips from its condyles. The tongue of deficient chicks may turn black caused by necrosis. Poults and ducklings may develop diarrhea in addition to the above symptoms. Niacin deficiency in chicks produces duodenal and pancreatic lesions comparable to those of thiamin deficiency. Chicken and turkey hens do not develop the above signs of niacin deficiency; however, niacin is needed to optimize egg weight and hatchability.

Treatment of Deficiency

Supplementing a deficient ration with required amounts of nicotinic acid has little or no effect on cases that have progressed to the extent that the tendon has slipped from its condyles (chondrodystrophy) or on advanced cases of enlarged hock disorder in adult tom turkeys.

Pyridoxine (Vitamin B_6)

Pyridoxal phosphate and pyridoxamine phosphate are cofactors involved in transamination and decarboxylation of amino acids as well as glycogen mobilization.

Clinical Signs, Signalment, and Pathology of Deficiency

Pyridoxine deficiency is relatively rare and most likely to occur with diets containing high levels of protein and especially methionine (29). Severely pyridoxine-deficient chicks show depressed appetite, poor growth, chondrodystrophy resulting in bone curvature, and characteristic nervous signs. Chicks show jerky, nervous movements of the legs when walking and often undergo extreme spasmodic convulsions that usually terminate in death. During these convulsions, chicks may run aimlessly about, flapping their wings and falling to their sides or rolling completely over on their backs where they perform rapid jerking motions with their feet and heads. These signs may be distinguished from those of encephalomalacia (vitamin E deficiency) by the relatively greater intensity of activity of the chicks during a seizure, which results in complete exhaustion and often

death. Clinical signs of pyridoxine deficiency in ducklings include poor growth and food consumption, hyperexcitability, weakness, microcytic hypochromic anemia, convulsions, and death.

A pyridoxine deficiency causes a defect in collagen fibers in cortical bone and articular cartilage matrix and increased solubility of proteoglycans and collagen. These structural defects apparently cause chondrodystrophy and osteoarthritis in deficient chicks.

In adult birds, pyridoxine deficiency causes marked reduction of egg production and hatchability, as well as anorexia and weight loss.

Biotin

Biotin is a cofactor in carboxylation and decarboxylation reactions involved in the metabolism of lipids, glucose, and some amino acids. Biotin bioavailability for chickens and turkeys varies greatly among practical feed ingredients, making diet formulation based on the content of ingredients difficult.

Clinical Signs, Signalment, and Pathology of Deficiency

The signs of a biotin deficiency are variable because of the marked influence of other dietary factors, especially the amount and type of fat. In chicks and poults, a deficiency causes slow growth, ruffled feathers, chondrodystrophy, and dermatosis of the feet and skin around the beak and eyes (Figure 29.12). In ducks, biotin deficiency affects the plantar region of the feet causing dehydration cracks and bleeding of the epidermis (90). Overall, many of these signs are similar to those of a pantothenic acid deficiency and a differential diagnosis requires the analysis of vitamin levels in the diet. Dermatitis of the footpads without involvement of the head (footpad dermatitis) has become an economically important disease of broilers and poults. Footpad dermatitis can be exacerbated by a biotin deficiency but is most often caused by environmental factors, such as wet litter, and is not usually prevented by extra dietary biotin (60).



Figure 29.12 Dermatosis at the corners of the beak. Biotin deficiency. (D.E. Swayne)

Defects in bone growth caused by biotin deficiency include shortened tibiae, higher bone density and bone ash, and an abnormal pattern of bone modeling characterized by the mid-diaphyseal cortex being thicker than the lateral side. These bone defects appear to be the result of altered eicosanoid metabolism caused by impaired elongation of dietary linoleic acid to arachidonic acid.

In broiler chicks and poults, a biotin deficiency may lead to sudden death without external lesions. Chicks become lethargic and develop hepatic and renal steatosis, decreased plasma glucose, increased plasma-free fatty acids, and increased ratio of C16:1 to C18:0 fatty acids in liver and adipose tissue (29). This condition is known as fatty liver and kidney syndrome (FLKS) and can occur with a marginal deficiency, especially when dietary fat is low, triggering lipogenesis from carbohydrates. Apparently, the diversion of biotin to lipogenic enzymes diminishes the amount available for the biotin-dependent enzyme pyruvate carboxylase, causing impaired gluconeogenesis, hypoglycemia, and death. Stresses that deplete glycogen, such as fasting, exacerbate the situation. Chicks with FLKS frequently do not have the characteristic signs of biotin deficiency. This may be a temporal phenomenon wherein the changes in tissue metabolism leading to FLKS occur rapidly in biotin-depleted chicks, but the classic signs of biotin deficiency require a longer period of time to develop (5).

Biotin has been suspected of having a role in sudden death syndrome in broiler chickens. Biotin deficiency alters the unsaturated fatty acid profile in tissue lipids in a manner suggestive of impaired conversion of linoleic acid to arachidonic acid. The latter is a precursor of the prostaglandins, prostocyclin I_2 and thromboxane A_2, which have marked effects on the vascular system.

Mortality of embryos from biotin-deficient eggs is increased during the first and third weeks of incubation. Many embryos that fail to hatch are chondrodystrophic, characterized by reduced size, a parrot beak, severely crooked tibia, shortened or twisted tarsometatarsus, shortened bones of the wing and skull, and shortening and bending of the scapula. Embryos may develop syndactylia, an extensive webbing between the third and fourth toes. If chicks hatch, they are usually ataxic as well as deformed.

Treatment of Deficiency

Injection or oral administration of a few micrograms of biotin prevents biotin deficiency signs in deficient chicks and turkey poults, but reversal of existing signs has not been explored.

Folic Acid (Folacin)

Folic acid is a cofactor of the enzyme system involved in the transfer of methyl groups of such important metabolites as choline, methionine, purines, and uric acid. Folic acid is required for normal nucleic acid metabolism and formation of the nucleoproteins required for cell replication. There are more biologically active forms of folate than any other vitamin, making their quantification in foods difficult.

Clinical Signs, Signalment, and Pathology of Deficiency

Folic acid deficiency in chicks is characterized by poor growth, very poor feathering, macrocytic anemia, leukopenia, and chondrodystrophy. Asynchronous cell division causes red blood cells to have large, multilobular nuclei. Poorly developed feathers with weak and brittle shafts give deficient birds a very unthrifty appearance. Folic acid is required for pigmentation in feathers of Rhode Island red and Black Leghorn chicks. Thus, folic acid, lysine, copper, and iron appear to be required for prevention of achroma of feathers. Deficient turkey poults display a cervical paralysis that is quickly fatal. Folate deficiency is exacerbated by low levels of other methyl donors, especially choline and methionine (53).

Embryos from deficient hens die soon after pipping the air cell and display a deformed upper mandible and bending of the tibiotarsus.

Treatment of Deficiency

A single intramuscular injection of folic acid returns hemoglobin values and growth rates to normal within 1 week. Addition of 5 mg folic acid/kg to feed gives similar results. Folate absorption is relatively well regulated (70) and high dietary levels are well tolerated.

Vitamin B_{12} (Cobalamin)

Vitamin B_{12} is involved in nucleic acid, carbohydrate, and fat metabolism. One of its main enzyme functions involves isomerization of methylmalonyl coenzyme A to form succinyl CoA.

Clinical Signs, Signalment, and Pathology of Deficiency

Vitamin B_{12} deficiency results in slow growth, decreased efficiency of feed utilization, mortality, and reduced egg size and hatchability (29). Signs of a folate deficiency also may occur because of trapping of folate. Vitamin B_{12} deficiency has been reported to cause myelin degeneration in chicks. Chondrodystrophy may occur in vitamin B_{12}-deficient chicks or poults when their diets lack choline, methionine, or betaine as sources of methyl groups.

Vitamin B_{12}-deficient embryos have a peak in mortality at day 17 of incubation and show reduced size, myoatrophy of the legs, diffuse hemorrhages, chondrodystrophy, edema, and fatty liver.

Treatment of Deficiency

Intramuscular injection of 2 μg vitamin B_{12}/hen increases hatchability of eggs from hens with a deficiency of vitamin B_{12}. Oral dosing also is effective. Similarly, B_{12} injections to young chicks followed by supplementation of the diet reverses the deficiency signs.

Choline

Choline has 3 primary functions in birds: structural as part of phospholipids; neurotransmitter as part of acetylcholine; and methyl donor, via betaine, in the synthesis of methionine, creatine, carnitine, and N-methylnicotinamide. Choline can be synthesized by chickens but the rate is insufficient for growth or egg production.

Clinical Signs, Signalment, and Pathology of Deficiency

In addition to poor growth, the most consistent lesion of choline deficiency in chicks and poults is chondrodystrophy (Figures 29.13 and 29.14). Young turkeys have a high requirement for choline and, therefore, will show a high incidence of severe chondrodystrophy. Chondrodystrophy is first characterized by pinpoint hemorrhages and a slight puffiness about the hock joint, followed by an apparent flattening of the tibiometatarsal joint caused by rotation of the metatarsus. The metatarsus continues to twist and may become bent or bowed until it is out of alignment with the tibia. When this condition exists, the leg cannot adequately support the weight of the bird. The articular cartilage is deformed and the Achilles tendon (tendo calcaneous) may slip from its condyles. Choline deficiency also may result in hepatic steatosis, especially in females.



Figure 29.13 Choline deficiency. Stunting, poor feathering, and short, thick, bowed legs typical of chondrodystrophy are seen in a bird that had been fed a choline-deficient diet. (D.E. Swayne)

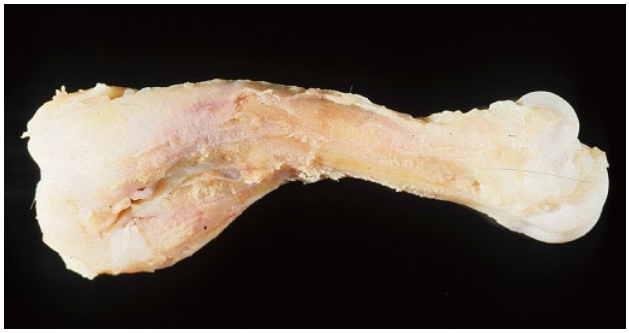


Figure 29.14 Choline deficiency. Chondrodystrophy and deformity of tibiotarsus from broiler chicken given a diet lacking adequate choline. (D.E. Swayne)

Treatment of Deficiency

If choline deficiency is noted in chicks or poults before severe signs of chondrodystrophy have developed, the deficiency can be cured by supplementing the ration with sufficient choline to meet the requirements. After the tendon has slipped in chicks or poults suffering from choline deficiency, the damage is irreparable.

Essential Inorganic Elements

The essential minerals needed in macro amounts in the diet are calcium, phosphorus, sulfur, magnesium, potassium, sodium, and chlorine. The essential trace elements are manganese, iron, copper, zinc, iodine, molybdenum, chromium, and selenium. Arsenic, boron, fluoride, nickel, rubidium, vanadium, and some rare earth minerals also may have essential functions, but mechanistic information is lacking, and deficiencies are essentially never observed. Analyses of individual mineral constituents in the body of chickens show that major portions of calcium, phosphorus, magnesium, and zinc are present in bones. Minerals function structurally, as catalysts in enzymes, and as regulators of osmotic pressure, acid-base balance, and cell signaling. Deficiencies of a mineral may result in perturbations of 1 or more of these functions. Excessive levels are also of great concern for many minerals. The maximum tolerable dietary levels set by regulatory agencies are often based on levels in meat and egg products that are potentially toxic to humans, which may be lower than levels that cause pathology to poultry (49).

The properties of different mineral sources used to fortify poultry diets greatly influence their bioavailability and consequently the amount needed to prevent deficiency or toxicity. These differences can be an order of magnitude or greater. Important properties include metallic versus salt, ionic versus covalently bonded, type of salt, organic versus inorganic, and hydration. A discussion of these important characteristics can be found elsewhere (2, 49) and the dietary concentrations provided below are for highly available mineral sources.

Calcium and Phosphorus

Calcium (Ca) and phosphorus (P) are closely associated in metabolism, particularly in bone formation. The major portion of dietary calcium is used for bone formation in growing chicks or poults and for eggshell formation in mature hens. Calcium also is essential for blood clotting and it is an integral constituent of cell signaling and regulatory pathways. Phosphorus is an essential component of phospholipids, purine nucleotides, and many other macromolecules, and is involved in the regulation of many cellular and metabolic processes including acid-base balance. The phosphorus in plant-based feedstuffs is poorly available because much of it is bound to phytic acid and is not released by digestive enzymes. The availability of phosphorus can be increased by dietary phytase of microbial origin. Efficient utilization of calcium and phosphorus depends on the presence of an adequate amount of vitamin D in the diet. An excess of calcium induces a phosphorus deficiency. Likewise, an excess of phosphorus induces a calcium deficiency (50, 55). Plasma calcium and to a lesser extent phosphorus are regulated very tightly via vitamin D, parathyroid hormone and calcitonin. Thus, moderate deficiencies or excesses do not markedly affect plasma levels but can reduce bone mineralization.

Clinical Signs and Signalment of Deficiency

Deficiencies of calcium and phosphorus in the diet of growing broiler chicks cause rickets. Differentiating rickets from a calcium deficiency from rickets caused by vitamin D deficiency or tibial dyschondroplasia requires histopathological examination (13, 29, 55). Tibiae from chicks fed a calcium-deficient diet for 2 weeks show widening of the proliferating prehypertrophic zone of epiphyseal cartilage and irregular contours in the boundary between the zones of proliferating and hypertrophic cartilage. Irregular cartilage columns and elongated epiphyseal vessels occur. By 4 weeks, the epiphyseal growth plate is widened, and in some cases, extended as a cartilaginous plug into the metaphysis. Histologically, the proliferating and hypertrophic zones become irregular and often contain areas of nonviable cells. The hypertrophied zone is markedly widened in some chicks by 4 weeks. Metaphyseal blood vessels invade along the lateral, but not the apical, region of the cartilaginous plug; cartilage columns of the metaphysis become thickened and irregular. A phosphorus deficiency results in similar histopathology but with a marked lengthening of the columns of degenerating hypertrophied epiphyseal cartilage and metaphyseal primary spongiosa. In severe cases of rickets, chicks display a spraddle-legged posture,

folding fractures, and bowing or rotation of the tibiotarsus. In a study of field rickets in broilers, calcium deficiency typically causes accumulation of proliferating chondrocytes, whereas a phosphorus deficiency causes accumulation of hypertrophic chondrocytes in the metaphyseal zone (12). Fluoride appears to interact with dietary P to induce rickets (61). Blood calcium levels are defended relatively well by the vitamin D-PTH endocrine system, but calcium deficiency progressively alters electro- and echocardiographic parameters in broilers, suggesting decreased cardiac function. Severely phosphorus-deficient chicks have increased respiratory rates, polycythemia, ascites, and decreased CO_2 and O_2 levels, presumably caused by poor rib strength and infolding, which interfere with respiratory movements of the rib cage.

In laying hens, calcium deficiency results in reduced egg production and thin-shelled eggs as well as calcium mobilization from both medullary and structural bone. Osteoporosis can occur even as medullary bone continues to accrete over the egg production life of the hen. Finally, bones become so thin that spontaneous fractures may occur, especially in vertebrae, tibia, and femur. This condition may be associated with a syndrome commonly termed "caged layer fatigue." Although a marginal calcium deficiency has often been found to be a triggering agent in caged layer fatigue, the syndrome is not caused by a simple deficiency of calcium but also involves other etiologic factors.

Excess Calcium or Phosphorus

Excess calcium causes a phosphorus deficiency and the development of rickets in broilers. Excess calcium also causes gout as indicated by the deposition of urates in the kidney, serous surfaces of the heart, liver, mesentery, air sac, and peritoneum (15). In Leghorn pullets, excess calcium causes urolithiasis, nephrosis, and visceral urate deposition. An excess of calcium interferes with the digestibility of other minerals in addition to phosphorus, including magnesium, manganese, and zinc; this may result in secondary deficiencies. High dietary levels of these minerals decrease the toxicity of calcium. Excess phosphorus causes a thinning of the egg shell independent of calcium status.

Magnesium

Magnesium (Mg) acts as a cofactor or an activator of many critical enzymes including the reactions involving adenosine triphosphate (ATP) that energize all major metabolic pathways. Magnesium levels in most feedstuffs are adequate, so magnesium is not often supplemented to poultry diets and deficiencies are rare (59).

Deficiency

Acute magnesium deficiencies are severe, beginning with lethargy, slow growth, and hypomagnesemia, followed by hyperexcitability if disturbed. Tibiae have decreased magnesium and increased calcium content and exhibit abnormalities including thickening of trabeculae, increased retention of cartilage cores, and the occurrence of elongated and inactive osteocytes in the metaphysis. Deficient chicks have thickening of the cortex, the presence of elongated inactive osteocytes, and enlargement of Haversian canals within the diaphysis. The epiphyseal plate, however, appears normal. The parathyroid appears hyperactive, perhaps in response to the hypocalcemia that is characteristic of magnesium deficiency.

Toxicity of Magnesium

Excess magnesium antagonizes calcium metabolism and increases its excretion (30, 59). This is manifest as detrimental effects including reduced growth rate and bone ash in chicks and decreased egg size, eggshell thinning, and diarrhea in hens. The maximum tolerable level of magnesium in the diets of poultry is about 0.5% for growing birds and 0.75% for laying hens (49). Diets with marginal levels of phosphorus enhance the sensitivity of hens to toxicosis.

Sodium and Chlorine (Salt)

Sodium (Na) is found chiefly in extracellular fluids. Sodium is connected intimately with maintenance of membrane potentials, cellular transport processes, and the regulation of the hydrogen ion concentration of blood. Chloride, the major mineral anion in extracellular fluids, plays a role in fluid, ionic, and acid-base balance. Many feedstuffs are deficient in sodium and chloride, so salt is supplemented to most poultry diets. Sodium and chloride in blood plasma are tightly regulated and changes in levels are more likely to indicate disturbances in acid base balance or osmotic changes than effects of diet.

Clinical Signs and Signalment of Deficiency

Chicks receiving diets deficient in sodium not only fail to grow but also develop softened bones, corneal keratinization, adrenal hypertrophy, and decrease in volumes of plasma and other fluids. Cardiac output drops, mean arterial pressure falls, the hematocrit increases, elasticity of subcutaneous tissue decreases, adrenal function is impaired, and a state of shock results, which if uncorrected, terminates in death.

Lack of salt in the diet of laying hens results in an abrupt decrease in egg production, reduced egg size, loss of weight, and cannibalism. In turkeys, low dietary salt impairs bone density (25), egg production, and hatchability. A chloride deficiency in chicks causes extremely poor growth rate, high mortality, hemoconcentration, dehydration, reduced blood chloride, and nervous signs characteristic of chloride deficiency. When startled,



Figure 29.15 Characteristic sign of chloride deficiency. (R.M. Leach)

chicks fall forward with their legs outstretched behind them and lay paralyzed for several minutes, then appear quite normal until frightened again (Figure 29.15).

Excess Salt

Large amounts of salt in the ration are toxic to chickens and turkeys. The lethal dose is approximately 4 g/kg bodyweight. Young chicks appear to be more susceptible to toxic effects of salt than are older chickens. Poultry are much less tolerant to salt supplied via the water than by the diet, and when water sodium is high, dietary sodium supplementation should be reduced. Signs of intoxication from sodium chloride or sodium bicarbonate are similar and include intense thirst, incoordination, swollen edematous shanks, respiratory distress, pronounced muscular weakness, inability to stand, spread legs, and convulsive movements preceding death. Necropsy reveals hemorrhages and congestion in gastrointestinal tract, muscles, liver, and lungs, ascites, right ventricular hypertrophy, pericardial edema and right ventricular failure (Figure 29.16) (29, 52). In broiler chicks, changing the diet from one with excess sodium to one with normal levels results in a return of mortality to normal levels after 1 week (52). In broiler breeders excess sodium causes decreased egg production, internal egg laying, and enlarged kidneys (29). High levels of salt also cause the excretion of dilute urine and wet litter, which can lead to footpad dermatitis (26).

Potassium

Potassium (K) is found primarily in the intracellular compartment of the body. The soft tissues of the chicken contain more than 3 times as much potassium as sodium. Potassium has an essential role in the maintenance of membrane potential and cellular fluid balance. Potassium participates directly in numerous biochemical reactions and is necessary for normal heart activity, reducing contractility of the heart muscle and favoring relaxation. Many feedstuffs contain adequate levels of potassium, so deficiencies are relatively rare.



Figure 29.16 Sodium excess. Cardiomegaly, especially involving the right ventricle, ascites, and fibrin masses in the body cavity and on the liver capsule, occurred in this chicken given excess sodium. (D.E. Swayne)

Clinical Signs and Signalment of Deficiency

The main effect of potassium deficiency is overall muscle weakness characterized by weak extremities, poor intestinal tone with distention, cardiac weakness, and weakness of the respiratory muscles and their ultimate failure. Severely affected individuals may exhibit tetanic seizures followed by death. Low levels of potassium in laying diets cause decreased egg production and eggshell thinning.

Dietary Balance of Macrominerals

The balance among dietary minerals has a profound effect on acid-base balance and certain developmental, metabolic, and physiologic functions in poultry (29). Diets rich in mineral anions, particularly chloride, tend to cause metabolic acidosis and result in disturbances of calcium metabolism, increased incidence and severity of tibial dyschondroplasia in immature fowl, and reduced eggshell calcification in laying hens. Diets rich in mineral cations, particularly calcium or sodium, result in the excretion of alkaline urine and the precipitation of

divalent mineral salts. Growing chicks given high levels of sodium bicarbonate develop visceral urate deposition (i.e., "visceral gout") that is especially pronounced in the kidney, which displays urate granulomas (tophi) in renal interstitium and tubular necrosis.

High levels of dietary electrolytes also increase fecal moisture and can cause problems with wet litter. Increasing dietary concentrations of sodium, potassium, or phosphorus causes linear increases in the water intake of laying hens and linear increases in the moisture content of their excreta. Each 1 g/kg increase in dietary mineral increases the moisture content of the excreta by 9, 12, and 5.6 g/kg for sodium, potassium, and phosphorus. Various sodium salts ameliorate heat stress in chickens, at least in part by increasing water intake (1, 29).

Manganese

Manganese (Mn) is an important activator of many enzymes and is also a component of arginase, pyruvate carboxylase, and manganese-superoxide dismutase. Functions such as superoxide dismutase activity and activation of glycosyltransferases are very sensitive to a deficiency of manganese. Glycosyltransferase enzymes are important for the synthesis of glycosaminoglycans, mucopolysaccharides, and glycoproteins in bone and cartilage. Manganese deficiencies are relatively common in poultry because the manganese in many feedstuffs is poorly absorbed.

Clinical Signs and Signalment of Deficiency

In laying chickens, manganese deficiency causes decreases in growth, egg production, egg weight, shell thickness, eggshell membrane glycosaminoglycan content, and sperm quality (29, 79, 80). Shells have translucent areas and abnormalities of the mammillary layer. In growing chicks, manganese deficiency impairs endochondral bone growth. Cells of the epiphyseal growth plate are arranged irregularly and the extracellular matrix is greatly reduced. Deficiency also decreases manganese-superoxide dismutase activity in tissues such as the heart and causes the ultrastructure of cone photoreceptor cells of the eye to be disorganized. In broiler chicks, growth rate and efficiency are not as sensitive to a marginal deficiency as other signs such as perosis. Tibial bones have decreased trabecular number, thickness, and area (%) together with increased trabecular bone separation. Ultrastructural modifications involve disruption of nuclear membrane and mitochondria outer membrane, loss of mitochondrion cristae, and alteration in endoplasmic reticulum in osteoblasts (36).

Manganese deficiency results in decreased hatchability of fertile eggs and chondrodystrophy in embryos. Chicks hatched from manganese-deficient eggs exhibit ataxia, particularly when excited.

Toxicity of Manganese

Manganese is generally considered to be one of the least toxic minerals (49), and poultry tolerate diets with up to 2,000 mg/kg without gross physical signs of toxicosis, although histological changes in the seminiferous tubules, indications of cerebral apoptosis, and immunosuppression occur at levels as low as 600 mg/kg (14, 37–40). Daily dosing of 100 g/kg body weight resulted in hepatotoxicity including apoptotic and necrotic changes characterized by cytoplasmolysis, cytoplasmic eosinophillia, pyknotic karyorrhactic and karyolized nuclei (56). These effects were accompanied by increased oxygen-derived free radicals (29). Manganese at toxic levels can be diagnosed by high plasma manganese concentrations.

Iodine

Iodine (I) levels in feedstuffs are extremely variable, depending on the location of their production. For this reason, iodine is supplemented to most poultry diets. Iodine is an integral part of the thyroid hormones triiodothyronine and thyroxin, which regulate metabolism, especially energetics. No other metabolic functions for iodine have been described in poultry.

Clinical Signs and Signalment of Deficiency

When the intake of iodine is suboptimal, the thyroid tissue enlarges and goiter results. Some feedstuffs have goitrogens, which impair iodine utilization and cause deficiencies in the presence of normal dietary iodine levels.

Iodine deficiency decreases hatchability, with mortality occurring late in incubation. Hatching time is delayed, embryo size is reduced, and yolk sac absorption is retarded. Severe deficiencies cause laying hens to stop laying and become obese (58).

Some egg producers feed high levels of iodine to enrich eggs and add economic value. High levels of dietary iodine decrease egg weight, albumen index, and Haugh units (34, 83). Iodine reduces the growth rate of chicks at 900 mg/kg and this effect is mitigated by dietary bromine (4). High levels of iodine also reduce male fertility. Iodine toxicosis is normalized within about 7 days of returning birds to a diet with normal iodine levels (34).

Copper

Copper levels in most feedstuffs are marginal for growth and reproduction so copper is supplemented to most poultry diets. Copper (Cu) is a component of a variety of intracellular and extracellular enzymes engaged in redox reactions including cytochrome oxidase, catalase, ceruloplasmin, lysyl oxidase, superoxide dismutase, and tyrosinase (29).

Clinical Signs and Signalment of Deficiency

In the absence of copper, low ceruloplasmin activity prevents the reduction of iron necessary for its transport and use for hemoglobin synthesis, resulting in anemia. Lysyl oxidase catalyzes oxidation of lysine residues in formation of the cross-linking in elastin and collagen. Weakened connective tissue caused by copper deficiency causes dissecting aneurysms, particularly of the aorta, and bone fragility. In broilers, a copper deficiency also causes hypertriglyceridemia, hypercholesterolemia, and prolonged prothrombin time. A deficiency of copper in laying hens causes reduced egg production, increased egg size, and abnormal eggshell calcification. Eggshell abnormalities include shell-less eggs, misshapen eggs, wrinkled eggshells, and reduced eggshell thickness. The palisade layer of the eggshell appears normal; however, the mammillary layer has enlarged mammillary knobs and increased spacing between knobs. This may be related to an abnormal structure of eggshell membranes caused by a decrease in lysine cross-linking (30).

Relatively high levels of copper (100–200 mg/kg diet) often are fed to poultry because of an antibiotic-like effect that improves indices of intestinal health, growth, and efficiency.

Toxicity of Copper

Excessive copper in the diet may cause free-radical production and oxidative damage in cells where it concentrates, resulting in degenerative lesions in various organs. Broiler chicks show atrophy of the thymus and bursa, swollen and edematous proventriculus, erosions in the koilin and in the proventricular–ventricular junction, pale kidneys, yellowish to brownish-orange liver often with linear pale areas and severe anemia (42). At very high levels, the koilin lining binds copper with high avidity and becomes rough, thickened, and warty in appearance. Histologically the ventriculus develops multifocal mild to severe fragmentation and detachment of the koilin with necrotic cell debris and heterophilic infiltration. Ventricular erosions and fissures in the ventricular lining may be accompanied by hemorrhages under the koilin layer. In the liver of broilers, excess copper causes mitochondria to swell, increase H_2O_2 production, and undergo oxidative injury (6, 65). The liver develops mild to moderate centrilobular to diffuse vacuolar degeneration and occasional acute coagulative necrosis of hepatocytes, scattered Kupffer cells containing golden-brown pigment in the cytoplasm, mild to moderate bile duct hyperplasia, and scattered erythrophagocytosis (42). The kidneys develop multifocal vacuolar degeneration of tubular epithelial cells. Ducks are more sensitive than chickens to copper toxicosis (49), and also show liver pathology including congestion, hemorrhages and necrosis of the hepatocytes with varying degrees of biliary epithelial cell proliferation (66).

Iron

Iron levels are variable in the ingredients used to feed poultry, so the levels of fortification are also variable. Iron (Fe) is an essential component of heme, the porphyrin nucleus of hemoglobin and the cytochromes, and is a component of several enzymes.

Clinical Signs and Signalment of Deficiency

Iron deficiency results in a hypochromic, microcytic anemia, reduced concentration of non-heme iron in plasma, and abnormal feather pigmentation in breeds having colored plumage. Iron-deficient chicks develop vacuolated aortic and lung smooth muscle cells that are surrounded by disorganized elastic lamellae with diffuse and fragmented networks of elastic fibers and microfibrils. A deficiency in laying hens also causes anemia in the developing chick embryo and reduced hatchability. Chicks that survive incubation are weak and listless; however, they recover when given supplemental iron (29).

Toxicity of Iron

High dietary iron is well tolerated in poultry, but not many other avian species. Characteristic signs of chronic iron toxicosis include decreased growth and efficiency of gain. The liver, heart, and pancreatic beta cells are most affected by iron overload (49). The primary diagnostic indication is hemochromatosis, which is the deposition of microscopically visible brownish-yellow granules of hemosiderin in lysosomes of hepatic cells. As storage increases, the lysosomes become damaged and ionic iron is released, causing oxidative damage to cell membranes and proteins. In advanced stages, the liver becomes cirrhotic.

Zinc

The ingredients used for poultry diets are often deficient in zinc, so zinc is commonly supplemented. Zinc (Zn) is an activator or a cofactor of more than 250 enzymes. Zinc also stabilizes "zinc finger" structural motifs, which are involved in protein-DNA interactions that regulate gene expression. Zinc plays a critical role in cell proliferation, differentiation, and survival. A marginal deficiency of zinc has a larger impact on growth and egg production than a marginal deficiency of any of the other trace minerals. Zinc also is supplemented in plethoric amounts to poults and chicks as a growth promoter, and to hens to induce molt.

Clinical Signs and Signalment of Deficiency

Deficiency of zinc results in decreased food intake, slow growth, poor feathering, dehydration, enlarged hocks (Figure 29.17), short, thickened long bones (chondrodystrophy), an awkward arthritic gait, scaling of the skin,

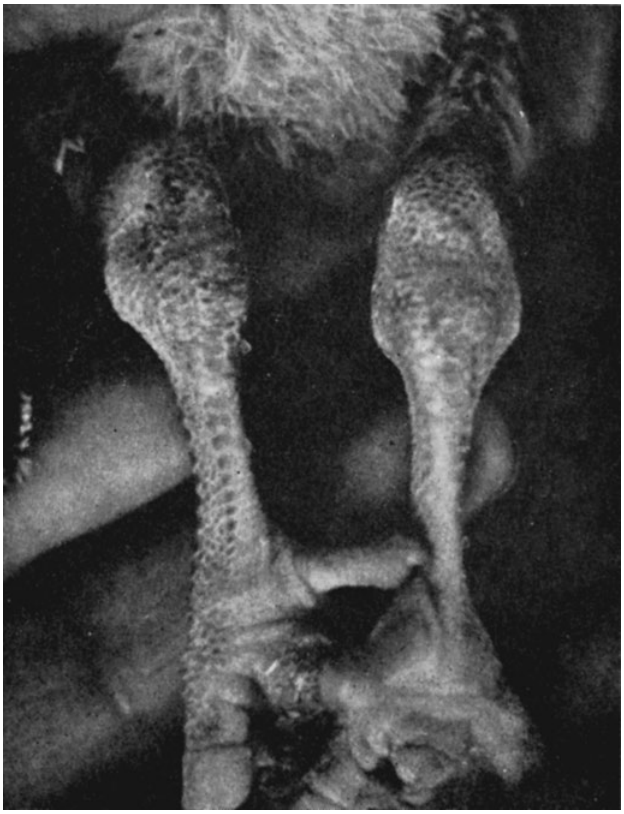


Figure 29.17 Enlarged hocks in poult caused by zinc deficiency. (R.J. Young)

and dermatosis of the feet (29). Histologic lesions include hyperkeratinization of skin of the shank and feet and parakeratosis of the esophagus. Nucleoli of the crop epithelium are enlarged and contain increased amounts of RNA. Severe epiphyseal growth plate lesions are characterized by reduced cellularity and abnormally shaped chondrocytes in areas remote from blood vessels. These changes are caused by decreased chondrocyte proliferation and increased cell apoptosis. Cells closer to blood vessels are normal, apparently because they are able to obtain sufficient zinc. Lymphoid organs become depleted of lymphocytes and reticular cells of the thymus become necrotic. The effect of deficiency is greatest on the cloacal bursa, followed by thymus, and then spleen. The zinc requirement of poults is higher than for chicks. Thus, at the same dietary zinc level, poults are more likely to show enlarged hocks and poor feathering.

Ducks exhibit poor growth and epidermal lesions of the feet, particularly interdigital webs. Pathology of the epidermis is evident in the interdigital web, mucous membrane of the tongue, and epithelium in other parts of the gastrointestinal tract. Hyperkeratosis and acanthosis characterize the tongue and interdigital web lesions. Intercellular spacing between prickle cells and basal cells is increased and number of desmosomes is diminished. Prickle cells have an abnormal structure, enlarged nuclei and nucleoli, and decreased content of free ribosomes, tonofilaments, and other structures. Lymphoid organs are affected similarly to those of chickens (9).

Toxicity of Zinc

In zinc toxicity, pathologic changes occur first in the pancreas and then in the ventriculus and thyroid. In hens, zinc (200 ppm as zinc oxide) results in pancreatic edema, dilated acinar lumina, vacuolated cytoplasm, and reduced zymogen granule density in the peripheral exocrine cells and occasional periacinar fibrotic infiltration. Thyroid follicles, especially those located centrally, are decreased in size and display cell hyperplasia and hypertrophy, with hyperplastic cells filling the lumen. Excessive dietary levels of zinc are sometimes fed to induce molt in laying hens. Zinc results in weight loss followed by an abrupt decline in egg production and onset of molt followed by rapid resumption of egg laying after dietary zinc concentrations are returned to normal.

In growing chicks, high levels of zinc result in a rough, pale ventricular lining, which may show evidence of fissures and, less frequently, ulceration. Inflammatory cells infiltrate the epithelium. Pancreas and thyroid lesions are similar to those of laying hens (29). Acute toxicity caused by very high zinc levels results in atrophy of the bursa, spleen, liver, pancreas, and ventriculus (43). The liver develops degeneration of hepatic parenchyma, dilatation of sinusoids, extensive hemorrhages, and inflammatory change. The kidneys develop interstitial nephritis, hyperemia, degeneration and necrosis, and the pancreas develops dilatation of acinar lumina, interacinar fibrous tissue proliferation, and vacuolation in the exocrine cell cytoplasm.

Selenium

Levels of selenium (Se) in feedstuffs are exceptionally variable and reflect the levels in the soil where they are grown. Severe selenium deficiencies are rare and limited to regions where feedstuffs are grown on very selenium deficient soils, but marginal deficiencies occur in many geographical areas. Selenium supplementation has a very low margin for error because of its high potential for toxicity and teratogenicity. Furthermore, the levels of selenium in the soils, feedstuffs, and water in some geographical regions are high and further supplementation is unwarranted. Selenium is a constituent of 25 proteins in the chicken, including glutathione peroxidase, which serves to protect tissues against oxidative damage, and iodothyronine deiodinase, which is needed for the conversion of thyroxine to its active form.

Clinical Signs and Signalment of Deficiency

Classical selenium deficiency diseases in chickens include poor growth and feathering, impaired fat digestion,

exudative diathesis, pancreatic atrophy, encephalomalacia, and nutritional muscular dystrophy. Myopathy of the ventriculus and heart occur in deficient poults and ducklings. Pancreatic lesions begin as vacuolation and hyaline body formation in the exocrine pancreas, followed by cytoplasm degeneration until acini are represented by rings of cells with a central lumen embedded in fibrous tissue (Figure 29.18). Hepatocytes develop apoptotic cells with condensed nuclei, mitochondria that are vacuolated with degenerated cristae, and fractured endoplasmic reticulum (84). The brain develops a disorganized medulla and cortex, thinner cortex, and wider medulla, and a marked increase in apoptotic cells (82). Ultrastructural damage in the brain manifests as vacuolation of the cytoplasmic reticulum, loss of nucleolus, fusion of nuclear membranes, chromatin margination, swelling of the mitochondria, and fusion of the mitochondrial tunica adventitia. In the testes there is a marked decline of spermatogenesis (24). The seminiferous tubules become shrunken, slightly swollen, disorganized, and buckled. There is desquamation of the germinal epithelium of the lumen. Inflammation of the duodenum also develops as indicated by infiltration of large numbers of macrophages, heterophils, and lymphocytes (87).

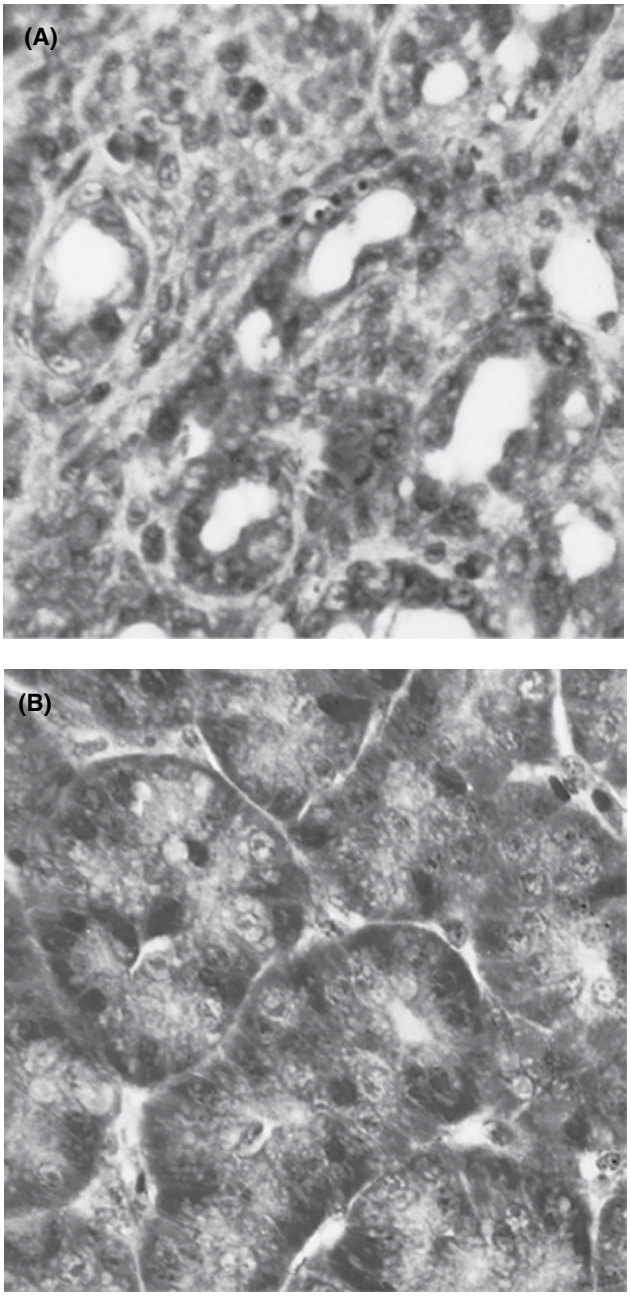


Figure 29.18 Pancreas from selenium-deficient chick. Acini consist of degenerating cells forming central lumen with extensive interstitial fibrosis (A). Control (B). (M.L. Scott)

Vitamin E and selenium have a mutual sparing effect in prevention of these diseases (see Vitamin E), though selenium is especially important for preventing exudative diathesis.

Treatment

Feeding or injecting selenium results in marked clinical recovery of pancreatic atrophy and exudative diathesis. High dietary levels of vitamin E (15–20 times the amount needed for prevention of other vitamin E-deficiency diseases) protect against the pancreatic degeneration caused by selenium deficiency (29).

Toxicity of Selenium

Excess inorganic selenium interferes with sulfur metabolism because of the formation of sulfur–selenium complexes and the substitution of selenium for sulfur in cysteine. Excess organic selenium, usually as selenomethionine, is incorporated readily into proteins because $tRNA_{met}$ does not distinguish selenomethionine from methionine, and selenomethionine is readily incorporated into proteins in place of methionine. These aberrations result in impaired protein synthesis, impaired function of proteins, and mutagenesis. A decrease in growth rate occurs with 5 mg/kg selenium in broiler chicks (49). Acute selenium toxicity in broiler chicks causes watery diarrhea, weakness, somnolence, and cerebellar edema (29). Pectoral muscle atrophy and claw and feather loss also have been noted.

Grossly, selenium toxicity causes varying degrees of congestion and hemorrhages in liver, kidneys, heart, trachea, and intestine (62). Hepatic lesions include vacuolic degeneration, pyknosis of cells of the mononuclear phagocytic system, hemorrhagic dystrophy, and parenchymal atrophy. Kidneys show diffuse tubulonephrosis followed by the necrosis of tubular epithelium. Myocardial and skeletal myodegeneration and damage of the spleen, thymus, and cloacal bursa also occur (51, 62).

The developing embryo is particularly affected by high selenium. Hatchability is typically poor and those that

hatch are often deformed. Legs, toes, wings, beaks, or eyes may be rudimentary or completely lacking. Down is often wiry and sparse.

Public Health Significance

Poultry products are an important part of the human diet and supply highly bioavailable forms of nutrients. Levels of many of the vitamins and minerals in meat and eggs are highly dependent on the levels in the diet. Birds fed diets that are deficient in vitamins or minerals do not supply intended levels of nutrition to human consumers. In fact, the vitamin and trace mineral levels of poultry products are often optimized at levels that are above the requirement for the animal. Animals deficient in nutrients are often immunocompromised, resulting in increased incidence of infectious diseases and, in some cases, evolution of more pathogenic disease organisms.

Often animals can serve as buffers for high levels of minerals or other nutrients found in plants and other foodstuffs, thereby reducing human exposure to potentially toxic nutrients including some heavy metals (49). However, levels of some nutrients (e.g., selenium, iodine, copper, fluoride, and vitamin A) may accumulate in meat or eggs to levels that might adversely affect human health. Prompt diagnosis and correction of toxicities is important for safeguarding the human food supply.

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30

Developmental, Metabolic, and Other Noninfectious Disorders

Rocio Crespo

Summary

Agent, Infection, and Disease. Disorders described in this chapter represent a heterogeneous group of conditions affecting 1 or more systems. Some of these conditions may be the consequence of increased metabolism, rapid growth rate, or high egg production that result in the failure of a body system to keep up with the demand. Their etiology and pathogenesis are often incompletely understood. Some examples are pulmonary hypertension syndrome in broiler chickens, tibia dyschondroplasia, osteoporosis, and fatty liver hemorrhagic syndrome in laying hens. Other disorders have a genetic cause that induces a failure in 1 of the body hormone or enzyme systems, such as dwarfism or retinal dysplasia. Yet, others are induced by environment or management conditions, such as heat stress, contact dermatitis, or urolithiasis. Economic losses are associated with increased mortality, reduced growth rate, drop in egg production, etc. depending on the condition and system affected. Metabolic diseases described in this chapter have been reported worldwide.

Diagnosis. At present, metabolic profiling has not been established widely as a routine diagnostic tool for poultry. As a consequence, many of these metabolic diseases are recognized through gross and histologic examination of the affected animals. Before determining a condition is a metabolic problem, it is essential to rule out infections as well as nutritional or toxicological causes because the pathology may be similar.

Intervention. Intervention strategies vary with the specific problem, but in general interventions are associated with manipulation of environment, nutrition, and genetics.

Introduction

Metabolic disorders have been a continuous challenge in poultry production for the last 70 years, exacerbated by huge improvements in the genetic potential for growth and feed efficiency. Metabolic problems in poultry production cause morbidity, mortality, or both, but are not related to infectious diseases. These diseases and conditions represent a heterogeneous group; in some cases the etiology is quite clear, whereas in others it is questionable or unknown. They vary in economic importance and frequency of occurrence. Emphasis has been placed on metabolic diseases of economic importance to the modern poultry industry. Diseases described in this chapter have been classified by primarily affected body system; however, the initial part of this chapter covers disorders induced by environment or management conditions and diseases that are not associated with a specific body system.

Developmental, metabolic, and other disorders rarely seen nowadays or only of historical importance have been removed from this chapter. Detailed information of these ailments is covered in previous editions of *Diseases of Poultry*.

Perinatal Conditions

Incubation and Hatchery-Related Problems

Poor incubation causes major losses to the industry, not only because of low hatchability or increased early mortality (114), but also because of growth depression, unevenness of the flock, increased susceptibility to infectious agents, and increased incidence of leg problems (278). It is important to identify the problem to minimize the losses in a flock. Temperature, humidity, ventilation,

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and egg turning are critical factors for hatching good-quality birds. Embryos or recently hatched birds are more susceptible to chilling or overheating than older birds. A typical effect of high incubation temperature, and sometimes low humidity, is "unhealed navels." Unhealed navel is a broad term that includes "black buttons," "strings," and ectopic viscera. Temperatures that are 3 °C higher than normal during the last week of incubation are associated with increased numbers of late dead embryos, unevenness in the flock, skin necrosis, and high incidence of leg problems including slipped tendons (278). A recent study has shown bone development in ducks to be influenced by elevated temperature incubation conditions (62). Effects of low incubation temperature are more pronounced during the first week of incubation. These include a high incidence of late dead embryos, pipped alive chicks, increased residual albumen, unsteady gait, and a high incidence of spraddle leg deformities. Low temperature while in the hatcher increases navel abnormalities such as improper closure of the navel, bloody navels, and urates covering the yolk sac. Starvation and dehydration may occur when birds are accidentally held in hatching machines for long periods.

Early chick mortality also is associated with rupture of the yolk sac. Late-hatching chicks may have a higher incidence of ruptured yolk sac. If relative humidity is too low in the hatcher, the yolk sac may adhere to the wall of the abdomen, reducing its mobility and increasing its susceptibility to injury. However, if the humidity during incubation is higher than normal, the hatchlings may have large yolk sacs. During hatchery manipulations birds may be squeezed too firmly, increasing the incidence of ruptured yolk sacs (336).

Prevention of hatchery-caused injuries and anomalies depends on high standards for operation and maintenance of equipment, especially ventilation systems. Environmental temperature, humidity, and pressures must be closely monitored and variations minimized. Unhatched eggs left in the hatching trays should be examined to establish specific causes of mortality or hatching abnormalities, and to aid in later analysis of poor flock performance.

Starve-Out

Mortality in young birds between 1 and 10 days of age that is not caused by infectious diseases is commonly referred to as starve-out, because it is generally associated with lack of water or nutrient intake. Temperature, light, and water and feed quality may contribute to early mortality. In commercial hatcheries, birds may be 24–48 hours old before they are removed, and additional time is spent in processing and transportation to the farm. As a result, most birds are delivered when they are 50 hours old or older, but will do well if they are placed in a suitable brooding environment and given adequate feed and water. However, mortality up to 6.14% has been reported in birds if placement is delayed to 72 hours after hatching, and up to 35.14% if they were not placed until 120 hours (102). There are no specific gross lesions associated with starve-out. In general, the starve-out birds are smaller, dehydrated, and have dark shanks with a prominent tibial vein. The crop, proventriculus, and gizzard may contain litter material, but not feed. Infectious diseases should be ruled out before making a diagnosis of starve-out.

Optimal temperature and light intensity in the house at placement and during the first week is crucial to stimulate birds to seek water and feed. Drinkers and feeders should be adequately spaced and easily accessible. The feed should be palatable. If crumbles or pellets are too large, birds will not be able to eat them. If the feed is too fine, it will stick to the beak. If the feed or water is too warm, the chicks will not eat or drink. Subcutaneous injection with a glucose solution has been tried to prevent starve-outs. However, no differences were found in the total mortality between fasted and glucose-treated poults at 2 weeks of age (248).

Environment-Induced Disorders

Heat Stress

It has been shown that heat stress negatively affects the welfare and productivity of broilers and laying hens. All classes of poultry experience heat distress when high temperature and humidity are above their comfort level. Birds, unlike mammals, do not have sweat glands. When environmental temperatures are between 28 °C and 35 °C (82 °F and 95 °F), birds use nonevaporative cooling (radiation, conduction, and convection) to dissipate heat. Birds manipulate nonevaporative cooling in two ways: (1) behavioral responses, such as increasing the surface area by hanging wings loosely at their sides or avoiding flockmates, and (2) vegetative reactions, which includes increase of heart rate and vasodilation (403). As the environmental temperature approaches the body temperature of the bird (41 °C or 106 °F), the rate of respiration increases and the bird open-mouth breathes to increase evaporative cooling or water evaporation. If panting (open-mouth breathing) fails to prevent body temperature from rising, birds become listless, then comatose, and soon die because of respiratory, circulatory, or electrolyte imbalances (362). The extent of the losses from heat stress are determined by the age, environmental history, maximum temperature to which the bird is exposed, duration of the high temperatures, rate of temperature change, and relative humidity of the air (131).

An increased respiration rate alters the acid-base balance because blood CO_2 concentration decreases (28). Consequently, laying flocks experience an increased number of thin-shell eggs caused by a reduction in blood ionized calcium with a higher pH. Panting or open-mouth breathing in heat-stressed birds may increase the incidence of respiratory infections because the natural filters in nasal passages are bypassed. Feed intake is also reduced in heat stress, which negatively affects growth rate. In laying flocks this results in reduced egg size, lower egg production, and poor egg quality.

Heat stress also has an immunosuppressing effect in both broilers and layers (207). Reduced lymphoid organ weights have been observed in heat-stressed layers and broilers (119, 296), with concomitant lower total circulating specific IgM and IgG antibody levels, reported in heat-stressed broilers during primary and secondary humoral responses (12). Heat stress can also cause an increase in heterophil:lymphocyte ratio in egg type chickens, caused by lymphopenia and increased heterophils (105, 294).

To prevent or alleviate heat stress, air circulation in the house should be increased by running ventilation at full capacity. Inside air can be cooled by using sprinklers or spraying down the floor, walls, ceiling, and outside roof with cool water. Adequate drinking water should be available and lowering water temperature will aid in heat dissipation.

Nutritional modifications usually involve optimizing the diet to meet the altered needs of stressed birds for energy and protein and providing certain additional nutrients which have specific beneficial effects. Energy content of the feed should be decreased (22 kcal/kg for each 2.5°C) (324). Nonenergy nutrient content (proteins, amino acids, vitamins, minerals) also should be high. Egg production is improved by increasing the intake of protein relative to energy (10). Addition of electrolyte solutions (with sodium, chloride, potassium, bicarbonate and/or vitamins [A, C, and E]) to the drinking water help replenish the electrolytes, correct the acid/base balance and prevent heat stress (324, 355). Fresh, cool drinking water also should be available to help reduce heat stress (363). Some drugs, such as nicarbazin or monensin (325, 363), have deleterious interactions when administered to heat-stressed birds, whereas others, such as virginiamycin (363), may alleviate heat distress.

Preventive measures consist of fans and foggers installation, proper construction of ventilation ducts, buildings and water pipes insulation, use of roof overhangs to prevent sunlight from shining directly into the house, and use of white or aluminum paint on the outside to reflect heat. In hot climates where low production and mortality from heat are constant problems, installation of foggers and sprinklers or evaporative coolers is essential. Lower stocking densities during the hottest months, to reflect realistically the ability of each poultry house to cope with extreme temperature, may also be needed. Enhancement of adaptability of birds to heat stress conditions, such as early thermal conditioning or feed restriction, also should be considered (66, 223).

Dehydration

Dehydration is generally caused by failure to find or reach water, failure to provide an adequate amount of water, or the presence of a deterring factor in the water. Faulty electrical systems may cause an electrical charge in the water, and birds will not drink. Chicks can survive several days without water but will die beginning on the fourth or fifth day, once the yolk sac nutrients are used. However, mortality will abruptly stop if water is provided. Laying birds need a constant water supply or production will drop. Birds will die if water restriction is severe. Signs of dehydration include insufficient weight (or even weight loss) for size and age, dark, dry, and wrinkled skin on the shanks, blue discoloration of the beak, dry and dark breast musculature, dark kidneys, accumulation of urates in the ureters, visceral urate deposition ("visceral gout"), and darkening of the blood. To prevent dehydration in chicks, water fountains should be placed directly on the litter and surrounding, but not underneath the brooder heater (i.e., in the comfort zones). Whenever large type or automatic drinkers replace small drinkers, the old type should be kept for a few days and gradually moved toward the new source of water supply to accustom birds to the change.

Asphyxiation

Crowding or piling of birds in a corner generally causes asphyxiation. This typically occurs when birds are moved to new quarters, when they are frightened, or when young birds are chilled. Asphyxiation of baby chicks can occur in chick boxes that are piled too high without an air space between each box, in boxes that do not have sufficient ventilation holes, or in boxes placed in a closed compartment such as the trunk of a car. Smothering can also occur in cage free laying hen flocks caused by panic (e.g., presence predators or vermin) or piling into a single nesting box. In controlled environment houses with no windows, asphyxiation may occur when electric power fails or a ventilation system is faulty. Mortality sometimes occurs at night in generally healthy flocks.

Necropsy of asphyxiated birds usually does not reveal specific gross or histologic lesions, but a thorough examination will eliminate other possible causes of death. In some birds, there are nonspecific changes such as congestion of the trachea and lungs. In older birds, feathers will be worn off where birds have been trampled.

Asphyxiation of chicks in the brooder house can be controlled by putting a circle of corrugated cardboard around the brooding area for the first week of life. This prevents piling in a corner during the night. When birds are moved to new quarters, the use of a dim light or lantern for the first few nights will decrease the possibility of piling-up and asphyxiation. Birds transferred to new quarters should be checked late in the evening for signs of piling. Frequent observation of the flock is very important in the first few days after acquiring a group of new chicks or grown birds. Placement of a video camera in the house may help elucidate the cause of smothering.

Problems Related to Vaccination

Killed vaccines and bacterins usually employ an oil adjuvant to stimulate a localized inflammatory reaction and improve the immune response. When oil-emulsion bacterins are administered subcutaneously into the neck, the adjuvant may infiltrate into the adjacent tissues causing dermatitis, neuritis, and myositis (1, 54). Neurological and/or musculoskeletal problems, which impair birds' ability to eat, have been documented when a killed vaccine is delivered into the neck musculature and/or subcutaneously too close to the skull. Killed vaccines administered intramuscularly into the breast or leg muscles may produce severe granulomatous myositis near the site of injection (75) (Figure 30.1). Birds may be reluctant to move, lose weight, and have reduced production; the meat may be downgraded at the processing plant because of trimming of affected areas. Recently, oil-emulsion-inactivated vaccines have been linked to outbreaks of hemorrhagic hepatitis and enteritis, and reported as hemorrhagic hepatopathy syndrome in commercial pullets, possibly because of an atypical reaction by the immune system (39, 326).



Figure 30.1 Layer chickens with severe myositis and muscle atrophy secondary to vaccination. Bar = 1 cm.

Live or attenuated vaccines and bacterins also have been reported to cause pathologic changes in birds. Young chickens given *in ovo* vaccines subcutaneously at the hatchery have sometimes shown neurologic signs, pyogranulomatous myositis, neuritis, and meningomyelitis (Figure 30.2). Because these vaccines do not have irritant substances, the tissue reaction is thought to be a misdirected vaccine (136). Improper sanitation or cleaning of vaccine equipment may result in bacterial contamination, and has caused nervous signs and increased mortality in young birds (244). Adequate training of vaccinating crews helps to minimize the inflammatory reaction in the tissues from incorrect injection of bacterins and vaccines.

Amyloidosis

Amyloidosis is a well-recognized pathological disorder in birds, characterized by deposition of proteinaceous material between cells in various tissues and organs of the body. Amyloidosis is generally divided into primary

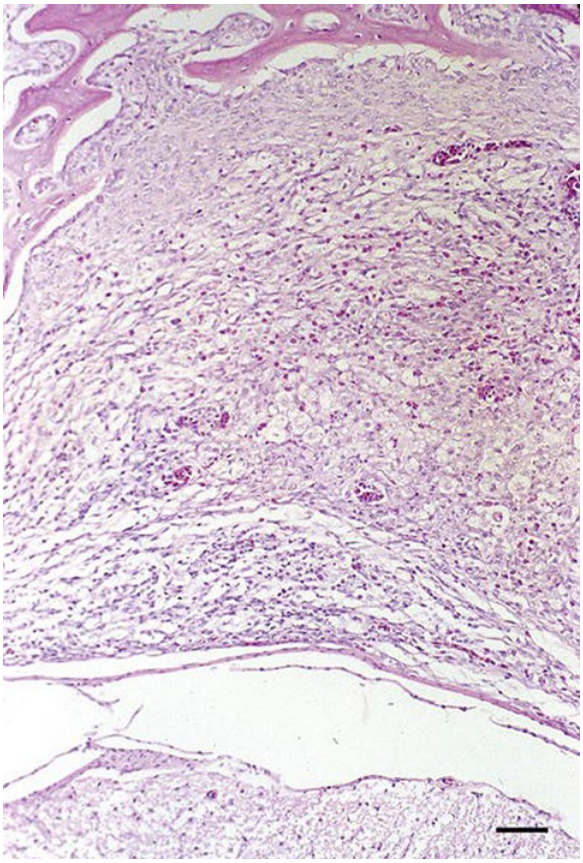


Figure 30.2 Histologic section of meninges around the spinal cord (bottom) of a 2-day-old broiler chicken that was vaccinated in the neck. Note the nonsuppurative meningitis, most likely associated with misdirected vaccination. H & E, bar = 10 µm.

amyloidosis and secondary amyloidosis (190). The former occurs as a consequence of various plasma cell dyscrasias, such as multiple myeloma, and other monoclonal proliferations of B lymphocytes. The latter occurs in association with chronic inflammatory diseases, such as tuberculosis and long-standing suppurative processes. More than 15 biochemical types of amyloid protein are recognized in mammals (199); however, only amyloid A has been detected in birds (204, 256). Amyloidosis caused by amyloid A is frequently associated with an underlying infectious or inflammatory condition (191). Landman *et al*. have published an excellent review of amyloidosis in birds (204). Among the domestic avian species, waterfowl are most susceptible to amyloidosis. Ducks as young as 4 weeks of age may be affected (339), although it is most common in adults.

Clinical Signs and Pathology

No specific clinical signs or gross lesions are associated with systemic amyloidosis. Clinical signs in ducks may include anorexia, lethargy, weight loss, decreased egg production in layers, swollen abdomen, and increased mortality. In brown egg-laying type chickens, locomotor problems caused by swollen joints and weight loss can be encountered, but often birds with amyloidosis are found dead with no prior clinical signs.

Brown egg-laying type chickens are particularly susceptible to amyloid arthropathy associated with *Enterococcus faecalis* (205, 206) and *Mycoplasma synoviae* (203) infections. Amyloid arthropathy associated with *M. synoviae* has also been reported in turkeys (345), but has not been reproduced experimentally. Other bacteria such as *Escherichia coli*, *Salmonella* Enteritidis, and *Staphylococcus aureus* also have been implicated in chickens (202). Amyloidosis has also been described in layer hens with mixed infection of fowlpox and *Staphylococcus hyicus* (257) and in mature chickens with hepatitis-splenomegaly syndrome (hepatitis E virus). Amyloidosis associated with mycobacteriosis is a common finding in waterfowl and other birds in zoological collections (132, 247). Management and genetic factors also may be important in the incidence of amyloidosis, especially in ducks raised commercially. It has been also observed that chickens develop amyloidosis after inoculation with multiple vaccines, including *Salmonella* Enteritidis and *Mycoplasma gallisepticum* vaccines (39, 251).

Amyloid deposition may be found in any tissue. Experimentally, amyloid fibrils administered intravenously induced amyloid deposits in every organ; however, in chickens that were administered amyloid fibrils orally, amyloid deposits were observed mainly in the spleen (252). These different distribution patterns are likely to reflect different stages in the progress of amyloidosis. In natural cases, the most commonly affected organs are liver, spleen, intestines, and kidneys. Gross lesions may be lacking or minimal when amyloid deposits are present in small amounts. However, with large deposits, gross lesions can be present including severe ascites ("water belly"), which is most common in ducks, and diffusely enlarged heavy liver with firm to rubbery consistency and pale or brown or gray smooth surfaces (Figure 30.3). Cut surfaces of the liver may have a smooth, waxy appearance and the capsule may be thickened caused by fibrosis. Occasionally the livers of some affected birds may have multiple hyperplastic nodules of various sizes that may have the normal color of the liver. Spleen can be severely enlarged and mottled white. Kidneys and adrenals may be enlarged and pale. Brown egg-laying type chickens with amyloid arthropathy may have enlarged joints with presence of orange-yellowish material in the joints (Figure 30.4).

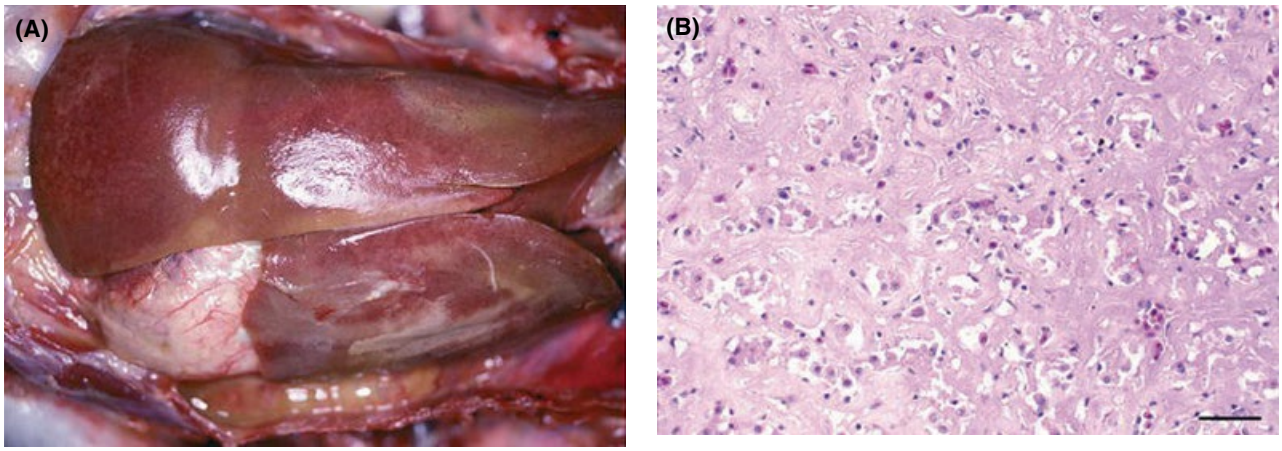


Figure 30.3 Hepatic amyloidosis in a 57-week-old duck. (A) The liver is severely enlarged and pale. Note the impression of the rib cage on the right lobe. (B) Histologic section of the liver. Note that the amyloid appears as homogeneous eosinophilic material deposited extracellularly. Most hepatocytes have disappeared. H & E, bar = 65 µm.



Figure 30.4 Amyloid arthropathy in 32-week-old brown chicken layers caused by *Enterococcus faecalis*. From left to right, normal control to most severely affected. There is accumulation of amyloid in the lumen of the tibiotarsal joint. Note that the articular cartilage is partially destroyed. (Barbara Daft)

During gross examination, application of Lugol iodine to a suspected organ stains amyloid a distinctly brown color. Application of diluted sulfuric acid changes amyloid from brown to blue. Microscopically, amyloid appears as homogenous eosinophilic material deposited extracellularly in many organs. Stains such as Congo red or the Shtrasburg method (347) can be used to detect amyloid A in formalin-fixed tissues.

Intervention Strategies

There is no treatment for amyloidosis, but prevention of chronic infections or stress in birds reduces its incidence. Treatment of the underlying inflammatory disease or eliminating the stress factor should be started as soon as possible to prevent or stop the progressive deposit of amyloid in the tissues. Feeding a high dose of vitamin A enhanced deposition of amyloid A in the joints of chickens, whereas methylprednisolone (an anti-inflammatory drug) and pentoxifylline had an inhibitory effect (333). Amyloidosis has long thought to be irreversible, because these proteins have low solubility and are resistant to proteolytic digestion (192); however, a recent study concluded that, in chickens, amyloid deposits may regress over time when chronic infection disappears (166).

Diseases of the Skeleton

There are numerous metabolic skeletal disorders; some have been associated with fast growth rates whereas others are correlated with gender.

Dyschondroplasia

Dyschondroplasia is a skeletal disease of birds in which there is impairment of normal endochondral bone formation. Dyschondroplasia is characterized by an avascular plug of abnormal cartilage in the growth plate of long bones that extends into the metaphysis. It is most commonly recognized in the proximal tibiotarsus; hence, the condition is often described as tibial dyschondroplasia (TD). Dyschondroplasia also occurs in the proximal and distal femur, the distal tibia, the proximal tarsometatarsus, and the proximal humerus, but is less severe. Reviews of the condition have been written by Farquharson and Jefferies (103), Leach and Monsonego-Ornan (213), Whitehead (385), and others.

Clinical Signs

Tibial dyschondroplasia is one of the most common problems in the poultry industry. Incidence of TD in broiler chicken flocks may be up to 30% whereas in turkey flocks the incidence has been reported to be as high as 79% (380). Most birds show no clinical signs. If masses of cartilage are large, birds may exhibit reluctance to move, stilted gait, and bilateral swelling of the femoral–tibial joints, often associated with bowing of the legs. TD lesions severity has been correlated with the degree of anterior bowing of the tibiotarsus and lameness in broiler chickens and turkeys (226, 380). Dyschondroplasia in the femoral head in broiler chickens has been associated with a widened and shortened femoral neck and, in some cases, with fractures of the femoral head (76, 77, 81).

Dyschondroplastic lesions can be recognized on radiographs and with a hand-held, low-intensity X-ray imaging scope (lixiscope) as early as 2 weeks of age in broiler chickens (365). Downgrading of carcasses and trimming of deformed legs at processing have been attributed to TD (35, 319). If broiler chickens are kept to roaster weights, lesions may be much more severe. In such birds, fractures below the abnormal cartilage in the tibia may cause severe crippling. Resolution of the abnormal cartilage may start as early as 48 days of age, but sequestra of abnormal cartilage separated from the growth plate and bowing of the tibia may persist to as late as 30 weeks of age, even though the proximal growth plate of the tibiotarsus in a chicken closes at 16–17 weeks of age.

In turkeys, TD is recognized as early as 5 weeks of age (293), peaking between 12 and 14 weeks of age (157). The incidence rate decreases after 15 weeks of age, until the age of closure of the proximal tibial physis at 22–24 weeks, but 5% or more of the toms may still retain some abnormal cartilage (380). Whereas there is no correlation between body weight and TD in early ages, TD severity is directly correlated with body weight between 14 and 15 weeks of age (301).

Pathology

Gross lesions of TD are characterized by abnormal masses of cartilage, usually cone shaped, below the growth plate, primarily in the proximal tibiotarsus

(Figure 30.5), but also at other sites. In mild cases, these cones of abnormal cartilage mainly develop below the posterior medial part of the growth plate. In severe cases, masses of cartilage develop from the whole growth plate and fill the whole metaphysis. Interestingly, dyschondroplasia within the proximal tibiotarsus appears to be bilateral, and the incidence and severity of the TD is similar in both legs (103).

Microscopically, TD is characterized by persistence and accumulation of prehypertrophic cartilage. The separation of the prehypertrophic cartilage from the proliferating cartilage is not sharply demarcated and few vessels penetrate the abnormal cartilage from the metaphysis. Normal growth plates or those with small dyschondroplastic lesions have few or no apoptotic chondrocytes, whereas in severe lesions numerous apoptotic cells with shrunken nuclei and little cytoplasm are present (303), suggesting that apoptosis is secondary to the formation of the cartilaginous plug. Other studies have shown quite the opposite, that TD lesions are associated with lack of apoptosis, which may be responsible for the retention of chondrocytes (267).

Ultrastructural and biochemical studies have demonstrated that the lesion begins in the prehypertrophic zone. The chondrocytes in the abnormal cartilage do not differentiate into fully hypertrophic chondrocytes (140, 292, 383), which are needed for cartilage vascularization, mineralization, and resorption. The abnormal chondrocyte mitochondria retain less calcium and phosphorus as compared with normal chondrocytes.



Figure 30.5 Medial view of sagittal sections of 2 proximal tibiotarsal bones from broiler chickens with tibial dyschondroplasia. The abnormal cartilage is only present in the posterior part of the metaphysis (right). Abnormal cartilage fills the whole metaphysis, and the proximal end of the bone is enlarged (left). (Craig Riddell)

Pathogenesis and Etiology

Tibial dyschondroplasia occurs spontaneously in many rapidly growing avian species, but its pathogenesis is not well understood. The cause of TD is believed to be multifactorial involving genetic, nutritional, and environmental factors. The initial phases of this mechanism appear to originate in the avascular transition zone of the growth plate, caused by an inability of the prehypertrophic chondrocytes to undergo terminal differentiation. In the absence of suitable biomarkers to monitor the initiation and progression of the naturally occurring disease, experimentally induced disease models are used to study the mechanism of pathogenesis in a controlled manner. Rath et al. (302) demonstrated that TD could be reproduced by feeding thiram, a dithiocarbamate fungicide. This experimental model is currently the most commonly used protocol to investigate the pathogenesis of TD.

Proteins such as α-enolase, G protein, calumenin, type II collagen precursor, and others, which are proteins associated with signal transduction, energy metabolism, and secretory functions all integral to cell viability, are down-regulated in the dyschondroplastic tissue (300). Research suggests that the dyschondroplastic cartilage is a hypoxic environment possibly caused by decreased proteolysis caused by the failure in expression or lack of activation of some extracellular matrix metalloproteinases (e.g., MMP-2 and MMP-13) (379). Some growth factors might not be able to exert their functions because they might be arrested in extracellular matrix storage sites.

Hypoxia leads to an increase in the transcription factor HIF-1α, causing increases in the levels of molecular chaperones Hsp90 and Hsp70 (118). Inhibition of Hsp70 in the TD-affected growth plates with dietary quercetin did not prevent the hypoxia that is characteristic of the TD-affected growth plate or development of thiram-induced TD and lameness (117). On the other hand, inhibition of Hsp90 restored normal chondrocyte columnar organization and vascularization (117, 170). Also, celastrol therapy, in addition to inhibiting Hsp90 mRNA and protein levels, up-regulated the expressions of receptor fetal liver kinase 1 (Flk-1) in thiram-induced TD (253).

Intervention Strategies

Restricting feeding reduces the incidence of TD (320). Lesion severity in the proximal tibiotarsus appears to be correlated with rapid growth. Daily fasting can reduce incidence of TD without causing growth depression (97). It has been suggested that diurnal rhythms may be important in reducing TD (100). An interrupted and increasing light program had no effect on clinical and subclinical TD in roaster chickens (315). An intermittent light program helped reduce the incidence of TD in some cases (412, 415). Although reducing the growth rate of

experimental birds decreased the incidence of TD, there was no direct correlation between growth of individual birds and the incidence of TD (197, 313).

Angular Bone Deformation

Angular deformation of the long bones of broiler chicken and turkey is a significant cause of economic loss because of culling and death of affected birds. Angular deformation includes many different types of bone twisting or bending and various terms such as long bone distortion, twisted legs, or crooked legs have been used. The general topic of deformation of the long bones in domestic poultry was reviewed by Riddell (309, 313) and Thorp (364, 365). The most common type of long bone deformation in broiler chicken is valgus deformation of the tibiotarsal–tarsometatarsal joint. The prevalence of valgus deformation is 2–4% in broilers, but it can be as high as 40% in severely affected flocks, whereas varus deformation of the tibiotarsal–tarsometatarsal joint affects no more than 3% of the birds in severely affected flocks (216). In the turkey, similar deformation is also common but is often associated with varus deformation of the femoro–tibiotarsal joint (314).

Clinical Signs and Pathology

Valgus or "knock-kneed" deviation results from the outward deviation of the tarsometatarsus. Varus or "bow-legged" deviation is the consequence of inward deviation of the tarsometatarsus. The major deformity is in the distal tibiotarsus, with similar but less severe angulation in the proximal tarsometatarsus (Figures 30.6 and 30.7). The valgus deviation is more common, but varus deviation may result in more restricted walking ability in poultry (48, 216). The defect may affect both legs but is often unilateral, with the right leg more commonly affected than the left leg (95, 319). In contrast, other studies (216) often described valgus angulation to be bilateral, whereas varus deviation was unilateral in most cases. Approximately 70% of affected birds are males (319). Most birds have either valgus or varus deformation, but the occasional bird will have valgus deformation of 1 leg and varus deformation of the other leg. These birds have been described as "windswept" (89).

Valgus angulation appears progressively between 2 and 7 weeks of age; on the other hand, varus deviation appears suddenly between 5 and 15 days of age (180, 216, 311). As the severity of the valgus angulation increases, the gastrocnemius tendon may become displaced and the distal tibial condyles become flattened. In the varus deviation, the gastrocnemius tendon is always displaced medially (216) (Figure 30.8). In some cases, the angulation progresses to displacement and separation of the tarsal bones from the shaft of the tibia. With severe angulation, birds are forced to walk on the posterior surface of the hock, which becomes bruised and swollen. In some instances, the distal shaft of the tibia will penetrate the skin.

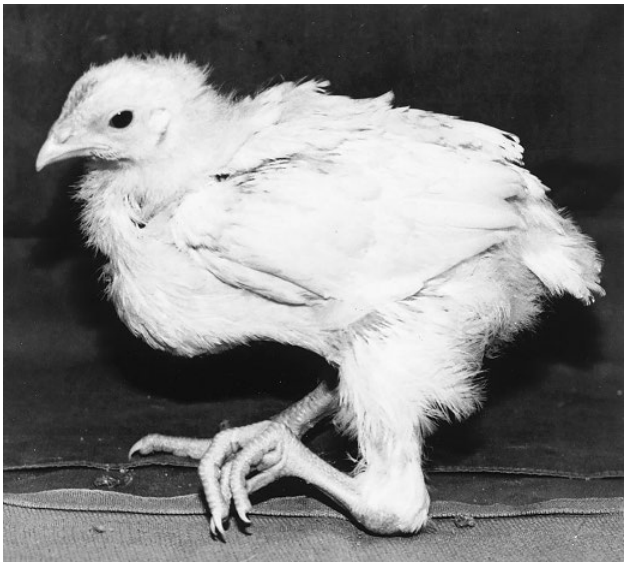


Figure 30.6 Broiler chicken with unilateral valgus deformation of the tibiotarsal–tarsometatarsal joint (311).



Figure 30.7 Tibiotarsal and tarsometatarsal bones from a broiler chicken with unilateral valgus deformation (311).



Figure 30.8 Medial displacement of gastrocnemius tendon caused by varus deviation of the tibiotarsal–tarsometatarsal joint.

Pathogenesis and Etiology

The pathogenesis of the deformation has not been defined; however, it does not appear to be associated with a nutritional deficiency. An increase in growth rate has been associated with an increased incidence of angular bone deformities (163, 311). However, reducing energy in the diet does not improve the quality of the cortical bone (217). The highest relative growth rate of the tibia and femur occurs in the first week posthatch (5). Therefore, the first few weeks of a chicken's life have a profound impact on leg bone development. Furthermore, researchers have shown the poor ability of fast-growing broiler strains to respond to mechanical load bearing or insensitivity to load, which would suggest that they are unable to adapt the skeletal system as rapidly as body weight increases (291). Different photoperiods also affect the incidence of angular bone deformations (45, 315), but gradual light–dark transition has little effect on leg bone development (373). It is unknown whether this is caused by a change in growth rate, amount of exercise, or a hormonal factor.

Valgus and varus deformities may each have a different etiologic pathogenesis (216) and these deformities may have genetic predispositions (212). In addition, genetic selection may influence the incidence of leg deviations. Le Bihan-Duval et al. (211) estimated that the susceptibility to valgus deformity was genetically independent of meat conformation, whereas varus deformation increased with body weight. Angular limb deformities are reportedly higher in turkeys subsequent to malabsorption syndrome (289).

An association between bone angulation and dyschondroplasia has been noted (293, 299, 311). Although TD may weaken bones and predispose to deformation, it may be secondary to the deformation (411). In a breeding study, it was observed that skeletal angular deformation was unrelated to TD (312).

Osteoporosis

Osteoporosis is an age-related disease of laying hens characterized by a decrease of normal mineralization of structural bone, resulting in increased fragility and susceptibility to fracture. It was first described in caged laying hens that had brittle bones and were unable to stand, but could eat and drink (50). The condition was then called "cage layer fatigue." Many factors contribute to osteoporosis, including: inadequate diet; lack of absorption of calcium, phosphorus, or metabolites of vitamin D; impaired bone formation; estrogen deficiency; and lack of exercise (64, 388). As much as 80% of birds have suffered breaks during the laying period (408). Bone fragility is responsible for up to 30% of fractures in commercial caged birds (130, 386), and most of these fractures occur during catching, transporting, and processing.

Clinical Signs and Pathology

Osteoporosis consists of loss in bone quality which predisposes the birds to fractures in various bones. Keel bone, followed by tibia and humerus, have the highest incidence of fractures in layers with noncage systems (408) whereas ischium, humerus, and keel bones show the highest incidence of fractures, followed by fractures of pubis, ulna, coracoid, and femur in caged birds (128). Osteoporosis can cause paralysis caused by the collapse of the vertebra (15), but in many cases in which the vertebra are not fractured, the loss of structural bone has been linked to exposure and pressure of the spinal cord and nerves (388). Osteoporosis is more severe between 25 and 50 weeks of age (108).

Sterna are often deformed, and there is characteristic infolding of the ribs at the junction of the sternal and vertebral components. The bone cortex is thin, but there are no changes in the external dimensions of the bone because cortical bone resorption is restricted to the endosteal surface (9). Parathyroid glands are enlarged. Many birds have regressive ovaries and are dehydrated, whereas some dead birds have an egg in the oviduct and have died suddenly.

Histologically, the cortices of bones are thin, with enlarged absorption spaces. Medullary bone is reduced in quantity, and largely consists of unmineralized osteoid. The ribs may be deformed by small fractures. Damage to the spinal cord is often associated with pressure on the nerves, which may cause paralysis.

Pathogenesis and Etiology

Sexual maturity onset causes a rise in estrogen which will stimulate accumulation of medullary bone in layers (388). On the other hand, structural bone resorption starts at sexual maturity and continues during the production life so that osteoporosis is more severe at the end of lay. Increased bone fragility is associated with

mineral loss and modifications to the collagen structure (193, 356). Beck and Hansen (14) suggested that estrogen synthesis and estrogen receptor populations may be associated with the age-related changes in avian bone.

Type of housing and handling during catching and slaughter may affect the incidence of osteoporosis. Confinement of laying hens in cages significantly reduces bone strength (194, 265) because reduced exercise is directly associated with structural bone loss and decreased bone strength. Numerous publications discuss the relationship between type of housing and bone strength (109, 219, 382, 387). Bones from laying hens kept in aviary systems are stronger than those from hens kept in enriched cages, litter, or wire systems, whereas hens kept in conventional cages had the weakest bones. Bones from egg layers became stronger after just 20 days of transferring the birds from cages to floor pens (262). However, there are no differences in molecular mechanisms for bone formation as a result of mechanical loading between birds using perches and those that do not (64). These authors hypothesize that the lack of upregulation may be caused by the already accelerated bone turnover in egg-laying hens or that birds may follow different pathways from mammals for bone turnover. Hens from cages had a greater incidence of freshly broken bones after handling at processing than hens from other housing systems (130).

Genetic selection to maximize egg production might have contributed to osteoporosis by producing strains of birds with poor bone quality. Bishop et al. (21) reduced the incidence of bone fractures and increased bone strength after 5 generations of selection in a commercial line of white leghorns. They found that cancellous and medullary bone volumes were poorly heritable parameters, but keel radiography density, humeral and tibial strength, and an index calculated from these 3 traits were promising predictors of bone characteristics and resistance to osteoporosis.

Intervention Strategies

Nutritional approaches may alleviate and even prevent osteoporosis. Adequate inclusion rates of calcium, phosphorus, and vitamin D are particularly important. Feeding 2% calcium 2 weeks prior to the start of lay followed by transfer to a higher calcium level during lay may reduce structural bone loss (106, 239). Feeding calcium in particulates, either as oyster shell or limestone granules, may extend the period of calcium absorption during the night, which reduces the depletion of medullary bone and benefits the eggshell quality (107, 108); however, it does not have much impact on the loss of structural bone. A combination of limestone with fluoride and/or vitamin K3 during the laying period had no greater benefits than limestone alone (107).

It has been hypothesized that formation of strong cortical bone and adequate medullary bone prior to egg production may be helpful in reducing osteoporosis during lay. Increased calcium in the ration prior to egg production may be necessary, but it has been suggested that if increased calcium is fed for too long before egg production, the parathyroid gland may be suppressed. Manipulation of lighting programs to delay sexual maturity has proven to have little effect on bone strength at the end of lay (158).

Treatment of pullets with alendronate just prior to the onset of lay decreased the loss of cancellous bone (368), but did not stop the loss of structural bone at the time of lay (410). Dietary supplementation with gallium prevented osteoporosis in hybrid laying hens experimentally (220).

Other Abnormalities of the Skeleton

Osteochondrosis

Osteochondrosis is a focal degenerative lesion of the growth plate, articular cartilage, or bone that is associated with ischemic and necrotic lesions of the growth plate, articular cartilage, or bone. Because this condition shares many features with TD, several investigators have used both terms as synonyms (292, 366). Osteochondrosis develops as a consequence of ischemic injury, either caused by mechanical forces (93, 241) or focal bacterial infection (218, 367). A variety of microscopic degenerative lesions including eosinophilic streaks or scars, occlusion and thrombosis of vascular canals, and necrosis in the growth plate and epiphysis have been described in growing meat-type birds (241, 311). Osteochondrosis has primarily been described in cervical and thoracic vertebrae of broiler chickens (165, 228), and in the femoral head (76, 81, 93, 182, 317) and the antitrochanter (82) of broiler chickens and turkeys.

Noninfectious Osteoarthritis

Osteoarthritis, also known as degenerative joint disease, is characterized by bone remodeling of a synovial-lined joint, and can include formation of osteophytes, degeneration or erosion of articular cartilage (Figure 30.9), fibrosis of joint capsules and inflammatory changes. Degeneration of the articular cartilage causes pain and lameness (96). Osteoarthritis can be observed in various species of birds, including poultry, psittacines, passerines, ratites, raptors, and other species. Risk factors include older birds, heavy body weight, genetic factors, infection, and articular urate deposition (71). In poultry, degenerative joint changes are more common in males than females (4). It has been recognized primarily in coxofemoral, femoro–tibiotarsal and tibiotarsal–tarsometatarsal joints of heavy breeders and meat birds (79, 82, 87), and in the spine of laying hens (413). The pathogenesis is not clear; however, morphologic and biochemical



Figure 30.9 Anterior and lateral views of distal femora from breeding turkeys with degenerative joint disease. Note the erosions and thinning of the articular cartilage.



Figure 30.10 Midline longitudinal section through the thoracic-lumbar region of the spinal column of a broiler chicken with spondylolisthesis, cervical end to right. Note the rotation of the body of vertebra T4, deformation of T5, and spinal cord compression (316).

changes of the articular cartilage are similar to those in mammals. Some may result from primary damage to the articular cartilage, whereas others may be sequelae to osteochondrosis (79, 82).

Spondylolisthesis

Spondylolisthesis or "kinky back" is the most common abnormality of the spine. It occurs when the anterior end of the articulating fourth thoracic vertebra dislocates, triggering the posterior end to override the fifth vertebra, causing compression of the spinal cord and posterior paralysis. This rotation causes a kyphotic angulation of the floor of the spinal canal and spinal cord compression (Figure 30.10). Another form of spondylolisthesis is characterized by step-like defects between adjacent thoracic vertebrae, producing stenosis of the vertebral canal (80).

By palpating the ventral surface of the spinal column during necropsy, one can readily detect the deformation of the spinal column. A diagnosis of spondylolisthesis is best confirmed by a midline longitudinal section of the spinal column to allow visualization of the spinal cord compression. Subclinical spondylolisthesis is common in broiler chickens. A few birds



Figure 30.11 Broiler chicken with spondylolisthesis (316).

affected with spondylolisthesis are found in most broiler flocks. In some flocks, the incidence of affected birds has reached 2%. The peak incidence occurs at 3–6 weeks of age. Affected birds are alert, remain sitting on their hocks with their feet slightly raised off the ground (Figure 30.11), and use their wings in an attempt to escape when approached. Severely affected birds often become laterally recumbent and often die from dehydration. The incidence of spondylolisthesis can be increased by genetic selection. It is postulated that spondylolisthesis is a development disorder influenced by conformation and growth rate. It can be decreased by slowing the growth rate.

Abnormal Spinal Curvature

Lordosis is the most common abnormal curvature of the spine in broiler chickens. It develops after hatching. Several other spinal deformities occur sporadically at a low incidence in commercial poultry. These deformities include scoliosis and rumplessness and have been reviewed by Riddell (310).

Spontaneous Bone Fractures

Bone fractures are one cause of downgrading and trimming of poultry carcasses. Fractures may occur spontaneously on the farm or during catching or transportation. In meat-type poultry, spontaneous fractures occur more frequently during the last part of the grow-out. In turkey breeders, complete fractures have been associated with preexisting stress or partial fractures in males (57). In laying hens, osteoporosis is the most common predisposing factor to bone fracture (see Osteoporosis).

Fractures of leg bones cause lameness. Birds may die if they do not reach feed or water or are killed by other birds



Figure 30.12 Two-day-old turkey poults with splayed legs.

in the flock. Predisposing factors include nutrition, poor bone quality, growth rate, heavy body weight, and lack of exercise. Handling of birds during catching and transport may create unnatural tensile and bending stresses on the bone, which may alter the cortex composition, predisposing the bones to complete fractures (57). Chickens that are held by both legs have fewer incidences of broken bones than those that are held by 1 leg only (129).

Rotated Tibia

Rotated tibia has been reported in turkeys, broilers, guinea fowl, and ratites (313, 319, 361). It consists of external rotation of the tibiotarsus, often to 90 degrees or greater, without angulation of the bone or displacement of the gastrocnemius tendon. The exact etiology of tibial rotation is unknown. However, genetic selection may explain why rotation is seen in broilers and no other chicken lines (160). Early rickets and malabsorption syndrome have been suggested as a predisposing factor in guinea fowl and turkeys respectively (16, 289)

Spraddle Legs

Birds with spraddle legs have 1 or both legs splayed laterally from the coxofemoral joint (Figure 30.12). It is usually associated with high humidity during incubation or newly hatched chicks being placed on slippery floors. The birds have their legs directed laterally and are unable to stand. Affected birds are culled, but this condition may not be manifested until birds are 2–3 weeks of age as the leg deformity becomes obvious.

Diseases of Muscles and Tendons

Deep Pectoral Myopathy

Deep pectoral myopathy has also been called green muscle disease. The condition has been described in meat-type breeding chicken, meat turkeys, and broilers (133, 146, 173, 174, 306). Both sexes have the defect, but males have a higher incidence of this condition. Condemnation of affected muscles can be higher than 3% in some meat-type poultry flocks (18).

Clinical Signs and Pathology

The lesion does not affect the general health of birds and is generally only found at processing. The lesion can be unilateral or bilateral. Chronic lesions result in dimpling or flattening of the breast muscles. Comprehensive descriptions of the pathology have been provided in turkeys (352) and in broiler breeder chickens (406). Lesions in both types of birds are similar. In early lesions, the whole deep pectoral muscle is swollen, pale, and edematous with necrosis in the middle third to three-fifths of the muscle. The overlying fascia is often opaque with edema between the deep and superficial muscles. In older lesions, the edema disappears and the necrotic muscle becomes more prominent and drier with greenish areas. In chronic lesions, the necrotic muscle has shrunk and is uniformly green, dry, and friable and enclosed by a fibrous capsule. It may shrink to a fibrous scar. The muscle posterior to the necrotic muscle becomes atrophied, pale, and sometimes fibrosed. The sternum adjacent to the necrotic muscle is roughened and irregular.

When examined microscopically, the fibers in the green necrotic muscle are swollen and uniformly eosinophilic with discoid necrosis. Nuclei are absent or faint. Blood vessels within the necrotic tissue often contain only nuclei of lysed red blood cells. Surrounding the necrotic tissue, there is an inflammatory reaction with heterophils, macrophages, and giant cells, and in chronic cases, a fibrous capsule. Viable, degenerated, and regenerating muscle fibers are often enveloped by the capsule. Brown pigments and cyst-like structures containing yellow material are also found within the capsule. In the muscle posterior to the necrotic tissue, fibers may be atrophied and replaced by fat, and in some instances, fibrosis is present. Vascular lesions consisting of thromboses, intimal proliferation, and aneurysm formation are found in and around the necrotic tissue. Ultrastructural studies on affected muscles have been conducted (174, 406).

Pathogenesis and Etiology

Deep pectoral myopathy is the result of ischemia secondary to the swelling in a tight fascia of a vigorously exercised muscle (228, 351, 353, 407). Surgical occlusion of arteries to the pectoral muscles in both turkeys and chickens resulted in infarcts similar in appearance to the lesions of deep pectoral myopathy (277, 354). Furthermore, temporary occlusion of the subclavian artery combined with electrically induced contractions of the deep pectoral muscle induced necrosis of the muscle in both lightweight and broiler strains of chicken.

Similar electrically induced contractions alone produced necrosis of the muscle in the broiler strains, but not in the lightweight chickens (407). The muscle necrosis can be produced by voluntary wing movements, but if the fascia around the deep pectoral muscle is surgically cut prior to exercise, it would prevent development of the lesion (351). Angiography demonstrated complete ischemia in the deep pectoral muscle associated with an increase in subfascial pressure following electrical stimulation of the muscle. After 24 hours, the ischemia only persisted in the middle of the muscle (228).

Modification of handling procedures may reduce the incidence (349, 404). Some evidence has been produced for a hereditary predisposition (143). This predisposition may be related to inadequate vasculature in muscles of meat-type birds (407), but not to body weight or breast width (144). No specific nutritional factors are known to influence the condition (133, 145). Slowing the rate of growth of turkeys does not reduce the incidence of deep pectoral myopathy (384).

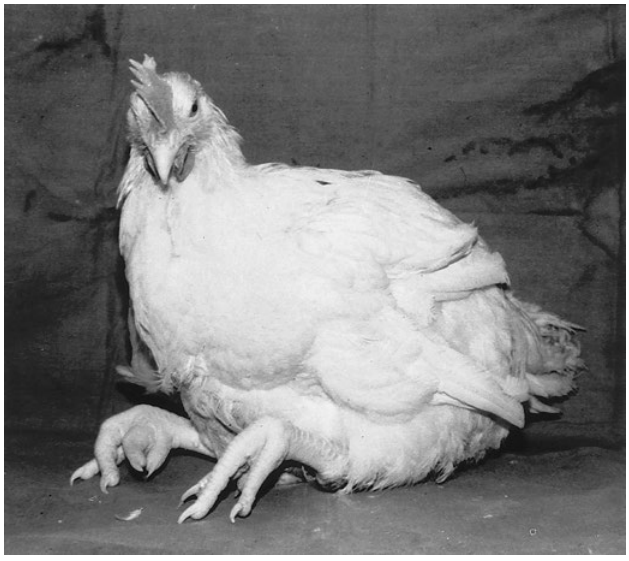


Figure 30.13 Roaster chicken with bilateral rupture of the gastrocnemius tendon. The hock-sitting posture with toes directed ventrally is characteristic. (Craig Riddell)

Rupture of the Gastrocnemius Tendon

Lameness caused by rupture of the gastrocnemius tendon has been recognized commonly in meat-type chickens and rarely in turkeys. It can cause considerable economic loss in broiler breeder flocks and in broiler chickens raised to roaster weights. The early literature was reviewed by Peckham (285).

Clinical Signs and Pathology

Up to 20% of a flock may be affected. Most outbreaks are in broiler breeder chickens older than 12 weeks of age, but may be seen in broiler chickens as early as 7 weeks of age. The rupture can be unilateral or bilateral. Onset of lameness is acute. Birds with bilateral rupture have a characteristic posture in which the bird sits on its hocks with its toes flexed (Figure 30.13). In affected birds, a swelling can be palpated on the posterior surface of the leg just above the hock. With acute lesions, hemorrhage can be seen through the skin. With older lesions, there is green discoloration. With chronic lesions, no discoloration may be apparent, but a firm mass of abnormal subcutaneous tissue can be palpated. Dissection of acute lesions reveals a blood-filled swelling under the skin on the posterior surface of the leg, within which the free end of the ruptured tendon can be found. The rupture generally occurs as an irregular transverse break just above the hock joint. In older and chronic lesions, the blood is partially or completely reabsorbed and fibrous tissue encloses the end of the ruptured tendon and surrounding tissue. Microscopic lesions are variable. In many acute lesions, there is hemorrhage only. In older lesions, there is fibrous tissue surrounding resolving hematomas and the ruptured tendon. Synovial hyperplasia and infiltration of heterophils and macrophages vary from very little to massive. The infiltration of inflammatory cells occurs within the tendon and in the synovial membranes and cavities, and may be associated with masses of heterophil debris and some bacterial colonies.

Pathogenesis and Etiology

Historically, rupture of the gastrocnemius tendon was associated with tenosynovitis, in particular caused by reoviruses (94). However, in many cases the rupture appears to have a noninfectious origin (56, 74). In cases associated with tenosynovitis, there was a marked inflammatory response, whereas in spontaneous rupture there was a minimal inflammatory response.

The tensile strength of the flexor *digitus perforatus* and *perforans* tendon to the third digit is less in meat-type chickens than in egg-type chickens, suggesting that this could predispose meat-type birds to tenosynovitis (375). This could also predispose to spontaneous rupture of tendons. Tissue of the gastrocnemius tendon in meat-type birds has a less organized appearance than that in egg-type birds (376). In addition, many meat-type birds have a hypovascular area in the gastrocnemius tendon just above the hock joint. This hypovascular area is associated with thickened chondrocyte plaques, chondrocyte death, and excessive lipid accumulation in the tendon. These changes may predispose to noninfectious tendon rupture (90). Little research has been conducted on the effect of nutrition on tendon strength. In 1 study, administration of glycine, vitamin C or E, or copper had no effect on tensile strength of tendons (377). In another study, restricted feeding had no effect on tensile strength of tendons, but the ratio of tensile strength to body

weight was less in chickens fed *ad libitum* than in those on feed (317). Prolonged sitting in broilers does not predispose tendons to ischemia and subsequent necrosis (60). In a recent study, tendon rupture was linked to male aggression and not body weight or exercise in hens (56).

Ligament Failure and Avulsion

Lesions of ligaments of the tibiotarsal–tarsometatarsal joint (85), in the posterior cruciate and other ligaments of the femoro–tibiotarsal joint in young adult broiler chickens (78, 83, 84, 91) and turkeys (87, 92), and in the intercondylar and collateral ligaments of the tibiotarsal–tarsometatarsal joint of turkeys (92, 184) and broiler chickens (88, 91) have been reported. Avulsion of the retinaculum on the distal tibia of turkeys also has been seen (53).

Clinical Signs and Pathology

Lameness has been attributed to lesions in the capital femoral ligament. Lesions found include stretching, partial or total rupture, and avulsion, sometimes with a piece of cartilage or bone from the femoral head insertion. Stretched ligaments sometimes contain hematomas or are infiltrated with fat. Microscopic lesions include fraying of collagen bundles and acellularity and hyalinization of the collagen in the tendon, along with necrosis, fissures, and hemorrhage in cartilage adjacent to the site of insertion (85). Lameness is also associated with lesions in ligaments of the femoral–tibial joint. The posterior cruciate ligament has been the most commonly affected, but the cranial cruciate, collateral, and caudal meniscofemoral ligaments also have been affected. In the cruciate ligament, total or partial rupture near the tibial insertion or avulsion from the tibial insertion occurs. Microscopic lesions are similar to those described for affected femoral capital ligaments. In addition, multicellular clusters and mucoid degeneration in the tendons and disorganization of subchondral bone with cysts and granulation at the avulsion site have been found (78). Some abnormalities of the menisci of the knee joint have been associated with ligament disruption (86). Lameness also has been associated with partial or total rupture of intercondylar ligament and with rupture or avulsion of collateral ligaments. Most microscopic changes in affected ligaments have been similar to those described for other affected ligaments (88, 184). In contrast to clinical signs associated with avulsion of other ligaments, avulsion of the retinaculum was not reported; however, there were increased condemnations of legs caused by hematomas and muscle discoloration at slaughter (53).

Pathogenesis and Etiology

Ligament rupture is often caused by trauma. Microscopic lesions similar to those described in ruptured ligaments have been described in intact ligaments of broiler-type chickens, indicating that these changes precede the rupture (83). In individual male broiler breeding chickens, tendon or ligament failure is often found at more than 1 site, suggesting a predisposition to ligament and tendon failure in these birds (84). Ligament failure may in part be age related, because the incidence appears to increase with age (91). Ligament lesions were less severe in turkeys fed a restricted amount of feed when compared with turkeys fed *ad libitum* (92). Rupture of ligaments may be secondary to stress induced by limb angulation (88, 184, 313).

Diseases of the Circulatory System

Pulmonary Hypertension Syndrome in Broiler Chickens

Pulmonary hypertension syndrome (PHS), also known as ascites syndrome, occurs worldwide in growing broiler chickens. It is characterized by excessive accumulation of transudate fluid within the peritoneal spaces caused by excessive elevated blood pressure within the pulmonary circulation (178). It is estimated that PHS accounts for 25% of overall mortality in the broiler industry and 5%–7% of condemnations (69). Interactions between management, environment, and genetic factors play a significant role in developing this disease. Recent reviews have been written by Gupta (135), Hassanzadeh (149), and Wideman et al. (402).

Clinical Signs

The peak incidence of ascites occurs between 5 and 6 weeks of age in broiler chickens. Affected birds are usually smaller than normal and listless with ruffled feathers and a pale, shrunken comb. Birds may be reluctant to move and are dyspneic and cyanotic (237). Affected birds may have abdominal distension caused by accumulation of ascitic fluid (Figure 30.14). Some birds may die suddenly before ascites develops (178, 186). Electrocardiograms of affected chickens shows an increased voltage of ventricular depolarization complex, consistent with dilation and hypertrophy of the right ventricle (266, 394). These changes in the electrocardiogram are preceded by modifications in the left ventricle consistent with enlargement and progressive left failure and compensatory right ventricular hypertrophy (269, 274).

Pathology

Gross lesions include ascites, right-side cardiac enlargement, often left-side ventricular dilation, and variable liver changes. The ascitic fluid is straw-yellow colored with or without fibrin clots (237, 409). Cardiac enlargement includes dilation of the right atrium, sinus venosus,

and vena cava as well as the right ventricle (Figure 30.15) and hypertrophy of both the right ventricle and right muscular atrioventricular valve. The ratio of right ventricular weight:total ventricular weight is greatly increased (162) and there is nodular thickening of the atrioventricular valves. Endocardiosis is more common in the left atrioventricular valve (273). Hydropericardium may be present. Arteries from ascitic broilers appear flaccid and lack elasticity (254). Lungs are edematous and congested. Livers in affected birds vary from congested or mottled to shrunken with a grayish capsule and irregular surface. Microscopic lesions have been described in the heart, major blood vessels, lung, liver, and kidney (139, 237, 254, 255, 409). The myocardial fibers are mildly disorganized, with occasional myocardial degeneration and calcification, edema, and some proliferation of loose connective tissue between fibers, focal hemorrhages, and infiltrations of heterophils. The arterial wall is thinner than in normal birds, or occasionally there is total loss of elastic elements. There is also reduced network density of the structural matrix of the vascular wall, as well as increased thickness of fibers in the vena cava. The lungs are often hyperemic with visible evidence of hemorrhage, edema, and hypertrophy of smooth muscle around the parabronchi, and collapse of the atria and air capillaries. Cartilaginous and osseous nodules may be found in the lungs (55). The liver has hepatocytic necrosis, dilation of hepatic sinusoids, and often fibrosis of the capsule and proliferation of connective tissue in the sinusoidal spaces and hepatic triad. Foci of lymphocytes and heterophils in the liver are common. Kidneys are congested and have vacuolar degeneration and necrosis. Lymphocyte aggregations may be present in the renal tubules.



Figure 30.14 Broiler chicken with abdomen distended with fluid secondary to right ventricular heart failure. (Craig Riddell)

Ultrastructural changes in PHS include myofibril disorganization, mitochondrial abnormalities and hyperplasia in the heart, thickening of alveolar and capillary walls in the lung, and thickened basement membranes and tubular degeneration in the kidney (233, 235, 238). There is also disruption of elastic elements in the wall of the vena cava (254). Abnormal calcium deposits have

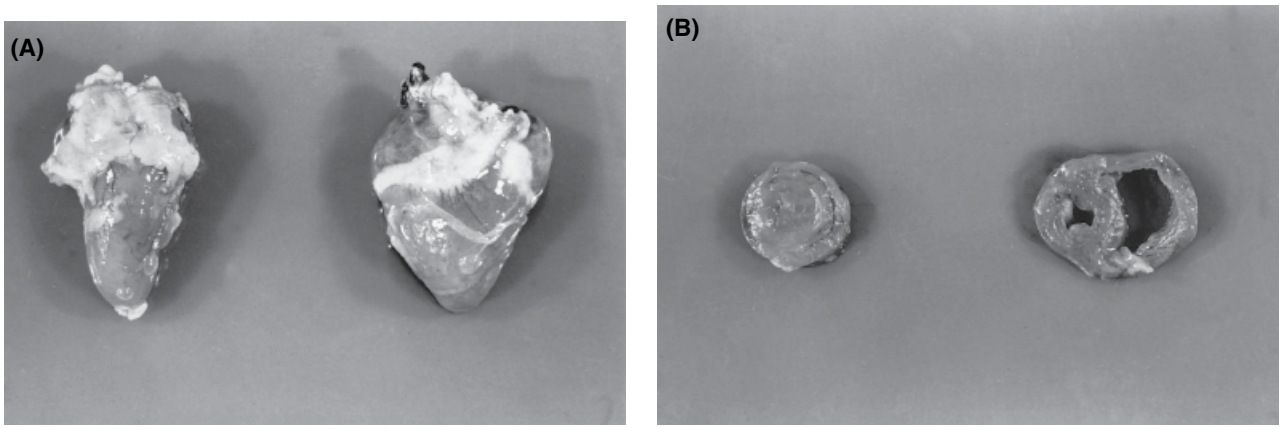


Figure 30.15 Right ventricular heart failure in a broiler chicken. (A) Enlarged heart (right) compared with a normal heart (left). (B) Transverse section through the enlarged heart (right) showing dilation and hypertrophy of the right ventricle compared with a transverse section through the normal heart (left). (Craig Riddell)

been demonstrated in the mitochondria of cardiac myocytes of birds with PHS (235). Increased serum troponin T found in live birds with PHS was another indicator of myocardial damage associated with PHS (236).

Physiologic blood parameters from affected birds show increased hematocrit, hemoglobin, and red and white blood cell counts. Heterophils and monocytes are increased at the expense of lymphocytes (237). There is also progressive hypercapnia and hypoxia (187).

Pathogenesis and Etiology

Clinical ascites represents the terminal consequence of a pathophysiological process initiated by high blood pressure within the pulmonary circulation (pulmonary hypertension) and results in right ventricular hypertrophy and failure. Despite the intensive investigation of the syndrome, the primary cause of ascites is unclear. Genetic and management factors are considered the main contributors to the increased incidence of ascites. High oxygen demand, low thyroid hormone activity, and increased metabolic rate of the modern broiler lines are 3 major factors responsible for the increase in ascites. Because there is a complex interrelationship between the cardiovascular, respiratory, circulatory, and other systems in the body, some of the observed changes in ascites syndrome may represent secondary compensatory responses.

Low partial pressure of oxygen or hypoxia in the blood is 1 of the major factors responsible for acute pulmonary vasoconstriction and pulmonary hypertension in broilers. PHS can be induced by environmental and experimental conditions that predispose to tissue hypoxia, such as high altitude, cold temperature, respiratory hypoxia, or increased metabolic rate (183, 280, 281). Hypoxemia results in increased cardiac output and polycythemia. The latter increases blood viscosity coupled with the larger and more rigid avian erythrocytes, which may have difficulty passing through the capillary bed of the lung (221, 234, 245). Elevated hematocrits also increase the risk of thrombotic occlusion of the pulmonary vasculature, which also can increase the pulmonary vascular resistance and contribute to the development of pulmonary hypertension (402). Additionally hypoxemia triggers arteriolar dilation to increase blood flow and restore adequate oxygen delivery to the organs and tissues (398). In turn, systemic hypotension stimulates the heart to increase the cardiac output, forcing even higher pulmonary arterial pressures.

Chickens susceptible to ascites have low thyroid hormone activity, predisposing them to a low capacity for oxygen consumption (70, 330) and possibly tissue hypoxemia. Additionally, thyroid hormone has been suggested as a modulator of the beta-adrenergic system, which is an important regulator of cardiovascular performance. Ascites-sensitive birds have a higher density of the beta-adrenergic receptors than ascites-resistant birds and the characteristics of these receptors are different from normal birds (151–153).

Increased metabolic rate implies a high oxygen demand to sustain metabolic needs. However, the modern broilers have a significant reduction in relative heart and lung size, and thus diminished cardiopulmonary capacity (187). It also has a thicker respiratory membrane and consequently a lower rate of oxygen diffusion from the lungs into erythrocyte hemoglobin (8). Increased metabolic rate also promotes oxidative stress that in turn causes lipid peroxidation mediated damage to the pulmonary vasculature, which further deteriorates oxygenation and aggravates hypoxemia (27). Dietary supplementation with coenzyme Q10 reduced PHS in broilers (116), probably by reducing free radicals.

Incubation conditions may influence the postnatal characteristics of PHS. Several studies have demonstrated that low oxygen levels during incubation influence the occurrence of ascites later in life. Embryos exposed to high carbon dioxide concentrations during the third week (38) and/or during the first 10 days (68) of incubation had lower incidence of ascites during the growth period than those incubated under normal concentrations. Similarly, birds incubated at high altitude (low oxygen) (154) showed less right ventricular hypertrophy and ascites mortality than those incubated at low altitude. In all of these studies, chickens exposed to less oxygen during incubation hatched earlier than those exposed to normal amounts, and the embryos might have experienced hypoxia for a shorter time. They also had higher plasma triiodothyronine and thyroxine (38, 154). Furthermore, embryos incubated at high altitude had higher plasma corticosteroid and lactic acid levels (154) and reduced binding capacity of myocardial beta-adrenergic receptors (152), suggesting an adaptation of the heart to hypoxia.

Wideman and French showed that animals that survived an ascites challenge produce offspring with reduced ascites syndrome (392). PHS-susceptible broilers have an elevated pulmonary arterial pressure, associated with increased precapillary (arteriole) resistance, when compared with PHS-resistant broilers (391). Cisar et al. found that the concentration of 2 mitochondrial matrix proteins involved in the aerobic metabolism was elevated in an ascites-resistant line broiler with ascites (43). Alternative genetic selection programs must look into parameters other than just body weight to reduce PHS (280, 417).

Accumulation of reactive oxygen may damage the cell membranes of different systems in the body. A direct correlation between low concentration of antioxidant in the lung and enlarged right ventricular weight has been found in broiler chickens (27). Lower levels of antioxidants also have been reported in the lung and liver (101). This suggests a deficiency in the control of oxidative stress.

Increased resistance to blood flow through the lung can cause pulmonary hypertension and consequently right ventricular failure and ascites.

Intervention Strategies

Several strategies have been used experimentally to prevent the accumulation of reactive oxygen and reduce the incidence of ascites. Supplemental L-arginine reduced the incidence of PHS mortality in experimental broilers, suggesting that L-arginine might be required as a substrate for nitric oxide, a powerful endogenous pulmonary vasodilator (396). Alternatively, nitric oxide may induce pulmonary hypertension by releasing reactive oxygen (6, 49). The manipulation of other important antioxidants, such as uric acid, flavonoids, or carotenoids, has not been investigated (8).

During the development of ascites, chickens develop systemic hypotension, which triggers retention of fluid and electrolytes (110). Furosemide, a diuretic that acts as a vasodilator, reduced PHS mortality in experimental broiler chickens, probably by reducing fluid and electrolyte retention, and pulmonary vascular resistance (393).

Systemic acid/base balance also influences the ventilation and perfusion through the lung. Intravenous infusions of 1.2*N* hydrochloric acid led to pulmonary vascular resistance and bradycardia, and could trigger pulmonary hypertension (395). Alternatively, the addition of 1% sodium bicarbonate to a broiler ration to cause alkalosis reduced the incidence of PHS in experimental birds in a hypobaric chamber (279).

In the field, there is no single treatment or preventive system for the control of PHS. Major broiler genetics companies use pulse oximetry to assess the adequacy of arterial blood oxygenation to select pedigree lines. Culling hypoxemic individuals improves the innate resistance of commercial broilers to PHS. Consequently, the incidence of PHS has declined in commercial broiler flocks reared at nominal altitudes (402). Additionally, management to reduce early growth rate in broiler chickens is used to reduce the risk of ascites. Manipulation of the photoperiod and feed restriction are common techniques for the control of ascites. Intermittent lighting programs during the early stages of life significantly reduce PHS (37, 150). The beneficial effect of intermittent lighting was more pronounced when applied from days 3 to 14 and/or from days 10 to 21 of age. Initial growth depression was followed by a compensatory growth with a similar final weight to the controls.

Feed restrictions may be used for early growth management (11). However, there are conflicting results in compensatory growth and carcass yield (183). Limiting the *ad libitum* food intake or skip-one-day programs may reduce PHS without compromising body weight (11, 183). The incidence of PHS is lower in broiler chickens fed mash diets as compared with those given pelleted diets (200, 346), without reducing body weight, weight gain, feed consumption, or feed conversion rate (26).

Dilated Cardiomyopathy in Turkeys

Dilated cardiomyopathy (DCM) has commonly been called round heart disease and less commonly cardiohepatic syndrome (308). The early literature on the syndrome has been reviewed by Czarnecki (61) and the reader is referred to this review for more detail and specific citations.

Clinical Signs and Pathology

The highest rate of mortality caused by spontaneous DCM occurs in young poults, commonly peaking at 2 weeks of age and normally disappearing at 3 weeks of age, but spontaneous DCM may be seen occasionally in turkeys up to 10–12 weeks of age. Mortality in flocks averages 0.5%–3% (112). Affected young turkeys may die suddenly or may have ruffled feathers, drooping wings, and labored breathing prior to death. Affected turkeys have increased end-diastolic volume and decreased ejection fraction and systolic blood pressure (115). On postmortem examination, affected young turkeys have greatly enlarged hearts caused by dilation of both ventricles. Often, the right ventricle is more dilated. Hydropericardium and ascites may or may not be present. Lungs are generally congested and edematous. Livers may be slightly swollen with rounded edges. In older turkeys from affected flocks the most prominent lesion is enlargement of the heart and hypertrophy of the left ventricle.

Microscopic changes in abnormal hearts are nonspecific and include congestion, degeneration of myofibers, focal infiltration of lymphocytes, and in older turkeys, increased fibroelastic tissue under the endocardium of the left ventricle. Vacuolization of hepatic cells, focal necrosis, bile duct hyperplasia, and intracytoplasmic PAS-positive globules in hepatocytes have been described in the swollen livers.

Pathogenesis and Etiology

Dilated cardiomyopathy in turkeys has been reproduced with similar environmental and management factors as PHS in chickens, such as hypoxic conditions in incubation (59), high altitude, and hypobaric chamber with cold weather (111, 185). The incidence of DCM can also be reduced by slowing the growth rate (30, 185), using an intermittent light program early in life (46), and providing a diet containing low sodium (0.10%–0.12%) and 0.38%–0.40% chloride (112). In addition, the drug furazolidone has been shown to induce DCM. However, furazolidone-induced DCM is significantly different from the naturally occurring disease, suggesting a different etiology (209).

Turkeys with DCM have a decrease in some enzymes involved in the energy supply, such as creatinine kinase, lactate dehydrogenase, the Ca^{2+} transport system, βreceptor-stimulated adenylyl cyclase (115), diminished concentration of adenosine-5′-triphosphate (ATP) (222), and reduced concentration of fatty acids, the main substrate for cardiac metabolism (209). Genetic studies have identified 2 possible proteins, troponin T and phospholamban, which may be involved in DCM (225). Troponin T is involved in the Ca^{2+} regulation of striated muscle during contraction, whereas phospholamban regulates muscle Ca during diastole. In turkeys with DCM induced by furazolidone, troponin T is reduced by 61% and phospholamban is only reduced by 18% (224). Troponin T has an abnormal structure in domestic turkeys (19, 20), which may predispose them to DCM.

Sudden Death Syndrome in Broiler Chickens

Sudden death syndrome (SDS) describes a condition in which healthy broiler chickens die suddenly for no discernible cause. The syndrome has been also called heart attack and flip-over. The latter term has been used because birds found dead from the syndrome are commonly found on their backs. The condition was first described as "edema of lungs" in England (155) and subsequently as "died in good condition" in Australia (171). Today, birds who have died from SDS are found in most broiler flocks throughout the world. The incidence varies from 0.5% to 4% (31, 40, 161, 319, 359). Reviews on SDS have been written by Olkowski and Classen (272) and Riddell (307).

Clinical Signs

Sudden death syndrome has been reported to occur from 1 to 8 weeks of age, with the greatest losses occurring from 2 to 4 weeks of age (319, 359). It is more common in males than females (270, 276). In some broiler flocks, the weekly incidence increases throughout the growing period, suggesting an error in diagnosis or a different syndrome (319). It is possible that birds dying from PHS may have been misdiagnosed as dying from SDS (307).

Affected chickens show no clinical signs or unusual behavior until less than a minute before death (260). Birds may squawk during the episode, with loss of balance, convulsions, and violent flapping (260). Most birds die on their backs with 1 or both legs extended or raised (318, 359).

Pathology

Birds that die from SDS are well fleshed with a full gastrointestinal tract. Livers are enlarged, pale, and friable, and the gallbladder generally is empty. Kidneys may be pale and the lungs are often congested and edematous (276, 359). The congestion and edema of the lungs may be a postmortem artifact, because it is not observed in freshly dead birds (318). Cardiac ventricles are generally contracted and thyroid, thymus, and spleen may be congested, with occasional hemorrhages in the kidneys (276). Relative liver weights of SDS broilers are significantly greater than the liver weights of control birds, with no significant differences in relative weights of lungs, heart, and intestines (29).

Microscopic lesions reported are nonspecific. Use of an allochrome stain and a hematoxylin-basic fuchsin-picric acid stain did not demonstrate any degenerative changes in hearts of birds dying from SDS (318). However, a more recent study described arteriosclerotic changes and myocardial necrosis mostly in the left ventricle of broiler chickens that had died suddenly without clinical signs (188, 275).

Biochemical data is limited, because of the lack of clinical signs prior death (260). Comparison of blood from birds just after death from SDS with blood from killed healthy birds revealed no consistent differences in serum levels of sodium, potassium, chloride, calcium, phosphorus, magnesium, or glucose (318, 418). Serum lactate dehydrogenase, glutamic oxaloacetic transaminase (168), and creatinine phosphokinase (297) were increased in SDS chickens compared with healthy chickens. Serum lactate dehydrogenase (LDH) and glutamic oxaloacetic transaminase (GOT) were elevated in SDS birds, but not creatine kinase (168). Birds that die with SDS have a tenfold higher concentration of lactic acid than unaffected birds (348).

Pathogenesis and Etiology

Sudden death syndrome has been described in most modern broiler-type chickens, but is not well understood. It has been suggested that SDS is a metabolic disease and that genetic, nutritional, and environmental factors may affect the incidence. SDS was associated with acute cardiac dysrhythmia, consistent with ventricular fibrillation (271). Birds that later died of SDS had a higher heart rate than the rest of the flock (274). Furthermore, a higher percentage of chickens that died with SDS had cardiac arrhythmias (270). LDH and GOT are considered indicators of circulatory disturbance in humans (168). It is possible male broilers are more susceptible to SDS, because they normally have higher serum LDH and GOT than females (418). Similar susceptibility to SDS was found between 6 strains of commercial broiler chickens, including Arbor Acres, Avian Farms, Cobb-500, Hubbard–Peterson, ISA, and Ross (125). The heritability of SDS is low (42).

In a nutritional study SDS was higher in birds fed crumble-pelleted feed vs. mash (295). Incidence was not affected by feed restriction or growth rate (319, 321). In a field survey, flocks on wheat-based rations had higher

SDS than those on corn-based rations (319). This difference also was noted in some experimental trials (22), but not in other studies (164, 246). Protein type and concentration in the diet influence the incidence of SDS. Chickens fed meat meal protein had a lower incidence of SDS than those on soybean meal (22). Also, higher protein in the finisher diet reduced the incidence of SDS (246). Addition of vitamins did not affect the incidence of SDS (164, 246, 360, 389), but it has been suggested that thiamine may influence the incidence (44). The concentration of calcium, phosphorus, and magnesium (179) or potassium (164) in rations had no effect on the incidence of SDS. SDS was reproduced by administration of lactic acid (198).

A lighting program with a short photoperiod at an early age decreased early growth rate as well as the incidence of SDS (45, 315). Extended dark periods (more than 8 hours) reduced SDS, but had a negative effect on body weight (332). Decrease in SDS may be associated with lower heart rates during dark periods (23); but in another study, light intensity did not affect the incidence of SDS (261). High stocking density may also influence the incidence of SDS (169, 319).

Intervention Strategies

There is no proper treatment and preventive measures for control of SDS. Because the condition is associated with faster growth rate, management strategies to prevent maximum potential for growth can reduce the incidence of SDS.

Aortic Rupture

Aortic rupture (AR) or dissecting aneurysm is characterized by sudden death in growing turkeys caused by internal hemorrhage. The condition is seen worldwide. Mortality in the past has been reported to reach 50%, but losses in affected flocks at present usually reach 1%–5%. AR also has been described in ostriches and emus (361).

Clinical Signs and Pathology

Aortic rupture occurs in turkeys, mostly males, between 7 and 24 weeks of age, with peak mortality between 12 and 16 weeks of age. Affected birds die suddenly in good body condition. At necropsy, the head, skin, and musculature may be pale. Upon internal examination, gross lesions consist of clotted blood in the abdominal cavity surrounding a portion of kidneys or whole kidneys. Clotted blood may be present in the oral cavity, trachea, lungs, and pericardial sac. Careful dissection of the posterior abdominal aorta and its branches in the vicinity of the kidneys reveals tears or ruptures in the aorta, between the celiac and ischiatic arteries (Figure 30.16), but is most common around the celiac artery. In some cases, at the site of the rupture the aorta was dilated, and the wall was thin and had lost its elasticity. Aneurysm and rupture of the coronary artery also have been described (343).

Histologically, the tunica intima and media may be thrown into deep folds and partially separated from the tunica adventitia. Fibers of the tunica media may show mild to severe degenerative changes and may be infiltrated with heterophils and macrophages. The media may be thickened caused by an increase in collagen and fibroblastic proliferation. Dissolution or disappearance of the elastic fibers of the media occurs at the site of rupture. Degenerative changes and areas of erosion and cellular infiltration may be present in the adventitia. There is often displacement of the internal elastic lamina. A marked intimal thickening or a large fibrous intimal plaque often occurs in the region of rupture. Sudan II or Oil Red O stains may reveal lipid accumulations in the affected intima and media.

Pathogenesis and Etiology

Copper deficiency, hypertension, hormonal influences, diet, lathyrism, zinc deficiency, pharmaceuticals, management practices, and parasites have been suggested as predisposing factors for AR (374). Low copper levels were found in the livers of turkeys and ostriches from field outbreaks of aortic rupture (126, 343, 378). However, copper deficiency is unlikely because AR occurs primarily in male turkeys and copper deficiency has not been consistently found in birds with AR. High blood pressure in young male turkeys may be a predisposing factor, but paradoxically, the administration of diethylstilbestrol decreased blood pressure and increased the incidence (195, 196). Several reports have emphasized the possible role of intimal plaques in the pathogenesis of aortic rupture in turkeys. It has been suggested that these plaques and the absence of an intramural *vasa vasorum* around the abdominal aorta result in impaired nutrition to, and degeneration of the media (258). Beta-aminopropionitrile, a toxic product that occurs in the sweet pea (*Lathyrus odoratus*), will produce AR in turkeys (285). Dietary reserpine reduced the incidence (113).

Sudden Death Syndrome of Turkeys Associated with Perirenal Hemorrhage

Sudden death in turkeys associated with perirenal hemorrhage (SDPH) sporadically affects male turkeys between 8 and 14 weeks of age where commercial heavy toms are grown (113, 251). Dead turkeys are in good body condition with food in the crop and gastrointestinal tract. They have congested and edematous lungs, splenomegaly, congested livers and digestive tract, and perirenal hemorrhage.

The most significant gross lesion is probably cardiac hypertrophy affecting the left ventricle and intraventricular septum (113). Because male turkeys have greater relative left and total ventricular weights than do females of the same age, this might explain the greater susceptibility

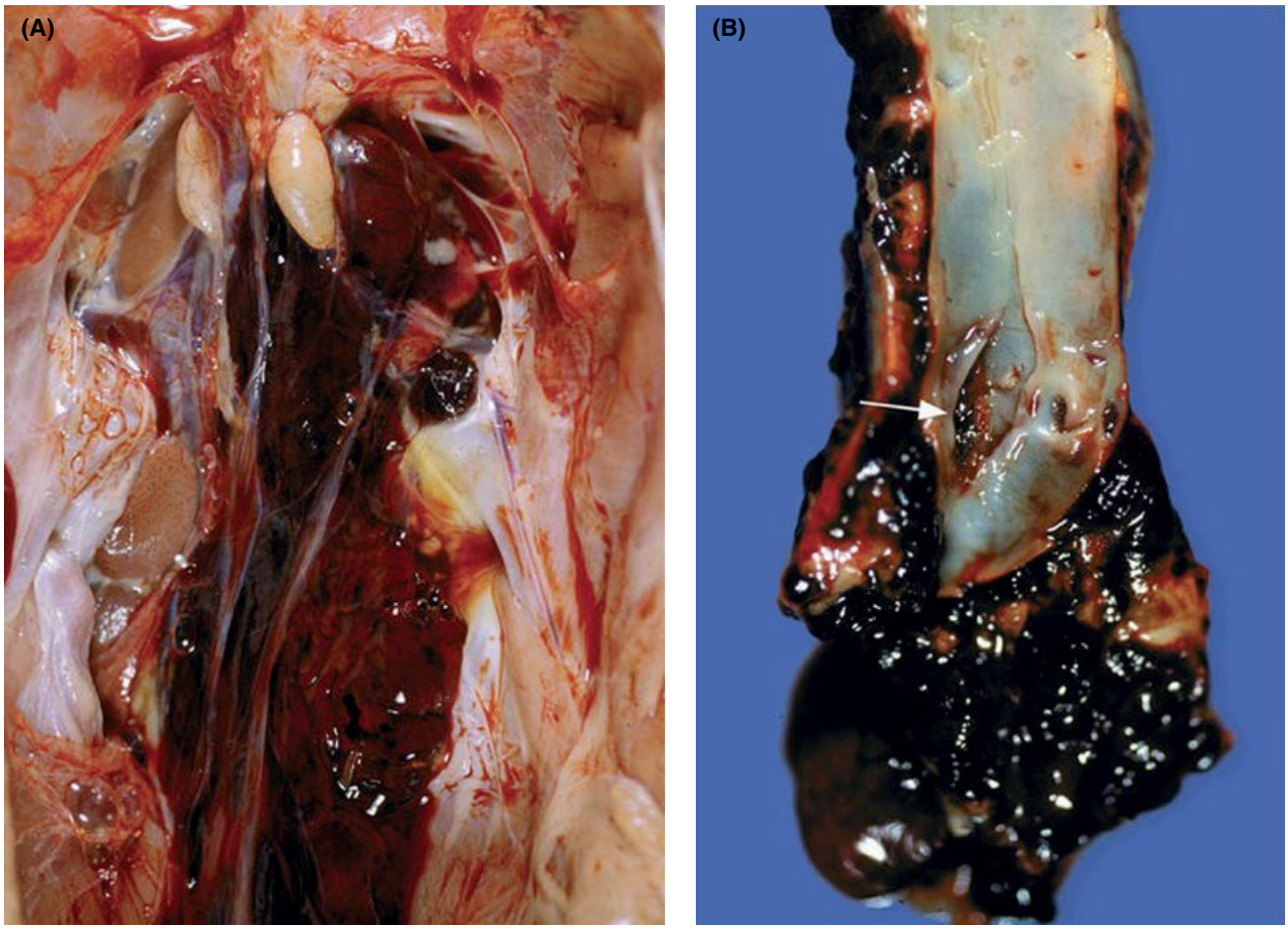


Figure 30.16 Aortic rupture. (A) Abdominal bleeding, around the kidneys, secondary to ruptured artery in a 16-week-old meat turkey. (B) Tear of the aorta wall (arrow) at the level of the ischiatic artery.

of male turkeys to SDPH (28). The etiopathogenesis of the condition remains unclear. It has been suggested that the cause of death in SDPH is an acute congestive heart failure secondary to cardiac hypertrophy. The observed perirenal hemorrhage is likely a consequence of this severe passive congestion (208). In the experience of the author, perirenal hemorrhage is also observed in many birds that died with AR. Furthermore, intimal thickening, fibrocartilaginous plaques, and subintimal vacuolation observed in the aorta (208) may represent early stages of aortic degeneration.

Fast weight gain, continuous lighting programs, crowding, and hyperactivity increased the incidence (251), whereas increased room temperature, toe clipping, and step up/step down lighting reduced the incidence (113).

Digestive System and Liver

Fatty Liver–Hemorrhagic Syndrome

Fatty liver–hemorrhagic syndrome (FLHS) affects chickens worldwide, including commercial layers and broiler breeders. FLHS is associated with high-energy diets along with restricted exercise and is most common during hot weather.

Clinical Signs and Pathology

The first sign of FLHS is an increase in mortality (36), with birds in full production found dead with pale heads. There is often a sudden drop in egg production. Mortality usually does not reach 5%. Dead birds are pale with large blood clots in the abdomen, arising from the liver and often partially enveloping the liver (Figure 30.17). The liver is generally enlarged, pale, and friable, with smaller hematomas within the parenchyma. These hematomas may be recent and dark red, or older and green to brown. Similar hematomas may be seen in clinically healthy birds in the same flock if they are examined during or after an outbreak. Large amounts of fat are present in the abdominal cavity and around the viscera. Most of the birds have active ovaries and often have an egg in the oviduct.

Microscopic examination of the liver shows hepatocytes distended with fat vacuoles, hemorrhages of



Figure 30.17 Fatty liver–hemorrhagic syndrome. A large blood clot is molded over the left lobe of the liver. Note the excess abdominal fat.

various sizes and organizing hematomas, and often small irregular masses of uniform eosinophilic material, likely derivative of plasma protein (405). Fat content of livers ranges from 40% to 70% dry weight. Increased lipid accumulation in the liver was observed in commercial layer hens stimulated with estradiol; however, there were no significant differences in free cholesterol or phospholipids (214). These results suggest that estradiol stimulated the synthesis of fatty acids and triacyglycerols in the liver. However, in a study of FLHS in backyard poultry, researchers found no correlation between the degree of vacuolization and reticulin loss (370). The reason for this discrepancy may be caused by hepatic hemorrhage initiated by different etiologies (e.g., toxic, nutritional deficiencies).

Common indicators of hepatic disease such as aspartate aminotransferase and other plasma enzymes were increased in birds from an FLHS-susceptible strain or commercial birds fed diets that induced FLHS (73, 416). Birds affected with FLHS had higher concentrations of estradiol (138), calcium and phosphorus (142) in the plasma than unaffected ones. No changes have been found in concentrations of progesterone (138), major plasma proteins, or glucose.

Pathogenesis and Etiology

The fat content in the liver normally rises with the initiation of egg production and is influenced by estrogen. Injection of immature chickens with estradiol has been shown to result in hepatic steatosis and hemorrhage (283). Similar injection of laying hens caused liver enlargement, death from liver hemorrhage, and neurologic disorders (358). In 1 experiment, treatment with synthetic estrogens was more damaging at 34 °C than at 21 °C (2), which corroborates with the observed increased incidence of FLHS during hot weather. Additionally, birds exposed to high temperatures are more likely to be in a positive energy balance.

Excessive consumption of high-energy diets, regardless of the source, in birds whose exercise is restricted results in excessive fat deposition. Excessive fat may disrupt the architecture of the liver and result in weakening of the reticular framework and blood vessels in the liver. A pathogenic relationship between hepatic steatosis and hemorrhage has been suggested (284). Lysis of the reticulin framework of the liver has been reported in FLHS. A strong association of reticulolysis with severity of liver hemorrhage has been described in experimental birds (231). Focal necrosis of hepatocytes leading to vascular injury has been described as another mechanism to explain the hemorrhage (167, 414). It has been postulated that excessive lipid peroxidation of unsaturated fatty acids in the liver may overwhelm cell repair mechanisms and result in tissue damage (357). However, chickens susceptible to FLHS were supplemented with ascorbic acid, tocopherol, or L-cysteine, and there was no improvement in FLHS (72).

Because energy balance is a risk factor in FLHS, many studies have focused on the influence of diet. FLHS increases as the total energy in the diet increases, regardless of the source. However, when isocaloric diets were compared, a diet that provided the energy in fat rather than in carbohydrates reduced the incidence (137). It was hypothesized that diets richer in fat might have reduced the hepatic metabolism by reducing *de novo* fatty acid synthesis in the liver. Furthermore, size of the liver, rather than lipid content in the liver or high-fat diets, was directly related to FLHS (331).

The possibility of toxins causing FLHS should not be ignored. Aflatoxin has been considered as a possible cause but produces different liver lesions. FLHS in chickens also must be differentiated from rupture of the liver associated with hepatitis E virus (see Hepatitis E Infections in Chapter 14). Livers are generally not fatty in ruptured livers of chickens associated with hepatitis E virus.

Other Disorders of the Digestive Tract

Pendulous Crop

Pendulous crop occurs at a low incidence in many chicken and turkey flocks. In severely affected birds, the



Figure 30.18 White broad-breasted meat turkey with pendulous crop.

crop is greatly distended and full of feed, particles of bedding, and fluid, which often has a foul smell (Figure 30.18). The lining of the crop may be ulcerated. Birds continue to eat, but digestion is impaired and they become emaciated and die. Carcasses of affected birds are generally condemned at processing. The possibility that diet may influence the incidence of pendulous crop is supported by the experimental production of pendulous crops with rations containing Cerelose as a substitute for starch. It was suggested that increased liquid intake in hot weather may be responsible. Additionally, hereditary predisposition has been proposed in turkeys. Neither of these factors appears to be important. For further discussion on the possible etiologies of pendulous crop, review the 8th edition of *Diseases of Poultry* (285).

Impaction

Impaction of the crop, proventriculus, or ventriculus (gizzard) has occasionally been reported in poultry, waterfowl, and ratites, but rarely in chickens. Gizzard impaction can cause high mortality during the first 3 weeks of life in turkeys. Proventricular and gizzard impaction are common in ostrich and other ratite chicks. Affected birds are emaciated, with empty intestinal tracts, but affected crops, proventriculi, and/or gizzards are full of a solid mass of interwoven fibrous material (Figure 30.19). This fibrous mass often extends into the upper duodenum, and in some birds, masses of fibrous material are found lower in the intestine. Impaction results from the birds eating litter or fibrous material that cannot be processed in the crop, proventriculus, or gizzard. In ratites impaction caused by foreign bodies such as rocks, metallic objects, pebbles, etc., is common. Crop impaction caused by feather eating was associated with improper management (249). Prevention is aimed at discouraging the eating of litter or fibrous materials by young poults and ratite chicks.



Figure 30.19 Gizzard impaction in 7-day-old turkey poult.

Intussusception and Volvulus

Intussusception and volvulus are occasionally seen in poultry. Intussusception occurs most frequently in the intestine, but sometimes in the proventriculus (335). Volvulus occurs when there is torsion of the intestine around itself or the root of the mesentery. In young birds, volvulus of the small intestine may be caused by twisting around the yolk sac. Intussusception and volvulus have been reported in chickens secondary to enteritis or abnormal peristalsis caused by nematode or coccidial infection. Intestinal torsion also may be associated with pedunculated neoplastic stalks. The clinical signs are anorexia and progressive weight loss, and death occurs over a few days. The affected and distal portions of the intestine are severely congested caused by circulatory compromise and the intestinal epithelium rapidly becomes necrotic. If an early diagnosis is made, resection of the affected intestine can be performed in a valuable bird.

Urinary System

An excellent review of renal pathology of the fowl was written by Siller (350). This discussion covers only metabolic conditions of major importance commonly seen in commercial poultry. It does not cover descriptions of

miscellaneous conditions such as congenital malformations and baby chick nephropathy.

An increased substrate load on the kidney that leads to dysfunction of this organ with precipitation of insoluble products within the kidney itself or other organs can cause urolithiasis or urate deposition. Urolithiasis and urate deposition together are responsible for significant losses in poultry. Historically, urate deposition has been described as 2 distinct syndromes, known as visceral urate deposition (VUD, "visceral gout") and articular urate deposition ("articular gout") (see Table 30.1). These 2 syndromes are different from the point of etiology, morphology, and pathogenesis. VUD is described under urolithiasis because they are related conditions in commercial poultry. VUD also can be seen in individual birds of any species as a sequela to acute renal failure.

Urolithiasis and Visceral Urate Deposition

Urolithiasis is characterized by severe atrophy of 1 or both kidneys, distended ureters often containing uroliths, and varying degrees of renal and visceral urate

Table 30.1 Differences between visceral gout and articular gout in birds. (Modified from Shivaprasad [341])

	Visceral Gout (Visceral Urate Deposition)	Articular Gout
Onset	Usually an acute condition but can be chronic	Usually a chronic disease
Frequency	Very common	Rare or sporadic
Age	One day and above	4–5 months and above. However, immature genetically susceptible chickens may be induced by high protein levels in the diet
Sex	Both males and females are susceptible	Mostly males
Gross lesions		
Kidneys	Kidneys are almost always involved and they look grossly abnormal with deposition of white, chalky precipitates	Kidneys are normal grossly Kidneys may become abnormal with white urate deposits if the bird gets dehydrated
Soft tissues	Visceral organs like liver, myocardium, spleen or serosal surfaces like pleura, pericardium, air sacs, mesentery, etc., are commonly involved	Soft tissues other than synovium are rarely involved; however, comb, wattles, and trachea may be involved
Joints	Soft tissues around the joints may or may not be involved. Surfaces of muscles, synovial sheaths of tendons and joints are involved in severe cases	Soft tissues around the joints are always involved, especially feet. Other joints of the legs, wing, spine, and mandible are also commonly involved
Microscopic lesions	Generally no inflammatory reaction in synovium or visceral surfaces. Kidneys and viscera have inflammatory reaction around tophus	Granulomatous inflammation in synovium and other tissues
Pathogenesis	Generally caused by failure of urate excretion (renal failure)	Probably caused by a metabolic defect in the secretion of urates by the kidney tubules
Causes	1) Dehydration 2) Nephrotoxicity: calcium, mycotoxins, (ochratoxins, oosporein, aflatoxins, etc.), certain antibiotics, heavy metals (lead), ethylene glycol, ethoxyquin, etc. 3) Infectious agents: nephrotropic infectious bronchitis virus and avian nephritis virus (chickens), astrovirus (chickens), paramyxovirus-1 (pigeons), *Eimeria truncata* (geese). In psittacines: *Salmonella* sp., *Yersinia* sp., *Chlamydia psittaci*, *microsporidia, cryptosporidia, Aspergillus* sp., polyomavirus, etc. 4) Vitamin A deficiency 5) Urolithiasis 6) Neoplasia (lymphoma, primary renal tumors) 7) Immune-mediated glomerulonephritis 8) Anomalies 9) Others?	a) Genetics b) High protein in the diet c) Others?

deposits. VUD (visceral gout) occurs when there is a failure of urinary excretion. Urolithiasis is primarily seen in laying flocks and has been associated with increased mortality and decreased egg production (227, 400).

Clinical Signs and Pathology

Overall mortality in affected flocks may exceed 2% for several months, and mortality in excess of 50% may be caused by urolithiasis (24, 227). Renal lesions have been recognized in clinically normal birds in flocks undergoing an outbreak, and 3.2%–6.3% of hens in some affected flocks had renal lesions at processing (227). Affected chickens die suddenly and may be in good condition and in full lay (24) or they may have a reduced muscle mass, small pale combs, and white pasting on pericloacal feathers (32).

Focal mineralization of the kidneys, progressive obstruction of the ureters by uroliths, causes kidney atrophy "upstream" of the site of ureteral obstruction and compensatory hypertrophy by the undamaged portions of the kidney. The ureters arising from the atrophied lobes are dilated and full of clear mucus and often contain white irregular concretions or uroliths (32). These uroliths are composed of compact masses of microcrystalline to fine pleomorphic crystals of calcium sodium urate, with random substitution of magnesium and potassium for the calcium and sodium, respectively (268). The kidney atrophy is often more severe in anterior lobes and is unilateral, but it may be bilateral. The surviving ipsilateral or contralateral lobes may be enlarged. Atrophied kidneys and dilated ureters are often accompanied by diffuse visceral urate deposits (24, 32, 227) (Figure 30.20). VUD is characterized by precipitation of urates in the kidneys and on serous surfaces of the heart, liver, mesenteries, air sacs, and/or peritoneum (Figure 30.21). In severe cases, surfaces of muscles and synovial sheaths of tendons and joints may be involved, and precipitation may occur within the liver, spleen, and other organs. The deposits on serosal surfaces appear grossly as a white chalky coating, whereas those within viscera may be microscopic.

Microscopic lesions in affected kidneys consist of dilation of ureter branches and tubules, tubular degeneration and loss of tubules, cellular casts, urate crystals, and varying degrees of fibrosis (24, 32). Much of the urate deposits are lost when tissues are processed for histology, but evidence of its presence is often seen as blue or pink amorphous material under the microscope. Feathery crystals or basophilic spherical masses may be seen within tissues under the microscope in some cases. Fixing and processing tissues in 90% or absolute alcohol will preserve urates.

Urolithiasis has been primarily recognized as a disease of laying birds, but reports indicate that lesions (Figure 30.20) and mortality may start during the rearing period (32, 51). In a sequential study of 1 outbreak, minor focal cortical tubular necrosis was found by microscopic study in grossly normal kidneys of 4-week-old pullets. In 7-week-old pullets, the kidneys were grossly swollen with tubular necrosis and casts, eosinophilic globules in glomeruli, and interstitial infiltration of heterophils and lymphocytes. Typical lesions of urolithiasis were found in 14-week-old birds (32).

Pathogenesis and Etiology

Wideman et al. (401) conducted renal function studies on chickens during outbreaks of urolithiasis and concluded that the physiologic impact of the kidney damage was the result of reduced renal mass, rather than of inappropriate renal handling of minerals or electrolytes.

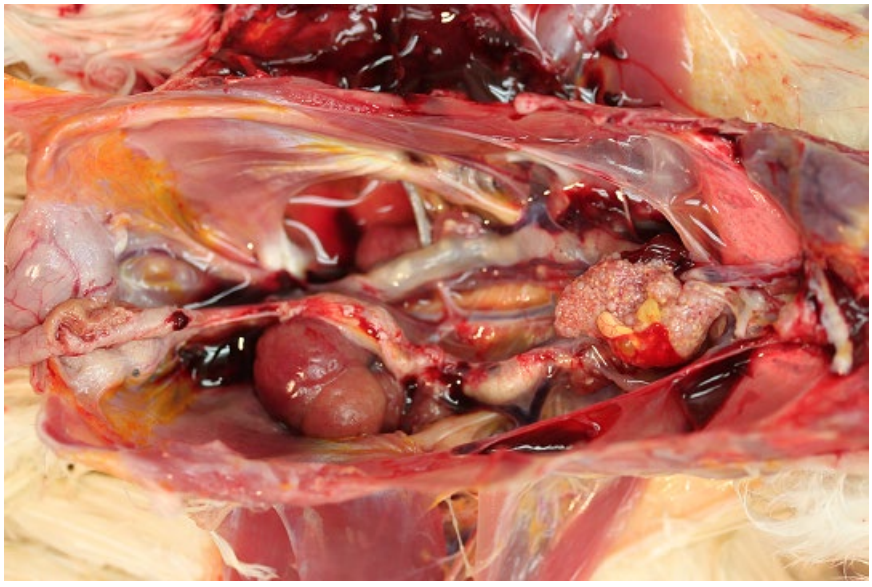


Figure 30.20 Urolithiasis in a chicken. There is severe atrophy of the right kidney and anterior lobes of the left kidney. The right ureter is distended with white material.



Figure 30.21 Visceral urate deposition over the heart and liver of an adult pigeon.

A significant reduction in number of glomeruli also has been reported in birds affected by urolithiasis (263). The uroliths may cause sudden death by blockage of ureters but probably occur secondary to kidney damage (227).

A marked difference in susceptibility to urolithiasis caused by high-calcium diets has been described between 2 strains of leghorn chickens (215). The more susceptible strain produced more alkaline urine and had a higher proportion of juxtamedullary nephrons (401). Excess dietary calcium induced urolithiasis in growing pullets and male breeders (121, 134, 250, 334, 390). Formation of uroliths may be caused by high levels of urinary calcium and decreased hydrogen ions in the urine (122). Dietary acidification with ammonium chloride, ammonium sulfate, or methionine has been shown to decrease the incidence of uroliths and gross kidney lesions in urolithiasis induced experimentally with high-calcium diets (120, 215, 397, 399). Ammonium chloride was not considered a practical treatment for use in the field because it caused increased water consumption, urine flow, and manure moisture (120), but other compounds did not have this disadvantage (215, 399). Ammonium sulfate was more effective than 2 forms of methionine in a single trial (215).

Outbreaks of urolithiasis and VUD in poultry also have been attributed to infectious causes, such as nephrotropic strains of infectious bronchitis virus (3, 32, 51, 58), astrovirus (34), and renal cryptosporidiosis (369). Exposure of pullets to the Gray strain of infectious bronchitis virus subsequent to feeding a high-calcium laying ration increased the incidence of urolithiasis and gross kidney damage (122). Noninfectious factors, such as vitamin A deficiency (350), treatment with sodium bicarbonate (65), and mycotoxins such as oosporein (286), induced urolithiasis and VUD. Water deprivation has been suggested as a cause on the basis of field observations (181).

Articular Urate Deposition

Articular urate deposition ("articular gout"), unlike the visceral type, is a sporadic problem of minor economic importance in poultry. Clinically this condition is characterized by leg shifting, lameness, and inability to bend the toes. It is characterized by tophi (deposits of urates around joints), particularly those of the feet, hence confusion with footpad dermatitis. Joints are enlarged and the feet appear deformed (Figure 30.22). When these joints are opened, the periarticular tissue is white caused by urate deposition, and white semifluid deposits of urates may be found. In chronic cases urate precipitates also can be observed in the comb, wattles, trachea, etc. Unlike VUD, the kidneys are usually grossly normal. Histologically there is granulomatous inflammation in the synovium. Therapy is palliative only. Removal of the deposits is not recommended because of the difficulty and profuse bleeding. Because it has been reproduced by feeding high-protein diets, the etiology is suggested as excess production of uric acid. Studies in a line of chickens bred for a high incidence of articular urate deposition, however, indicate that they may have a defect in tubular secretion of uric acid (7, 47).

Integumentary System

Feather Pecking and Cannibalism

Feather pecking is a major welfare problem. It is a behavior expressed by dominant birds on subordinates. Pecking may vary from pecking without removal of feathers to plucking of the feathers. Birds with damaged feathers have poor thermoregulation and greater energy demands than unaffected birds (287). Egg production in affected laying hens usually drops.



Figure 30.22 Articular urate deposition ("articular gout") in a mature layer chicken causing enlargement and deformity of toes and feet. Large amount of urate crystals is visible when the skin is removed.

Vent pecking is a separate form of pecking that also can be observed in well-feathered birds (327). It generally occurs soon after birds have come into lay. It is more common when birds in floor systems lay their eggs on the floor in crowded areas. It occurs immediately after oviposition, and exposure of the mucous membrane stimulates pecking by other birds. Vent pecking is responsible for at least 80% of all prolapses (123). It is also possible that vent pecking may trigger the onset of salpingitis and perhaps egg peritonitis (282).

If the feathers or tissue are severely damaged, hemorrhage may occur. Appearance of blood on the exposed skin may induce more pecking and lead to the death or culling of the bird. Cannibalism is the most severe outcome of both feather and vent pecking (371).

Etiology

Individual and strain differences in the incidence of feather pecking suggest a genetic component. Light breeds of the Mediterranean class (e.g., white leghorns) have been much more prone to feather pecking than the heavier breeds of the American and Asiatic classes (381). Studies in feather pecking behavior have demonstrated a genetic heritability of this trait (210). Some researchers have found links between feather pecking and feather pigmentation (189). Cannibalism is reportedly more common in the modern brown hybrids than in the white layer lines (327), but no estimates of heritability of cannibalism have been developed (210).

There is an ongoing debate regarding the causes of feather pecking. Feather pecking may be redirected foraging behavior (food searching and food consumption) as well as dust bathing (322, 327). Other possible causes of feather pecking may be fearfulness and stress (33, 99, 264), or lack of synchronization of activity within the group (304, 305). Some conditions reported to stimulate feather pecking are bright light, pelleted or compressed feed, absence of foraging materials, high-density rearing systems, nutritional and mineral deficiencies, and irritation from external parasites (99). Feather pecking also may be related to accelerated sexual maturity and increased egg production (52, 172). Feather pecking is more common in females than males (172), and the presence of males is an important factor in reducing the problem (123).

Because feather pecking and cannibalism tend to reoccur within the same group or in adjacent cages, it is recognized to be a learned behavior. Increased group sizes in larger cages or floor systems can increase the risk of cannibalism and feather pecking (210). Feather pecking and feather damage are more severe in hens housed in cages than in floor systems, but cannibalism is less likely to occur in hens housed in cages than it is in hens housed in pens (25, 329).

Intervention Strategies

Outbreaks of feather pecking and cannibalism occur unpredictably in birds, despite the measures taken to reduce their risk. Beak trimming is performed as a means to minimize injury caused by feather pecking and cannibalism. However, there is an increasing public concern that beak trimming is associated with pain. It has been

banned in some countries and bans are under consideration in others (210). Plastic devices have been used in preventing feather pecking in game bird rearing (13, 104), but these devices are not satisfactory for use in chickens (328).

Feather pecking and cannibalism can be prevented by providing adequate diet, replacing mash diets with pelleted feed, rearing the birds on floor litter rather than slats, reducing light intensity, providing perches as a refuge for pecked birds, and avoiding overcrowding (175, 327). Environmental enrichment with substitute pecking devices allows the birds to exercise their natural behavior and reduce pecking among birds (176). Substitute pecking devices that can increase social pecking, such as plastic rods or shoelaces, and systems that may be ignored by birds, such as beads or motorized devices, should be avoided. Simple devices such as hanging white or yellow strings are particularly attractive stimuli to chickens (177). Inclusion of oat hulls and other insoluble dietary fiber in diets decreases the incidence and severity of feather pecking (148). Access to perches and substrate for foraging and dust bathing during rearing can reduce feather pecking and cannibalism during lay (372).

Dermatitis

Erosive lesions affecting the skin on the plantar surface of the feet and the posterior surface of the hocks, thigh, or breast overlying the sternum have been recognized in turkeys and broiler chickens. Breast, thigh, hock, and even footpad conditions have been an important cause of downgrading of chicken and turkey carcasses (124, 242, 337). The relationship with animal welfare and performance are other important reasons for monitoring these lesions. Depending on the location, these skin lesions have been called by different names. Lesions in the footpads are known as pododermatitis or footpad dermatitis (337), whereas lesions on the breast are known as breast burn in chickens (127) and breast buttons in turkeys (124). Ulcers and erosions of the skin covering the thigh and hip of broiler chickens have been described as scabby hip (147, 290).

Clinical Signs and Pathology

Dermatitis on the foot and hock appear as dark black scabs filling ulcers on the plantar footpad (Figure 30.23), digits, and caudal tibiotarsus–tarsometatarsus joint (127, 230). In turkeys, early changes of pododermatitis occur during the first week of life and become more severe over a short period of time; by 6 weeks of age the changes typical of pododermatitis are fully developed (240). Early changes include enlargement of foot scales, cracks, abrasions, and a superficial scab, eventually proceeding to a deep ulcer. Histologic lesions include defective keratin in the stratum intermedium, particularly



Figure 30.23 Severe pododermatitis in a 16-week-old turkey. Bar = 1 cm.

adjacent to the ulcer, and infiltration of heterophils in adjacent epidermis. The center of the lesion is occupied by a necrotic mass of cellular debris, which may enclose plant material and bacteria. The base of the mass is underlined by heterophils and often macrophages and a line of giant cells. Many birds also have similar ulcers filled with black scabs on the posterior of the hock and on the breast.

Breast lesions in turkeys are characterized by a granulomatous response with no giant cells, and connective tissue proliferation below the ulcers (124), probably related to the chronicity of the lesion. Scabby hip syndrome is characterized by ulcers and erosions covered by scabs on the skin of the thigh of broiler chickens associated with femoral head degeneration (290) and high flock density (147).

Pathogenesis and Etiology

Skin lesions have been associated with poor litter conditions, nutrition, and genetics.

Field outbreaks of contact dermatitis have been associated with poor litter conditions (127), wet litter (141,

229, 230), and coarse bedding materials (156, 259). Cool temperatures prior to 12 weeks of age increased the incidence of breast buttons, whereas no association was found with the feathering condition (259). An epidemiologic study (242) found that dermatitis lesions were more frequent with increased stocking density, increased age, particular feeds, male birds, and winter. In meat-type chickens, many of these skin lesions have been associated with poor structural integrity of the skin caused by differences in collagen structure, known as oily bird syndrome (298).

Supplementation with biotin, riboflavin, pantothenic acid, and sulfur amino acids that have been shown to affect the structural components of the skin did not significantly reduce pododermatitis (337). Incidence of pododermatitis has also been reported to be related to hatchery conditions (63, 67).

Intervention Strategies

Improvements in litter management and the use of nipple drinkers in poultry houses have contributed to reducing the incidence of these conditions (243). Improved ventilation and floor drainage allow for higher stocking densities in meat chicken without increasing the incidence of pododermatitis; on the other hand lower stocking densities is needed in order to reduce the frequency of pododermatitis (232).

Diseases and Conditions of Other Systems

Reproductive System

Cystic Right Oviduct

In the female chicken embryo, 2 Müllerian ducts start to develop into oviducts. The left duct develops into a functional oviduct, whereas the right duct regresses. If this regression is not complete, partial development will result in a cystic right oviduct. Cystic right oviducts are common incidental findings in postmortem examination of chickens. They vary in size from small, 2 cm diameter, elongated cysts to large, fluid-filled sacs up to 10 cm or more in diameter (Figure 30.24). Small cysts are of little consequence, but large cysts compress the abdominal viscera. The large sacs can result in a bird with a pendulous abdomen and should be differentiated from ascites.

Abnormal Eggs and Depressed Production

Poor egg quality and depressed egg production are common problems causing great economic losses to the poultry industry. They can be caused by a multitude of factors involving nutrition, management, environment, and disease.

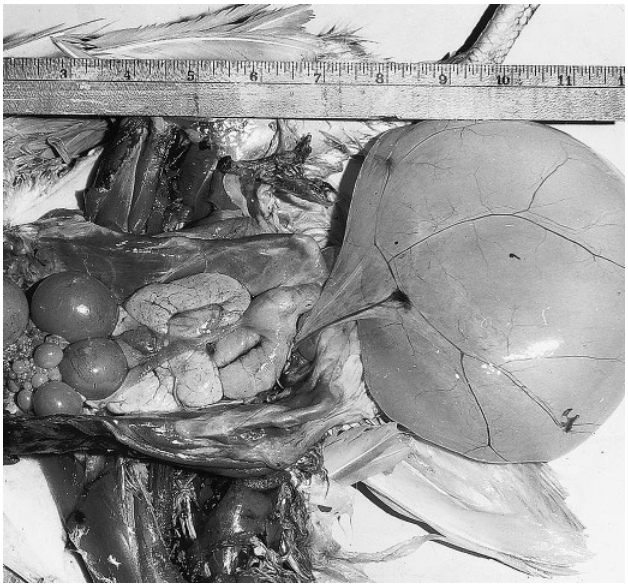


Figure 30.24 Cystic right oviduct in a chicken. (M.C. Peckham)

In some birds, soft-shelled eggs or fully formed eggs may be found in the peritoneal cavity (Figure 30.25). This problem is commonly known as internal layer. This indicates that the yolk progressed normally through the oviduct to a certain point and then reverse peristalsis discharged the egg into the body cavity. A bird with a large accumulation of eggs in the peritoneal cavity may assume a penguin-like posture.

Egg-bound is a condition in which an egg is lodged in the cloaca but cannot be laid. It may result from inflammation of the oviduct, partial paralysis of the muscles of the oviduct, or production of an egg so large that oviposition is physically impossible. Young pullets laying an unusually large egg are more prone to this problem. Prolapse of the oviduct, usually along with the cloaca, may be seen as a sequela of dystocia.

Prolapse

Cloacal prolapse may involve the intestines, reproductive tract (oviduct or phallus), and ureter(s). The prolapsed tissue has a smooth surface and is shiny and congested. Cloacal prolapse may be associated with diarrhea, impaction, or nutritional imbalances. It occurs commonly in young ostriches and it has been associated with *Cryptosporidium* spp. infection (17). In laying hens, cloacal prolapse may result from oviposition. In poultry, picking of the prolapsed tissue may result in cloacal rupture and evisceration.

Phallus prolapse is occasionally seen in anseriformes and ratites and associated with infection or immunodepression. The exact cause of prolapse is unknown. In geese, phallus erosions and prolapse have been associated with *Neisseria* spp. infection (see Miscellaneous and Sporadic Bacterial Infections in Chapter 23). In ratites,

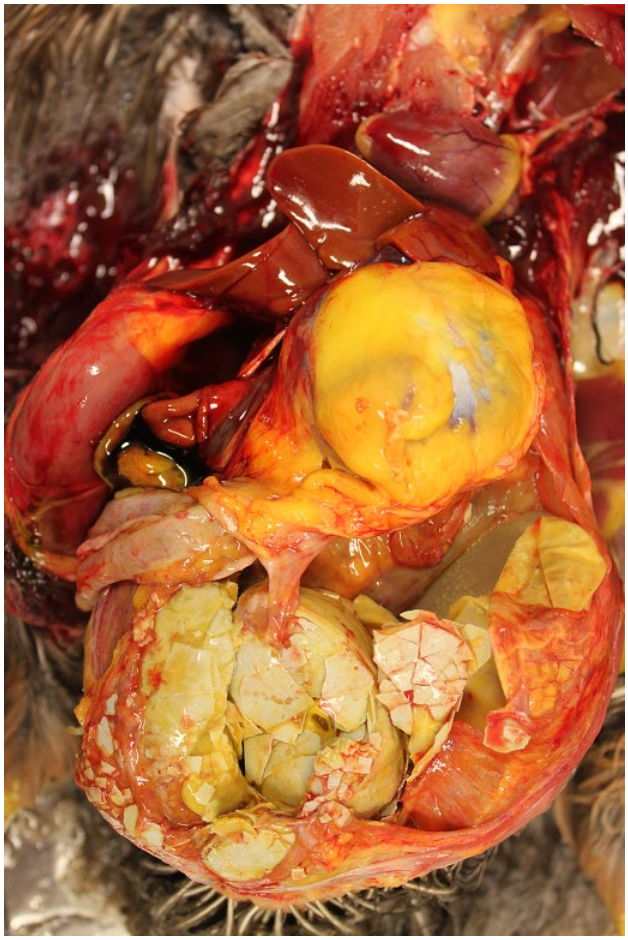


Figure 30.25 Internal layer. Both soft-shelled eggs and fully formed eggs are in the abdominal cavity.

phallus prolapse usually occurs at the end of the breeding season and after sudden weather changes (41, 159). One report associated *Cryptosporidium* spp. infection with cloacal and phallus prolapse in ostriches (288). Frostbite or bacterial infection may occur as a sequelae of phallus prolapse.

Special Senses

There are many conditions and diseases that affect the eye, which have been reviewed by Shivaprasad (342). Diseases involving the ear in birds in general have not been well studied; but they do occur. Common conditions that affect the ear of poultry have been reviewed recently by Shivaprasad (340).

Developmental Anomalies of the Eye

Retinal dysplasia or abnormal development of the retina is most probably inherited as an autosomal recessive trait. It has been described both in layer and broiler commercial chickens (342, 344). The disease is present in chicks when they are a few days old and becomes apparent when they are 5 or 7 days old, but it becomes more evident when the birds are about 2 months of age. Clinically, the chicks with retinal dysplasia are smaller than their counterparts, wander aimlessly, and are unable to find feed and water. Ophthalmoscopic examination of the eyes in these chicks showed lack of papillary reflexes and normal posterior and anterior segments. The incidence of blindness is generally less than 1%, and postmortem examination did not reveal any gross lesions in the eyes. Microscopic examination of the eyes revealed degeneration of photoreceptors (rods and cones) in the earlier stages followed by rosette formation, disorganization of retinal layers, synechiae of the retina, reactive and proliferative retinal pigmented epithelial cells, and inflammation of the choroid in later stages. If the birds survived for several weeks, progressive changes such as retinal detachment, cataract formation, fibrosis, and metaplastic changes to cartilage were seen.

Several other developmental anomalies, including albinism (323), keratoglobus or corneal ectasia (201), microphthalmia (98), cyclopia, triple eyes, buphthalmia, anophthalmia, corneal edema, and optic nerve hypoplasia (338, 342) are described in young chicks and turkey poults. These defects can have genetic origins, but most are the result of suboptimal incubation conditions (342).

Cataracts

The opacification of the lens is referred to as cataracts. It is uncommon among most domestic poultry. The condition has been described in chickens, turkeys, and quail. Cataracts might be caused by noninfectious etiologies such as suboptimal incubation conditions, heredity, old age, and nutritional deficiencies (e.g., vitamin E), as well as infectious conditions including viral diseases such as avian encephalomyelitis. Lens opacity is normally bilateral, resulting in blindness. Microscopically the lesion is characterized by degeneration of lens fibers, epithelial hyperplasia, formation of bladder cells, and liquefaction in advance stages.

Eye-Notch Syndrome

Eye-notch syndrome refers to a widespread lesion in the eyelid of caged layers. The condition appears to start as a small scab or erosion on the lower lid, which develops into a fissure with a tag of flesh attached to 1 side. Microscopically, it is characterized as blepharoconjunctivitis. The significance and cause of the condition are unknown.

Acknowledgment

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Mycotoxicoses

Frederic J. Hoerr

Summary

Agent, Infection, and Disease. Mycotoxins are toxic metabolites of fungi that colonize and invade cereal grains and poultry feed. Mycotoxins are ubiquitous in poultry feed worldwide. Well characterized mycotoxins include the ergot alkaloids, aflatoxins, trichothecenes, and other mycotoxins produced by *Fusarium*, ochratoxins, oosporein, citrinin, and fumonisins. Mycotoxins cause losses through reduction of growth, fertility and egg production, and the quality of poultry food products. A spectrum of pathology occurs that may target liver, kidney, immunity, hematopoiesis, reproduction, digestion, integument, and the musculoskeletal system. The public health threat from mycotoxin residues in poultry meat and eggs is low for transient or low-level exposure, but increases with prolonged, higher-level exposure.

Diagnosis. A mycotoxicosis can be suspected when the flock history is suggestive of overt intoxication, or when production parameters are substandard without an obvious cause. A definitive diagnosis requires identification and quantification of mycotoxins. Detection methods include chromatography, mass spectrometry, fluorometric tests, and enzyme-linked immunosorbent assays.

Interventions. Treatment primarily involves removing or diluting toxic feed with unadulterated feed. Substandard management and ongoing disease are especially detrimental to poultry stressed by mycotoxins. Prevention focuses on reducing the mycotoxin risk in feedstuffs and feed manufacturing through practices that prevent mold growth and mycotoxin formation. All components of the feed system require good hygiene to prevent mycotoxins from forming in crusted moldy feed or feed ingredients. Mycotoxin prevention in feed also involves antifungal agents to prevent the growth of fungi, and detoxification of mycotoxins using binding agents, organic compounds from fermentation, plants, and essential oils, and microbial feed additives.

Introduction

Definition and Synonyms

A mycotoxicosis is a disease caused by a toxic metabolite of a fungus (mycotoxin). Hundreds of mycotoxins are recognized and characterized chemically and biologically, and many occur in poultry feed. The historical importance of mycotoxins in poultry health and the foundational clinical reports and research were detailed and referenced in previous editions of this text (38, 39). This edition only references more recent information.

The mycotoxins best characterized for causing problems in poultry are the ergot alkaloids, aflatoxins, trichothecenes and other mycotoxins produced by *Fusarium*, ochratoxins, oosporein, citrinin, and fumonisins.

Occurrence

Mycotoxins are detected worldwide in animal feeds and feed ingredients, with regional differences in the relative prevalence of the various toxins (25). Mycotoxins are detected in most survey samples tested and multiple mycotoxins may occur in a sample. A survey of 8,452 feedstuff samples from 63 countries detected deoxynivalenol (DON) (81% of samples), fumonisin (71%), zearalenone (52%), aflatoxin (26%), T-2 toxin (19%), and ochratoxin (18%), with 76% of the samples testing positive for more than 1 toxin (60). Many are detected below the respective regulatory threshold concentration, but higher levels capable of causing overt intoxication occur. Another survey method is culturing fungi from feedstuffs and assessing for mycotoxigenic potential (7, 49). A survey of 100 poultry feed samples detected toxigenic fungi capable of producing aflatoxin (3%), cyclopiazonic acid (76%), fumonisin (86%), griseofulvin (42%), moniliformin (18%), ochratoxin A (5%), penitrem A (30%), and sterigmatocystin (10%) (49).

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Economic Significance

Surveys confirm that mycotoxin exposure is common but losses attributable to intoxication are difficult to document. The economic benefit of mycotoxin prevention and amelioration programs reinforces the conventional wisdom that mycotoxins often impact poultry health and well-being. Mycotoxin losses resulting from lowered production efficiencies are attributable to the presence of the toxin, the reduced market value of the affected feedstuff or poultry commodity, and secondary effects on production, such as disease interactions (12).

Public Health Significance

Poultry products present a low risk for human toxin exposure because mycotoxins have low distribution to skeletal muscle, the dominant product in poultry food supply. Mycotoxin distributions to liver, kidney, and eggs are of greater concern. Mycotoxins are rapidly metabolized and excreted in urine or feces. Residues in liver and kidney are transient and in low concentrations relative to the exposure dose. Chronic exposure to mycotoxins causes changes in color and size of liver and kidney, a visible indicator that warrants removal from the food chain at slaughter.

In modern production, mycotoxin exposure of poultry is self-limiting because production economics requires that the problem must be addressed. Of concern are aflatoxin residues in poultry tissues occurring at higher levels in areas with no regulatory limits on aflatoxin levels in poultry feed, and thus pose a risk to consumers' health (40). Both poultry and poultry farmworkers have a risk of airborne mycotoxin exposure (85).

The US Food and Drug Administration issues compliance and guidance documents for mycotoxins, setting maximum concentrations for feed and commerce to protect the food supply (2). The European Commission Recommendations and Directives specify maximum tolerable levels of mycotoxins that may be fed in consideration of poultry health and residue avoidance in the food supply (11, 15, 47).

Ergotism

Etiology and Toxicology

Ergotism is characterized by vascular, neurologic, and endocrine disorders, and is caused by toxic alkaloids produced by *Claviceps* spp., which attack cereal grains. Ergot is detected in rye and other cereals and weed seed in cooler grain-rearing climates worldwide. A maximum level of 1000 mg/kg of rye ergot (*C. purpurea*) sclerotia has been established by the European Commission for feed containing unground cereals (15), whereas in the United States, wheat containing 0.05% ergot is classified as "ergoty" (3).

Claviceps purpurea is implicated because of its wide host range among cereals. The mycotoxins form in the sclerotium, a visible, hard, dark mass of mycelium that displaces grain tissue. In the normal cycle, the sclerotium falls to the ground, germinates, and produces spores that infect the flower of the new crop, and the cycle repeats. The sclerotium is channeled into the food chain during harvest.

Lysergic acid is the chemical skeleton of the ergot alkaloids, which produce convulsive and sensory neurologic disorders, vasoconstriction and gangrene of extremities, and altered neuroendocrine control of the anterior pituitary gland. Pelleting feed increases the toxicity of ergot through increased liberation of toxins.

Natural Disease

Ergotism in poultry presents as decreases in spontaneous activity, feed intake, growth, and egg production, and diarrhea. Vesicles and ulcers develop on the beak, comb, wattles, and toes, and the combs and wattles can become atrophied and disfigured. Young poultry are more sensitive.

Experimental Disease

Ergotism causes reductions in appetite and growth, and mortality, with poor feathering, nervousness, and loss of coordination sometimes seen. At necropsy, the heart and liver can be enlarged. Broilers are more sensitive to ergot than leghorns (10, 54).

Metabolism and Residues

Ergotamine tartrate accumulates in trace amounts in broiler tissues when fed at high concentrations. About 5% of the alkaloid is excreted unchanged and the remainder is excreted as a mixture of metabolites.

Fusarium Mycotoxins

Incidence and Distribution

The genus *Fusarium* produces many mycotoxins injurious to poultry through chemical and radiomimetic injury, cardiac toxicity, and skeletal, digestive, and reproductive disorders. *Fusarium* toxins that are commonly detected in grain and feed include trichothecenes, fumonisins, zearalenone and moniliformin, beauvericin, enniatins, and fusaproliferin occurring alone and in combinations (29). Fusarial mycotoxins may occur in combination with aflatoxin or ochratoxin.

Trichothecenes

Etiology and Toxicology

Trichothecene mycotoxins are produced by common soil and plant fungi found worldwide, including *Fusarium* and its perithecial stages, *Calonectria* and *Gibberella*, and other genera. *Fusarium* produces about one-half of the more than 100 trichothecenes, with greatest toxin production at high humidity in a temperature range of 6 °C –24 °C.

Trichothecenes have a tetracyclic sesquiterpene nucleus with a characteristic epoxide ring. Poultry are usually exposed to nonmacrocyclic trichothecenes, which includes type A trichothecenes (T-2 toxin, neosolanial, diacetoxyscirpenol [DAS], and others) and type B (nivalenol, DON, vomitoxin, fusarenone-X, and others). Toxicity resides in the epoxide ring, which is stable during prolonged storage or when heated. Trichothecenes damage structural lipids and inhibit the synthesis of protein and DNA, and many are chemical irritants.

T-2 toxin, DAS, DON, and nivalenol occur in feedstuffs worldwide, including corn and cereal grains and feed (74). DON was the most prevalent mycotoxin detected in a global mycotoxin survey (60) and occurs naturally along with zearalenone, aflatoxin, and other mycotoxins. Grains contaminated with DON may be diverted to poultry feeds because DON has low toxicity for poultry in comparison to swine, which experience feed refusal and emesis.

Natural Disease

Trichothecene mycotoxicosis reflects chemical burn and radiomimetic-like toxicity, that are expressed as feed refusal, necrosis of oral mucosa and skin in contact with the toxins, acute digestive disease, and depressed bone marrow and immune functions. Decreases in egg production and shell quality, neurological signs, malformed feathers, rickets, gout, and generalized hemorrhages can develop. Recovery occurs when an unadulterated diet is provided.

Experimental Disease

Experimental trichothecene mycotoxicosis in poultry has reproduced essential features of the natural disease using purified type A toxins administered alone or in combination, and using toxigenic fungal cultures. Purified trichothecenes are less toxic than equivalent concentrations in more complex toxigenic fungal cultures.

Collectively, these toxins cause feed refusal, impaired growth and reproductive capability, and whole-body pathology. The spectrum of lesions includes: chemical injury to skin and alimentary mucosa; radiomimetic injury to bone marrow, lymphoid tissues, gastrointestinal tract, and feathers; hepatosis; and thyroid alterations. Neurotoxicity occurs inconsistently. Quail are more resistant than chickens, and ducks are more sensitive, but all develop similar signs and lesions.

Erosive and exudative injury to the oral mucosa occurs as focal yellow oral plaques that progress to confluent yellow-gray, raised accumulations of exudate. Underlying ulcers are located near major salivary duct openings on the palate, tongue, and floor of the mouth. Thick crusts accumulate along the interior margin of the beak (Figure 31.1). Histopathology reveals mucosal necrosis and ulceration, with superficial crusting and submucosal inflammation.

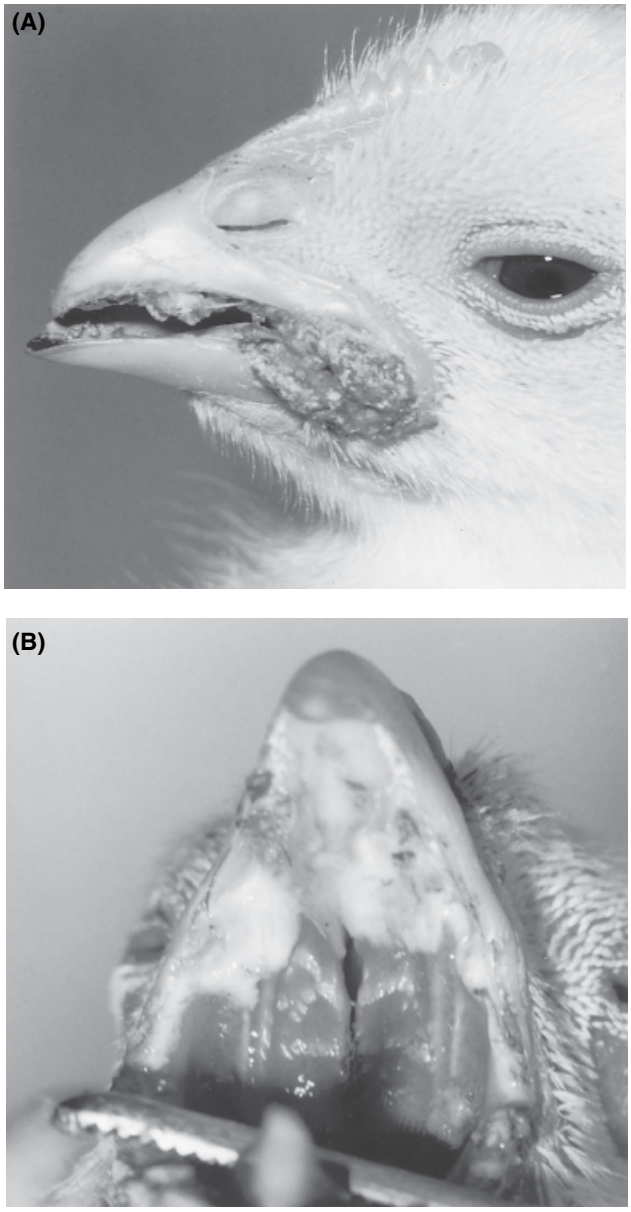


Figure 31.1 Fusariotoxicosis. Trichothecene mycotoxins cause chemical irritation of the upper digestive tract mucosa. (A) Crusts at the beak commissure of a broiler chicken fed diacetoxyscirpenol for 8 days. (B) Beak and palate ulceration and crusting in a broiler chicken following 14 days consumption of diacetoxyscirpenol (4 mg/kg diet).



Figure 31.2 Feathers from a chicken fed T-2 toxin for 24 days (right) are narrow because of radiomimetic injury to the developing barbs; control (left).

The systemic histopathology of acute toxicosis by type A trichothecenes (T-2 toxin, DAS) occurs as rapid necrosis and depletion of lymphoid and hematopoietic tissues and then a rapid recovery. The liver and gallbladder develop necrosis and hemorrhage, and mild proliferation of bile ductules. Mucosal necrosis occurs in the proventriculus, gizzard, and intestine. The feather epithelium undergoes necrosis which leads to feather malformation.

Extended toxicity adversely affects body weight, skin pigmentation, and feathers, bone marrow (with anemia and leukopenia), lymphoid organs, liver, and thyroid gland (Figures 31.2 and 31.3). Coagulation can be impaired and serum vitamin E concentration is decreased. Egg production and hatchability are decreased relative to the toxicity of the different trichothecenes. Immune responses are depressed and associated with increased severity of concurrent infectious diseases (16, 30, 91).

Deoxynivalenol and nivalenol (type B trichothecenes) are less toxic to poultry, affecting digestive and lymphoid tissues, but at doses higher than those that cause feed refusal and emesis in swine. DON causes reduced weight gain associated with decreases in the height of the intestinal villi, and alters transport function in lower small intestine (9, 94, 95). A cortisone stress response occurs (4) resulting in lymphoid organ atrophy. Immunosuppression can delay recovery from coccidiosis (31, 32). Gout occurs in laying hens.

T-2 toxin and other trichothecenes are metabolized in the liver and excreted with metabolites mostly in the bile and feces within 48 hours of ingestion. A small amount of T-2 toxin is excreted into the egg and is detectable in yolk and albumen. DON is rapidly eliminated in feces and transmitted to eggs at trace to undetectable concentrations. However, it was detected as a natural adulterant in home-produced eggs (79). DON is not detectable in skeletal muscle.

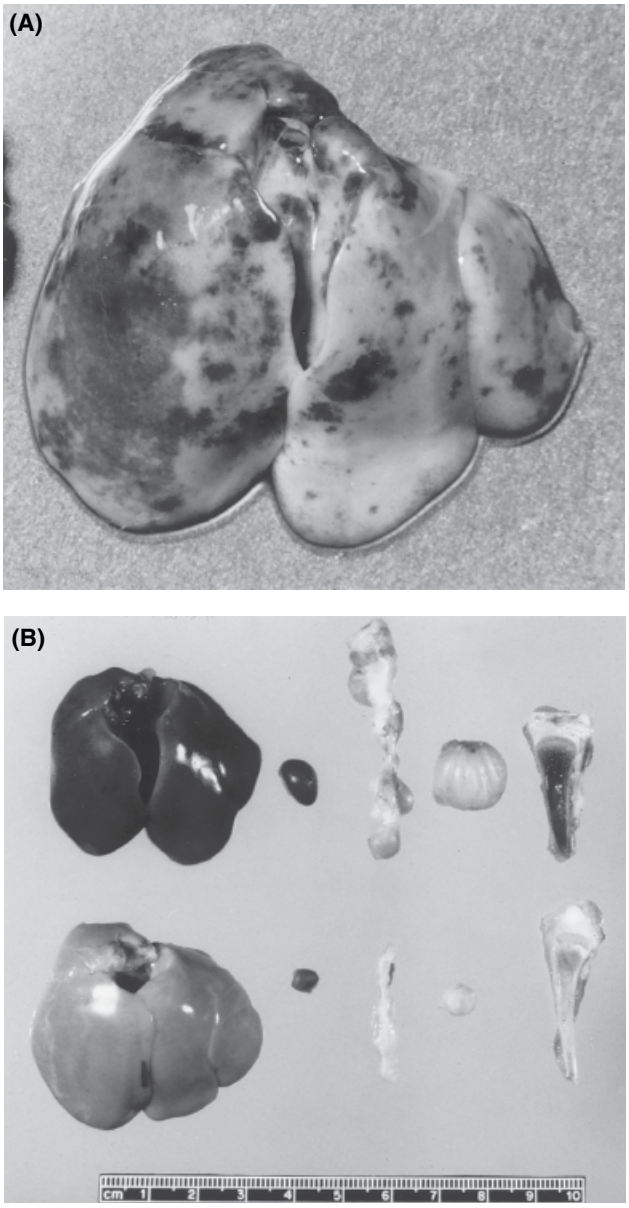


Figure 31.3 Experimental trichothecene mycotoxicosis in broiler chickens. (A) Hemorrhage in the liver occurring 20 hours after consumption of feed mixed with a culture of *Fusarium sporotrichiella* that produced T-2 toxin and neosolaniol. (B) Daily oral doses of T-2 toxin caused yellow discoloration of the liver (top row) compared with control (bottom row). The radiomimetic effects are evidenced by atrophy of the spleen, thymus, and bursa of Fabricius and yellow discoloration of the bone marrow.

Moniliformin

Etiology and Toxicology

Moniliformin is produced by *Fusarium verticillioides*, *F. subglutinans*, and other *Fusarium* spp. and is cardiotoxic and nephrotoxic in poultry. *F. verticillioides* causes ear rot, kernel rot, and stalk rot of unharvested corn and occurs in stored high-moisture shelled corn, soybeans

and other cereal grains. *F. verticillioides* also produces fumonisins, zearalenone, fusariocin A, and other toxic fractions. Purified moniliformin is less toxic than more complex fungal cultures.

Natural Disease

Field reports indicate that *F. verticillioides* (*F. moniliforme)* and moniliformin mycotoxicosis is problematic but verification is lacking. Moniliformin was detected in 26 of 50 (52%) of poultry feed samples from central Europe, nearly all of which also contained fumonisin B1 (48), indicating the potential for intoxication of poultry.

Moniliformin toxicosis presents as reduced rate of lay and delayed peaks in production. Uneven feed consumption occurs along with diarrhea, feed passage, fecal-stained egg shells, and blood smears on egg shells. Contaminated corn can be high in moisture, low in protein, and high in crushing strength, which contributes to larger particle size and maldigestion.

Experimental Disease

Experimental moniliformin toxicosis occurs as cardiac toxicity, ascites, and nephrosis. A combination of fumonisin and moniliformin may cause sudden death associated with decreased blood glucose and immunodepression. Younger chickens are more sensitive than hens.

Lesions are enlargement of the heart, ascites, and digestive and cutaneous hemorrhages and edema. Muscle fiber degeneration and necrosis can be partially alleviated by selenium supplementation. Kidney develops nephrosis with mineralized casts. Liver develops vacuolar degeneration and focal necrosis of hepatocytes; bile duct proliferation and fibrosis occur with ongoing intoxication.

Fumonisins

Etiology and Toxicology

Fusarium verticillioides (F. moniliforme) also produces the fumonisins, which are the cause of equine leukoencephalomalacia (moldy corn poisoning) and porcine pulmonary edema syndrome. Several fumonisins are produced (B1, B2, B3), but fumonisin B1 is the most common. Fumonisins are also produced by other species of *Fusarium*. The toxicity of fumonisin B1 is related to disrupted sphingolipid synthesis.

Natural Disease

Fumonisin and aflatoxin were detected in the feed of hens that developed: black adhesive diarrhea; reductions in feed intake, egg production, and body weight; lameness and increased mortality. The diarrhea was reproducible in chicks and in laying hens fed the suspect toxic feed and with diets spiked with fumonisin.

Experimental Disease

Fumonisin B1 toxicosis occurs as diarrhea, catarrhal enteritis impaired weight gain (Figure 31.4) and feed conversion, and transient decreases in egg production. Poults are more sensitive than chicks, but poultry are

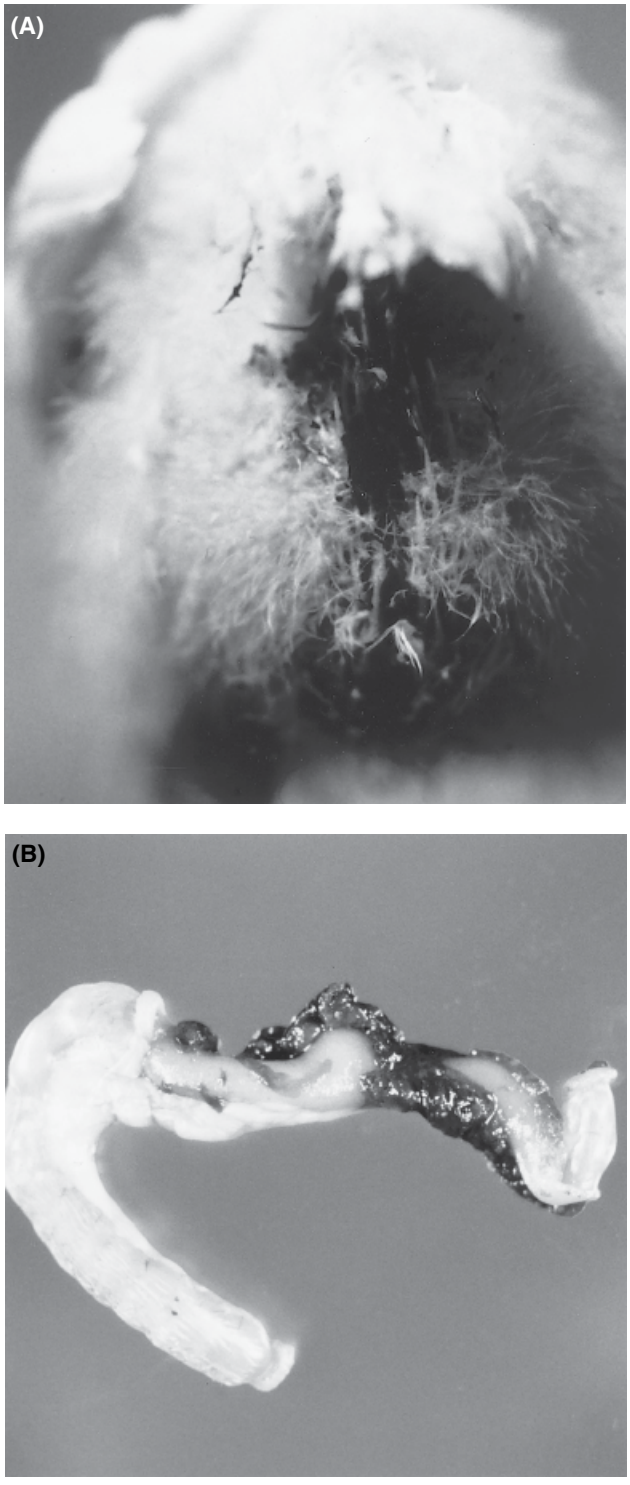


Figure 31.4 Diarrhea (A) and catarrhal enteritis (B) in broiler chickens fed cultures of *Fusarium moniliforme* that produced fumonisin B1.

resistant to fumonisins in comparison with horses and swine. Although concentrations that are toxic to poultry are higher than those likely to occur naturally in grain, *F. moniliforme* remains a threat because it can produce other toxins.

Lesions include: enlargement of the liver and variable enlargement of the kidney, proventriculus, and gizzard; atrophy of lymphoid organs; and rickets. Histologic lesions in the liver are multifocal necrosis, bile ductule hyperplasia and hypertrophy of Kupffer cells, and in the intestine, villous atrophy and goblet cell hyperplasia. Combined fumonisin and moniliformin exposure causes lesions reflective of both toxins, with ascites and enlargement of the heart, and lymphoid depletion. Fumonisin B1 is metabolized by the liver and excreted in feces. Oxidative stress occurs in the liver along with accumulation of sphingosine and sphinganine (64).

Fusarochromanone

Etiology and Toxicology

Long bone deformities in broiler chickens have been induced by cultures of *F. moniliforme, F. roseum*, *F. equiseti, Aspergillus niger* and *A. flavus*. Toxigenic *Fusarium* cultures cause tibial dyschondroplasia from the toxic fraction TDP-1, also known as fusarochromanone.

Experimental Disease

Chicks fed fusarochromanone as toxigenic cultures of *Fusarium* develop dyschondroplasia in the tibial growth plate in 4 days. A partial sparing effect is provided by increasing dietary intake of copper and zinc. *Fusarium* strains that produce fusarochromanone also cause immunodepression.

Zearalenone

Etiology and Toxicology

Grains infected with the fungus *Gibberella zeae* (*F. graminearum*, *F. roseum* "Graminearum") are a source of zearalenone, an estrogenic mycotoxin. Zearalenone and zearalenol occur naturally and zearalenol is more active estrogenically; however, zearalenone is the most prevalent and most studied in poultry. Zearalenone occurs in corn and other grains. Swine are very sensitive and develop estrogenic syndrome and reproductive failure. Chickens tolerate zearalenone better than turkeys or swine and provide an outlet for grains unfit for swine. Zearalenone is less toxic for chickens, but its presence confirms that toxigenic *Fusarium* is present and may be an indicator of mycotoxin risk.

Natural Disease

Zearalenone was detected in feed of broiler breeders that developed a decrease in egg production, accompanied by ascites, cystic inflammation of the oviduct, and lowered serum progesterone.

Experimental Disease

Turkeys are the most sensitive to zearalenone, with reproductive- and sex hormone-sensitive tissues targeted. Leghorn hens are tolerant of zearalenone, but egg production and egg quality may decrease. Lesions include swelling of the cloaca, oviduct enlargement, and reproductive tract cysts. Males experience decreased comb and testicle weight, and lower fertility. Male turkey poults show precocious strutting behavior and develop caruncles, dewlaps, and soft tissue swelling of the vent. Lymphocyte cytokine expression is impaired (86).

Metabolism and Residues

Zearalenone distributes chiefly to liver and gallbladder and is excreted in feces as zearalenone and zearalenol. Egg residues are minimal or undetectable and are restricted to yolk.

Other *Fusarium* Toxins

Fusarium verticillioides and other *Fusarium* spp. can produce depsipeptide mycotoxins beauvericin and enniatins, and the sesterpene mycotoxin fusaproliferin, which have various antibacterial, antifungal, insecticidal, and cytotoxic effects (65, 72, 76, 78). These toxins occur in cereal grains in combination with other fusarial mycotoxins and have potential interactive effects. Previous reports of *F. verticillioides* (formerly *F. moniliforme*) cultures that produced undefined toxins causing immunodepression, reduced egg production, and suspected thiamine deficiency, are suggestive of a role by these toxins.

Aflatoxins

Etiology and Toxicology

Aflatoxins are highly toxic and carcinogenic mycotoxins produced by *A. flavus*, *A. parasiticus*, and *P. puberulum*. Poultry feeds and ingredients are vulnerable to fungal growth and aflatoxin formation. Aflatoxin-producing fungi and contaminated feedstuffs are recognized and regulated worldwide because of the threat to human health and the food animal supply (2, 82).

The chemical structure of aflatoxins is based on 2 fused dihydrofuran rings with moieties, and members are designated by their blue (B) or green (G) color reaction to fluorescent light and by their chromatographic values. Aflatoxins are chemically stable in feed. Aflatoxin B1 is the most toxic and primarily affects the liver. Aflatoxin and several of its metabolites are carcinogenic

and chronic aflatoxicosis results in neoplasia in many species, in the liver and other organs.

Poultry are highly susceptible to aflatoxins with variable species sensitivity. Ducklings and turkey poults are the most sensitive to aflatoxins, quail show intermediate sensitivity, whereas chickens are the most resistant (21).

Natural Disease

Aflatoxicosis occurs in poultry worldwide. The direct and indirect effects of aflatoxicosis include: increased mortality from heat stress, loss of egg production, anemia, hemorrhages, liver condemnations, paralysis, lameness, and impaired growth; nervous signs, impaired ambulation, paralysis and mortality (quail); and immunodepression with increased susceptibility to infectious disease in many species. Cases of concurrent aspergillosis (mycosis) and aflatoxicosis (mycotoxicosis) confirm that *Aspergillus* spp. are a threat to poultry production in the feed, litter, and environment.

The first cases of aflatoxicosis were reviewed in detail in the previous editions of *Diseases of Poultry*. Early accounts of spontaneous aflatoxicosis were subsequently recognized to have toxic contributions from cyclopiazonic acid, sterigmatocystin, and other toxins.

Lethal aflatoxicosis in ducklings occurs as inappetence, reduced growth, abnormal vocalizations, feather picking, purple discoloration of legs and feet, and lameness. Ataxia, convulsions, and opisthotonus precede death. At necropsy, liver and kidneys are enlarged and pale. Chronic cases have hydropericardium and ascites, shrunken firm nodular liver, bile-distended gallbladder, and hemorrhages. Microscopic lesions in liver are fatty change in hepatocytes, proliferation of bile ductules, and fibrosis, accompanied by vascular and degenerative lesions in pancreas and kidney.

Turkeys develop inappetence, reduced spontaneous activity, unsteady gait, recumbency, anemia, visceral congestion and edema, mucoid enteritis, and death. Lethal aflatoxicosis causes dark red and yellow discoloration of the liver (Figure 31.5). Microscopic lesions are vacuolar (fatty) degeneration of hepatocytes, karyomegaly, and necrosis of centrilobular hepatocytes. Chronic toxicity occurs as hepatocyte regeneration, proliferation of bile ductules, reticuloendothelial cell hyperplasia, and degenerative lesions in the heart, kidney, and intestine.

Aflatoxicosis in chickens closely resembles that in ducks and turkeys (Figure 31.6).

Experimental Disease

Aflatoxicosis impairs all important production parameters including weight gain, feed intake, feed conversion efficiency, pigmentation, processing yield, egg production, and male and female reproductive performance.

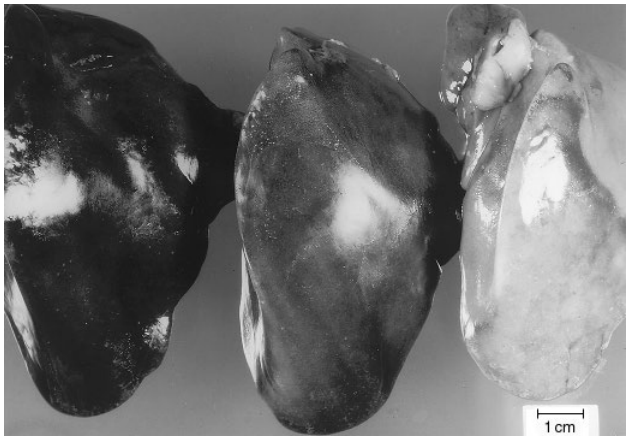


Figure 31.5 Lethal aflatoxicosis in turkeys caused liver discoloration from dark red (left), caused by congestion and necrosis, to yellow (right) from fat accumulation in hepatocytes. Aflatoxin B1 was detected in the feed (200 ppb).

These occur directly from intoxication or indirectly from reduced feed intake and stress.

Susceptibility of poultry to aflatoxins varies among species, breeds, genetic lines, age, and sex. In general, ducklings, turkeys, and pheasants are susceptible, and chickens, Bobwhite and Japanese quail, chukar partridge, and guinea fowl are resistant. The sensitivity of poultry species to aflatoxin B1 is linked to efficiency of hepatic cytochrome P450-mediated bioactivation and deficient detoxification by glutathione S-transferases (69).

The pathology of experimental aflatoxicosis is like the naturally occurring disease. Acute intoxication in ducks causes pale, yellow-green discoloration, and atrophy of the liver, with the left lobe being more affected. Microscopic lesions are fatty degeneration of hepatocytes, necrosis, and hemorrhage. Proliferation of bile ductules develops by the second day and progresses rapidly. The kidney develops membranous glomerulopathy and interstitial fibrosis (Figure 31.7)

Aflatoxicosis in chickens causes yellow to ocher discoloration of the liver, with multifocal hemorrhage and a reticulated pattern on the capsular surface, and white foci as hepatic lipid content increases. Histologic lesions are fatty degeneration of hepatocytes, karyomegaly and prominent nucleoli in hepatocytes, proliferation of bile ducts, and fibrosis. Basophilic, vacuolated, regenerative hepatocytes and inflammation by heterophils and mononuclear cells occur in the portal zones. Turkeys develop less severe fatty change, but bile duct proliferation and nodular regeneration of densely eosinophilic hepatocytes are found.

Aflatoxin causes microcytic hypochromic anemia in young poultry from decreased iron absorption and retention, accompanied by lymphopenia.

Serum biochemistry reflects injury to liver and kidney. The degree of decrease in total protein and albumin and

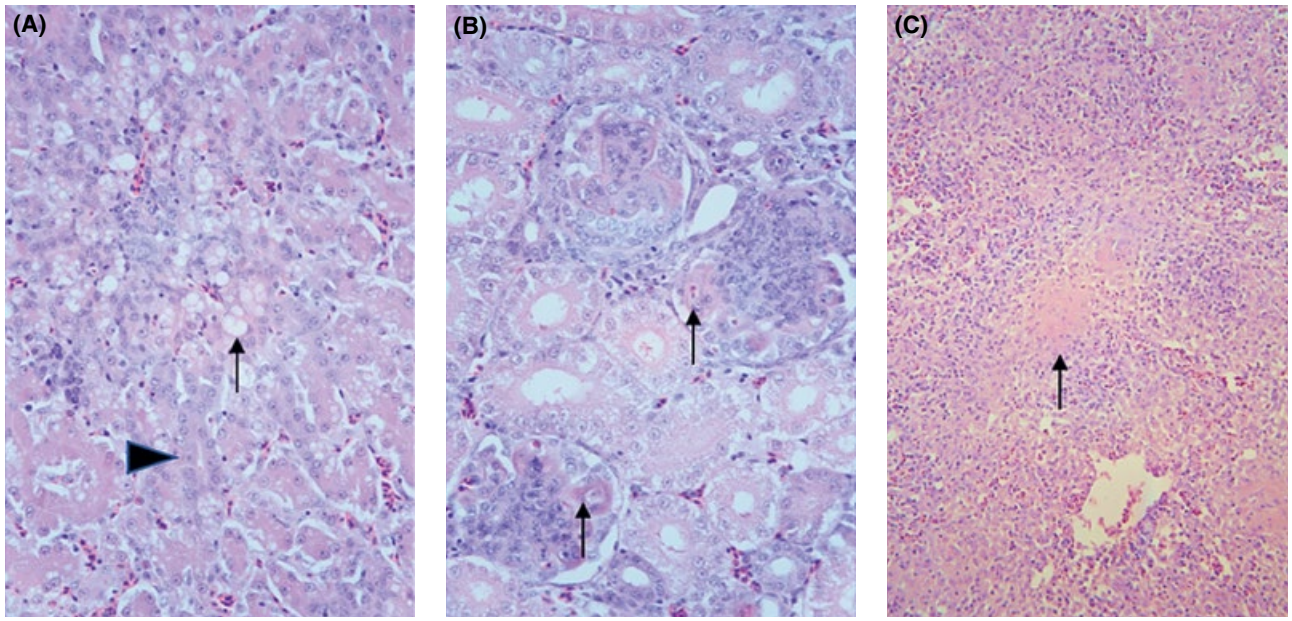


Figure 31.6 Aflatoxicosis in broiler chickens, 14 days of age, that consumed feed that contained aflatoxin B1; 1 sample had 700 ppb aflatoxin B1 and 1 had 1,400 ppb. (A) Liver has coarse vacuolar degeneration (fatty degeneration) of hepatocytes (arrow) and bile duct proliferation (arrowhead). (B) Kidney has membranous glomerulopathy (arrows). (C) Spleen has fibrinoid necrosis and fibrin accumulation in the periarteriolar sheath (arrow).

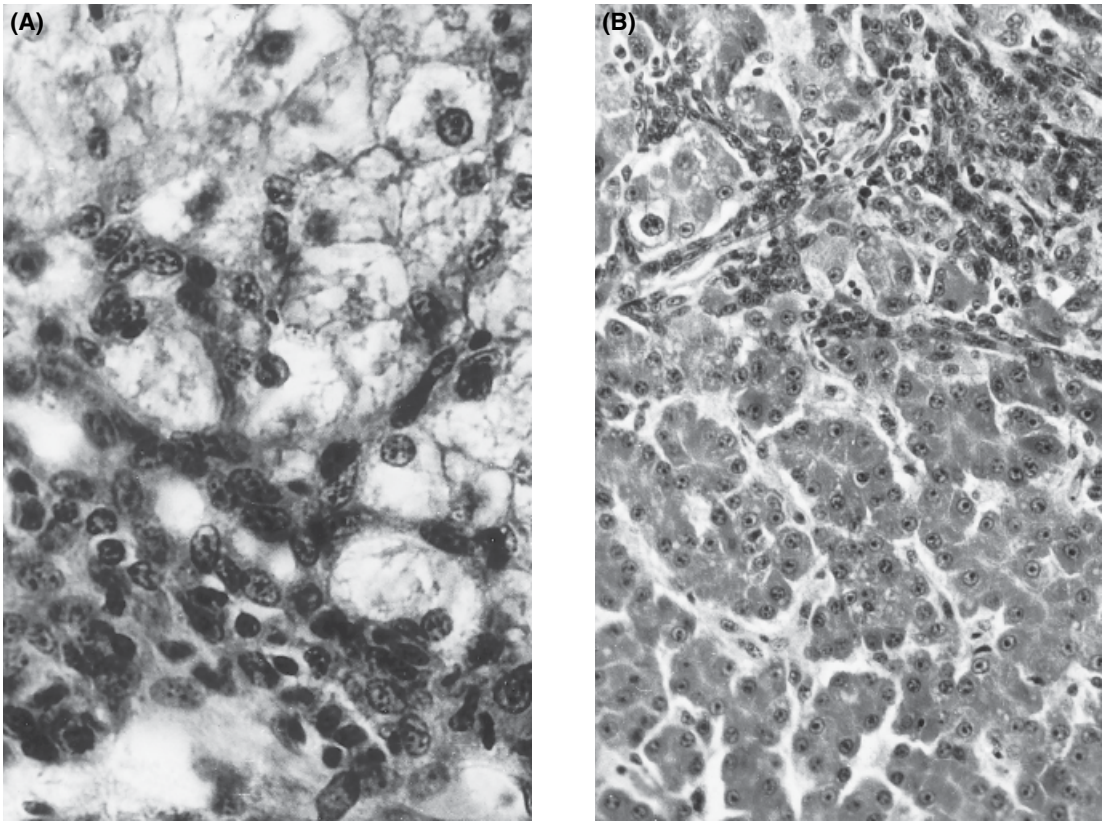


Figure 31.7 Aflatoxicosis in ducks fed toxic peanut meal. (A) Early liver lesion showing degenerative changes in parenchyma and bile duct hyperplasia. (B) Nodular hyperplasia of liver parenchyma and bile duct hyperplasia are present. (C) Kidney. Proximal tubules are dilated, epithelium is undergoing necrosis, and some nuclei have enlarged bizarre forms with prominent nucleoli (arrow).

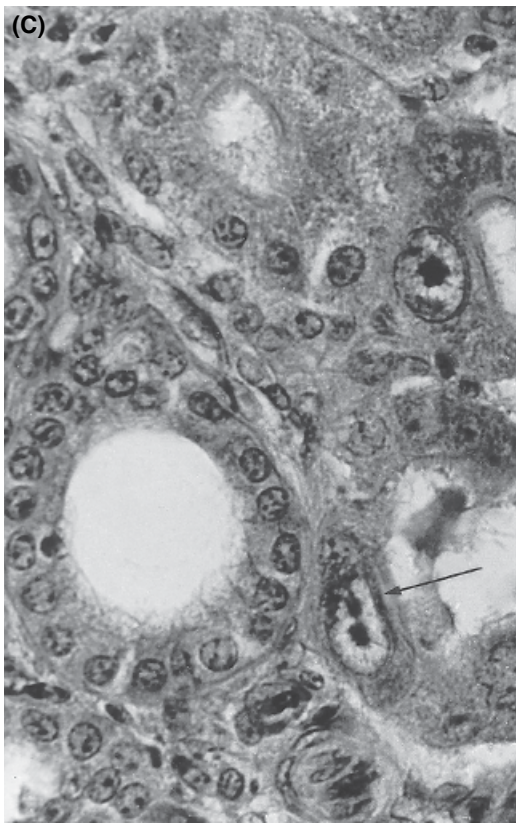


Figure 31.7 (Continued)

the increase in ß-glucuronidase and clotting time are correlated with resistance to aflatoxin.

In hens, chronic aflatoxicosis affects renal function and induces calcium, phosphate, and sodium losses through the urine (57). Aflatoxin promotes bruising during poultry processing by increased capillary fragility and by interfering with prothrombin and other coagulation components.

Skin pigmentation is impaired by aflatoxin by blocking intestinal absorption of carotenoid pigments and sequestration of pigments in the liver.

Aflatoxin interacts with nutritional components. The severity of aflatoxicosis is enhanced by diets low in fat, protein, riboflavin and vitamin D_3, and high in tannic acid. Increasing the crude protein can ameliorate a toxic low-dose exposure to aflatoxin (14). Aflatoxin influences calcium and phosphorus metabolism by altering the metabolism of vitamin D and parathyroid hormone. In broilers, steatorrhea develops from a deficiency of pancreatic amylase and lipase related to pancreatic retention, leading to loss of performance (56). Aflatoxin impairs intestinal crypt epithelium, altering villus:crypt ratio and digestion (5, 100).

Vitamins A, C and E, and alpha-lipoic acid have an ameliorating role in aflatoxicosis through the reduction of oxidative stress (1, 45). Aflatoxin decreases the glutathione peroxidase level (a selenium-dependent enzyme) in the liver (89), and sodium selenite supplementation has a sparing effect on aflatoxicosis (84, 100).

Aflatoxin adversely affects reproduction and hatchability. Males have decreased semen volume, testis weight, spermatocrits, and testosterone concentration, caused indirectly by reduced feed intake. Microscopic examination reveals abnormal spermatozoa and cessation of spermatogenesis in seminiferous tubules.

Loss of hatchability caused by embryonic death is a sensitive indicator of aflatoxicosis; more sensitive than decreased egg production. Egg production is impaired by reducing synthesis and transport of yolk precursors in the liver, resulting in decreases in yolk and egg size. Egg production may require several weeks to return after toxin exposure.

Aflatoxicosis is associated with increased susceptibility to infectious disease, with exacerbated cecal coccidiosis (26), Marek disease, salmonellosis, inclusion body hepatitis, and infectious bursal disease virus. Impaired response to vaccination occurs for Newcastle disease, infectious bronchitis, infectious bursal disease, and fowl cholera.

Aflatoxin-induced atrophy of the major lymphoid organs occurs with apoptosis of lymphocytes (63) and reduced mitogenic responses of B and T lymphocytes. Aflatoxin is toxic for embryonic B lymphocytes and immune dysfunction occurs in progeny of hens exposed to aflatoxin. Cell-mediated immune response, clearance functions of macrophages, and serum complement activity are impaired.

Pharmacological Interactions

Aflatoxicosis decreases the absorption of enrofloxacin and the plasma half-life of ceftiofur and chlortetracycline, and increases tissue residues of enrofloxacin and ciprofloxacin (43, 44).

Metabolism and Residues

Aflatoxins distribute to edible tissues in low concentrations and clear rapidly after nontoxic diets are provided (41). With sustained ingestion, higher toxin residues occur in liver and muscle in a dose-related pattern (40).

Metabolites of aflatoxins concentrate in gizzard, liver, and kidney and are excreted in bile, urine, and feces in 4 days. Aflatoxin B1 metabolizes into conjugated aflatoxins B2a and M1 in the liver, and is further reduced to aflatoxicol. The dominant enzyme responsible for bioactivation and metabolism of aflatoxin B1 is the hepatic cytochrome P450 enzyme group (23, 24, 68, 70). Selenium supplementation increases the percentage of conjugated aflatoxin.

The half-life of aflatoxin B1 in laying hens is about 67 hours, and the feed:egg transmission ratio is about

5000:1. Most aflatoxin is excreted through the bile and intestine, but aflatoxin B1 and aflatoxicol are detectable in ova and eggs for 7 days, or longer (27). Aflatoxin B1 can accumulate in reproductive organs with transfer to egg yolk and albumen, with residues detectable in the yolk sac and liver of hatched progeny.

Ochratoxins

Etiology and Toxicology

Ochratoxins target the kidney and are among the most toxic mycotoxins to poultry. Ochratoxins are produced chiefly by *Penicillium viridicatum* and *A. ochraceus* on cereal grains and feed throughout the world. Ochratoxins are isocoumarin compounds linked to L-b-phenylalanine and are designated A, B, C, and D. Ochratoxin A is the most common and most toxic, and is stable. Some ochratoxin-producing fungi produce other mycotoxins toxic to poultry, including citrinin. Ochratoxin is the major determinant in porcine endemic nephropathy, a chronic wasting disease of swine in Denmark and Ireland. Ochratoxin in combination with fumonisin B1 and penicillic acid is a risk factor in Balkan nephropathy of swine and poultry in Bulgaria (77).

Ochratoxin occurs in cereal grains and feed and may occur with contamination by aflatoxin. High temperature and moisture promote ochratoxin formation in feed.

Natural Disease

Ochratoxicosis presents as clinical diseases and processing plant problems, affecting chickens, turkeys, and geese, stemming from contaminated corn gluten meal, corn, bakery products, and pelleted feed. The clinical signs include reductions in weight gain and pigmentation, enteritis and diarrhea, and intestinal breakage during processing from intestinal fragility.

Lesions include discoloration of kidney and liver, visceral gout, and enteritis. Histologic lesions in the kidney include edema, degeneration of proximal and distal tubules, and interstitial fibrosis.

Experimental Disease

Experimental ochratoxicosis causes feed refusal and reductions in growth, feed utilization, and pigmentation. Combined exposure to ochratoxin and aflatoxin causes synergistic reduction of performance of broilers. Ochratoxicosis causes delayed sexual maturity and reductions in egg production, egg quality, fertility, and hatchability. Embryos develop renal lesions, and hatched progeny have reductions in growth and immunocompetence (98, 99). Ochratoxicosis causes primarily renal disease but also influences hepatic, immunologic, and hematopoietic functions. Poultry are sensitive to ochratoxin and ringneck pheasants and Japanese quail are highly sensitive.

The lesions of acute lethal ochratoxicosis are swelling and discoloration of the kidney and liver, urate accumulation in the ureters, and gout. Histologic lesions occur in the kidney as acute tubular nephrosis with cast formation and interstitial heterophilic inflammation. Vacuolar change and necrosis occur in hepatocytes. The bone marrow has suppression of hematopoiesis, and lymphocyte depletion occurs in lymphoid organs.

In subacute toxicity, the weights of liver and kidney increase. Kidney enlargement is associated with hyperplasia of tubular epithelium, membranous glomerulopathy, and interstitial inflammation. Lymphocytic depletion from primary lymphoid organs may be severe, accompanied by impaired cellular and humoral immune functions (37, 97). Vaccination efficacy is impaired and disease susceptibility is increased for coccidiosis, salmonellosis, and colibacillosis. Defective cortical bone formation caused by osteopenia is described.

The spectrum of histologic lesions in kidney and liver, skeletal muscle, pancreas, and bone, are reflected in serum biochemical changes and decreased renal function tests. Ochratoxin causes anemia, leukopenia, and prolonged coagulation caused by a reduction in clotting factors.

Metabolism and Residues

In chickens, ingested ochratoxin A distributes chiefly to the kidney and to a lesser degree to liver and muscle, and is rapidly eliminated with a half-life of about 4 hours. Kidney and liver are monitored for residues, although bile provides a useful matrix for detection of exposure (6). Residues in kidney may occur in the absence of renal lesions. Residues persist for 4 days or less when toxic diets are replaced. Ochratoxin A distributes to eggs within 3–5 days of feeding, and disappears within 5–6 days after withdrawal of toxic feed (96).

Citrinin

Etiology and Toxicology

Citrinin is a natural contaminant of corn, rice, and other cereal grains and produced by many species of *Penicillium, Monascus,* and *Aspergillus*. Citrinin is detected most frequently in Canada and northern Europe. First purified as a yellow crystalline compound from *P. citrinum* in 1931, its antibiotic properties against staphylococci and other bacteria were recognized before the nephrotoxicity was discovered. Citrinin is one of the causes of yellow rice

mycotoxicosis described in Japan, and is implicated in porcine endemic nephropathy, which also involves ochratoxin. Citrinin is heat sensitive.

Natural Disease

Citrinin mycotoxicosis presents as diuresis associated with wet litter and reductions in weight gain resulting in small, uneven chickens at processing age. The kidneys may be swollen and showed degenerative changes in the tubules.

Experimental Disease

Citrinin is nephrotoxic and causes diuresis in poultry, with turkeys being the most sensitive. Removal of the toxin allows a return to normal renal function. Citrinin acts directly on the kidney to increase urine flow rates and free water clearance with increases in sodium, potassium, and inorganic phosphate excretion.

Citrinin is physiologically active at the level of the renal tubule, but gross and microscopic lesions may be absent or mild. When present, lesions include epithelial degeneration and necrosis in proximal and distal tubules. The liver may have focal necrosis and hemorrhage. Lymphocyte necrosis and depletion in lymphoid tissues, anemia, and leukopenia develop. With ongoing exposure to citrinin, dose-related nephropathy occurs with loss of tubular parenchyma and fibrosis, accompanied by hyperkalemia, metabolic acidosis, reduced blood pH, and base excess.

In broilers, citrinin residues are detectable only in blood and liver. In laying hens with prolonged exposure, citrinin is detectable in muscle, trachea, and egg albumin.

Oosporein

Etiology and Toxicology

Oosporein is a red, toxic, dibenzoquinone metabolite of *Chaetomium* spp. originally extracted from *Oospora coloran*. In poultry, oosporein causes high mortality from gout. Toxigenic cultures of *Chaetomium* spp. are isolated from feeds and grains.

Natural Disease

Oosporein mycotoxicosis has occurred in poultry in both North and South America and is characterized by mortality associated with nephrotoxicity and gout.

Experimental Disease

Experimental oosporein mycotoxicosis causes visceral and periarticular urate deposition (gout) related to impaired renal function and elevated plasma uric acid.

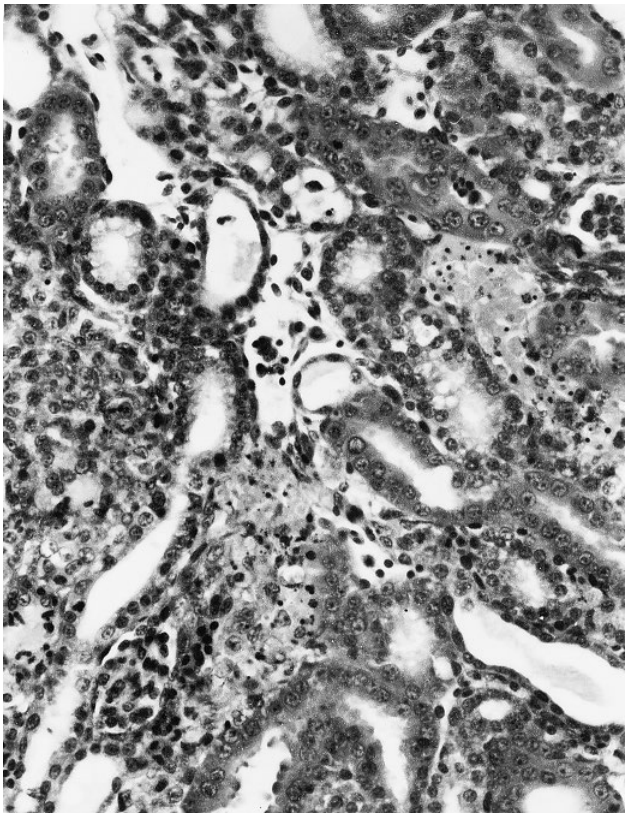


Figure 31.8 Kidney from a chicken fed oosporein for 3 days. The renal cortex has necrosis of proximal tubules.

The proximal tubular epithelial cells are targeted (Figure 31.8). Water consumption increases with fluid and fecal droppings. Feed consumption and egg production decrease at doses that cause nephrotoxicity and gout.

Lesions include dehydration, swollen pale kidneys, and extensive visceral gout. This may be accompanied by focal necrosis in the liver, distention of the gallbladder with bile, increased diameter of the proventriculus associated with mucosal exudate, and bile staining of the gizzard and intestinal contents. Articular gout develops with ongoing toxicity. Plasma uric acid concentrations are increased and serum chemistry changes are reflective of renal toxicity.

Other Mycotoxins

Cyclopiazonic Acid

Cyclopiazonic acid is an indole-tetramic acid neurotoxin produced by some of the same strains of *A. flavus* that produce aflatoxins and by some *A. oryzae* strains (13). Some features of turkey "X" disease in the United Kingdom in 1959, notably enteritis and opisthotonus, were not fully accountable to aflatoxin and are explained by the presence of cyclopiazonic acid, which was identified in stored

samples from the original episodes. Ten of 45 strains of *A. flavus* isolated from feed produced both cyclopiazonic acid and aflatoxin in culture. The toxin is also produced by *Penicillium* spp. and is a contaminant of meats, cheeses, peanuts, corn, and millet.

Cyclopiazonic acid affects weight gain, feed conversion, and male reproduction, and causes mortality. It has additive toxicity with aflatoxin and T-2 toxin. Lesions occur in the proventriculus, gizzard, liver, and spleen. Mucosal necrosis occurs in the gizzard, and the proventriculus is dilated by thickened mucosa caused by hyperplasia and ulceration. Yellow foci of necrosis may occur in the liver and spleen and there is generalized lymphoid depletion. Oxidative stress is measurable in liver and kidney through increased organ-specific enzymes at cyclopiazonic acid exposures too low to affect body weight (55).

Cyclopiazonic acid residues occur in chicken muscles at 14% of an oral dose, 48 hours after dosing, and in eggs, with higher concentrations in albumen.

Sterigmatocystin

Sterigmatocystin is a biogenic precursor to aflatoxin B1, and although less toxic, is produced in higher concentration and is hepatotoxic and hepatocarcinogenic. It occurs less commonly than aflatoxin and is associated with visibly moldy products. Sterigmatocystin is produced on feed and grain by *Aspergillus* spp. and other cereal fungi (83), and detected in North America, Europe, and Japan.

Sterigmatocystin mycotoxicosis occurs in hens as decreases in feed intake and egg production, and loss of pigmentation in brown-shelled eggs, with lesions of pale fatty liver with hemorrhages. Experimental toxicity affects liver, pancreas, lymphoid organs, and kidney. Histologically, the liver shows necrosis of periacinar hepatocytes with heterophil inflammation and hemorrhage. Pancreas has cytoplasmic vacuolation from zymogen-granule loading in exocrine cells. Lymphocyte necrosis and depletion occur in lymphoid organs, accompanied by leukopenia. Kidney has necrosis of tubular epithelium. Serum chemistries reflect these target organ lesions.

Other Mycotoxins and Toxigenic Fungi

Rubratoxins A and B are hepatotoxic mycotoxins produced by *P. rubrum* and *P. purpurogenum* and were recognized before aflatoxicosis was defined. In 1958, an investigation of poultry hemorrhagic syndrome yielded these toxigenic fungi from feed and litter of affected chickens, as well as *A. flavus* and *P. citrinum*, producers of aflatoxin and citrinin, respectively. In chicks, toxigenic cultures of *P. rubrum* and *P. purpurogenum* caused bloody diarrhea, hemorrhages in muscles and viscera, and erosions and free blood in the proventriculus and gizzard. Purified rubratoxin (20% A, 80% B) causes decreased growth, hemorrhages in multiple organs, liver enlargement, and atrophy of lymphoid organs.

Penicillic acid is a metabolite of *Penicillium* spp. and *Aspergillus spp*. and is important because of high concentrations in corn and poultry feed. Penicillic acid has low toxicity to poultry when fed solely at concentrations likely to occur naturally, but growth and feed conversion are affected when fed with low doses of aflatoxin.

Alternaria spp. are common in corn and can produce multiple toxins, including tenuazonic acid. Early investigations of poultry hemorrhagic syndrome revealed marked toxicity by a culture of *Alternaria*. Tenuazonic acid is moderately toxic causing hemorrhage in multiple tissues, and hemorrhage and erosions in the proventriculus and gizzard.

Patulin is produced by several species of *Aspergillus*, *Penicillium*, and *Byssochlamys*, and patulin-producing *Penicillium* has been isolated from chick feed. Patulin produces watery crop content, acute ascites, and gastrointestinal hemorrhage, misshapen eggs, and reduced calcium content in the shell.

Kojic acid is produced by *A. flavus,* is mildly toxic to liver and kidney, and may cause anemia at high exposure level (33).

Diagnosis

Mycotoxicosis is a consideration in the differential diagnosis when the flock history, signs, and lesions are overtly indicative of an intoxication. Subclinical mycotoxicosis should be considered when production parameters are not meeting expectations without an obvious cause. The presence of visible mold in the feed or ingredients, or feeding system, is an obvious clue. Less specific findings include: feed refusal; lesions in the mouth, proventriculus or gizzard diarrhea or feed passage; bruising of muscle or hemorrhages in visceral tissues; ascites, gout, or lesions in liver, kidney, or heart; and evidence of immunodepression such as impaired vaccinal immunity, coccidiosis, respiratory disease, and opportunistic bacterial infections (Figure 31.9). The onset of a problem may coincide with newly acquired ingredients or feed. The mycotoxin source of exposure may be contaminated equipment at any point in the feed supply system. Clinical signs and lesions caused by mycotoxins are suggestive but not pathognomonic; therefore, mycotoxin involvement is a diagnostic rule-out. For example, oral lesions are taken as evidence of trichothecene mycotoxins (T2-toxin), but similar lesions are caused by high concentrations of fines in the feed, toxicities by copper sulfate and quaternary ammonia disinfectant, candidiasis pox, and hypovitaminosis A.

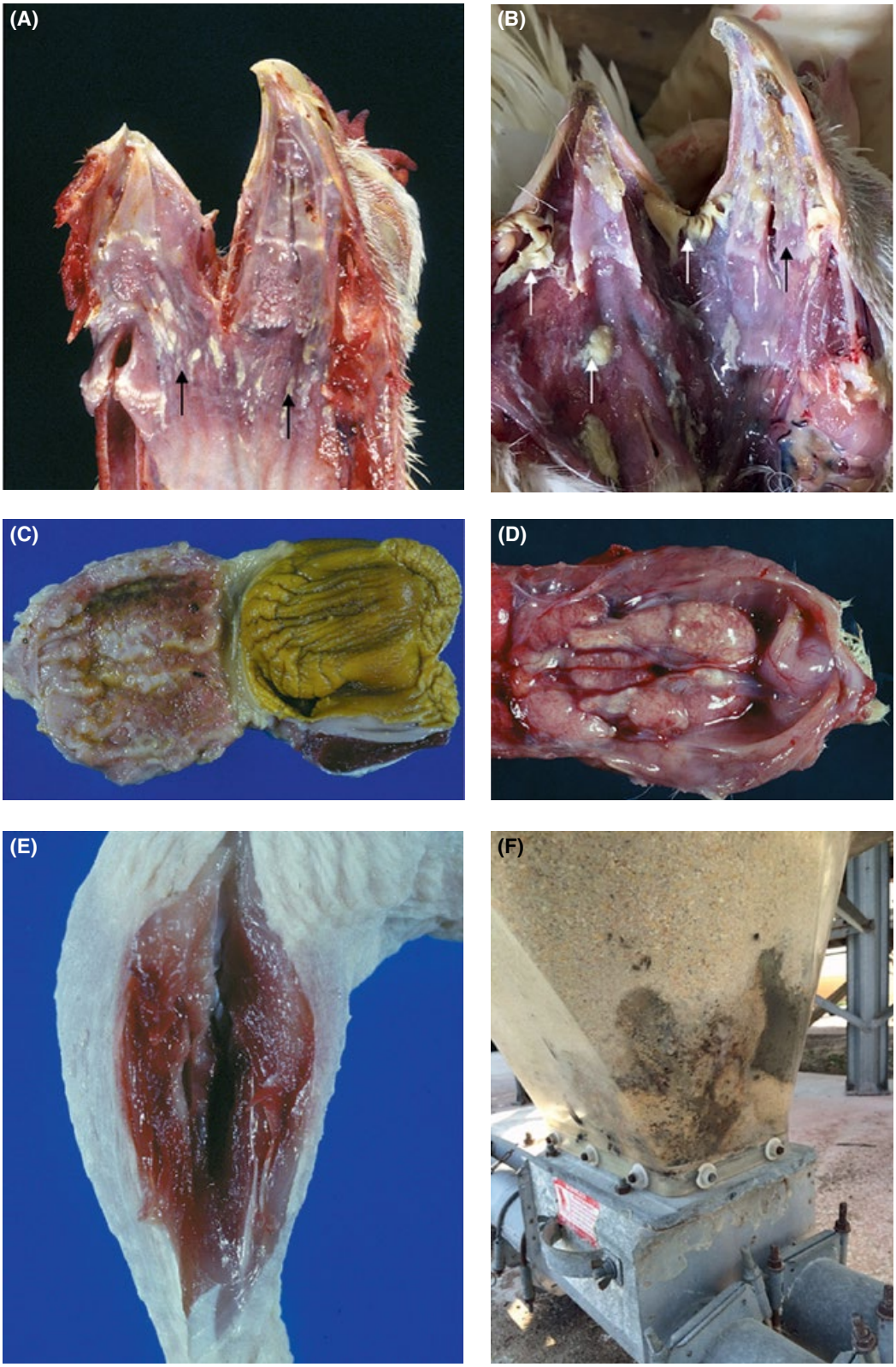


Figure 31.9 Mycotoxicosis merits inclusion in the differential diagnosis when lesions are found in the mouth (A,B), proventriculus, or gizzard (C), or when gout or swollen kidneys are identified (D). Mycotoxins can prolong clotting time and predispose to bruising or hemorrhages (E). These lesions are not pathognomonic but warrant testing for mycotoxins and inspection of the feeding system in the diagnostic investigation. (F) Fungal growth is visible in the boot of this feed bin, creating a potential hotspot of mycotoxin formation that can dislodge and distribute through the feeding system. Of the hens examined in a mortality survey of this flock, 30% had ulcerative oral lesions.

A definitive diagnosis of mycotoxicosis requires identification and quantification of specific toxins. This is difficult in poultry production because of the rapid and high-volume use of feed and ingredients. At least, a positive analysis can confirm mycotoxin exposure with the understanding that mycotoxin hot spots may result in sporadic severe intoxication.

Analytical techniques for mycotoxins are beyond the scope of this text, but in general, begin with pretreatment extraction and clean-up to separate the target toxin from the matrix. Detection methods include chromatography (thin-layer, gas, and liquid), mass spectrometry, lateral flow chromatography, quantitative fluorometric tests, and direct competitive enzyme-linked immunosorbent assay (ELISA) technology (66, 81). Simultaneous analysis of multiple mycotoxins is achieved by liquid chromatography with tandem mass spectrometry (46). This is applied to mycotoxins produced by fungal genera (*Fusarium*, *Aspergillus*, *Penicillium*) and by mycotoxin groups, such as those of regulatory interest (66). The diversity of matrices in which mycotoxins are found (grains and other feed ingredients, mash or pelleted feed) presents an ongoing challenge. Depending on the test used, qualitative, semi-quantitative, or quantitative test results may be reported.

Laboratories differ in their respective capabilities to conduct tests for mycotoxins and should be consulted before sending samples. Identification of mycotoxin residues in blood or tissues is possible but not routinely available.

Although a mycotoxicosis may be suspected, a complete laboratory evaluation can exclude other significant diseases. Birds that recently died and those obviously sick should be selected for examination. When any toxicity is suspected, the following tissues should be collected in clean containers and stored frozen until tested for mycotoxins and other toxins: crop content, proventriculus and gizzard content, liver, kidney, fat, and bile. Analysis of selected mycotoxins in body fluid and tissue may be available in certain laboratories (17).

Visibly moldy feed appears unwholesome and indicates the potential for mycotoxin formation. It can be unpalatable and have reduced nutritive value, with decreases in vitamins, amino acids, and the energy level of fats. *Aspergillus*, *Penicillium*, and *Fusarium* are mycotoxin-producing fungal genera that occur in most poultry feeds, so the potential for toxin formation is evident.

Properly collected feed and ingredient samples should be promptly submitted to a feed testing laboratory for analysis. Mycotoxin formation may occur unevenly in a batch of feed or grain, and multiple samples from different sites, including areas with caked or moldy feed, increase the likelihood of confirming a mycotoxin formation zone (hot spot) (66). Sampling may be the largest source of variability associated with mycotoxin testing. Samples should be collected all along the chain of ingredient storage, feed manufacture, and transport, feed bins, and feeders within poultry houses. Fungal activity increases as feed moves from the feed mill to feeders and is associated with an increase in fines (small particles of feed) and higher zinc concentrations.

Samples of 500 g (1 lb) should be collected and submitted in separate containers. Clean paper bags, properly labeled, are adequate. Sealed plastic or glass containers are suitable only for short-term storage and transport because feed and grain rapidly deteriorates in airtight containers. A written record of sample collection and direct labeling of specimen containers help to ensure sample integrity.

Rapid on-site screening test kits are available for several mycotoxins (aflatoxin, T-2 toxin, DON, fumonisin, ochratoxin, and zearalenone). Grain can be screened for *A. flavus* or *A. parasiticus* contamination by examination for blue-green fluorescence under a black (ultraviolet) light. The fluorescence is attributable to kojic acid and indicates potential aflatoxin contamination, which must be confirmed by chromatographic analysis.

Treatment

Toxic feed should be removed and replaced with unadulterated feed. Dilution of the problematic feed or ingredient with nontoxic alternatives may reduce the exposure level and the toxicity. Poultry usually recover from mycotoxicoses soon after an uncontaminated diet is available, but this may not compensate for production losses. Substandard management and ongoing disease are especially detrimental to poultry stressed by mycotoxins and merit correction. Feed formulation and water-based treatment can compensate for vitamins, trace minerals (selenium), protein, and lipid requirements that are increased by mycotoxins.

Prevention

Toxigenic fungi and mycotoxins are common in poultry feedstuffs and may cause subclinical effects at toxin concentrations too low to register as diagnostically significant. An integrated prevention program to prevent mycotoxin formation and inhibit the effects of mycotoxins already formed can be effective for poultry health and production.

Feed Manufacturing and Management

The goal of prevention is reducing the mycotoxin risk in feedstuffs and feed manufacturing through management practices that prevent mold growth and mycotoxin formation. This involves testing to confirm the acquisition of ingredients at low risk for mycotoxins.

Certification of ingredients by risk level is standardized for aflatoxin, DON, fumonisin, zearalenone, and ochratoxin A (34). Proper storage of ingredients, and feed processing, shipping, and handling procedures can minimize mycotoxin formation. Distillers dried grain solubles (DDGS), a byproduct of the ethanol fuel industry, are a global feed commodity. The fermentation process removes starch from the grain substrate, but mycotoxins remain with a threefold concentration. DDGS have formulation limits in poultry rations and the toxins are generally diluted in finished feed; however, there is potential for substantial mycotoxin exposure (50, 87).

Manufacturing and maintaining feed of low moisture (<12%), minimizing feed residence time on the farm, and cleaning the feeding system help in preventing mold growth. Mycotoxins form in moldy, crusted, built-up feed in feeders, feed mills, and storage bins. Regular inspection of feed bins identifies flow and separation problems that enhance fungal activity and mycotoxin formation. Temperature extremes cause moisture condensation and migration in bins and create high-risk situations for mycotoxin formation. Bin inspection and cleaning to remove feed residue is a practical control point. Tandem feed bins on farms allow cleaning between successive feed deliveries and reduces mold growth in stored feed. Adequate ventilation of poultry housing removes moisture available for fungal growth and toxin formation in feeders. Feeder equipment that minimizes surface-area contact with feed decreases the potential for mycotoxin formation.

Pelleting feed destroys some fungal spores, decreases the fungal burden, and is made more effective by the addition of an antifungal agent.

Antifungal Agents

Antifungal agents added to feeds to prevent fungal growth have no effect on toxins already formed but may slow further mycotoxin formation. Regulatory approval of these various compounds in feeds differs among countries. Organic acids are effective against *Fusarium*, *Aspergillus*, and *Penicillium*, in order of declining susceptibility, with reduction in efficacy caused by large particle size of ingredients and buffering by other ingredients. Other agents showing efficacy include phosphates (tetrasodium pyrophosphate and alkaline sodium polyphosphate), spice oils and extracts, ammonium hydroxide, formaldehyde, and copper sulfate.

Detoxification with Binding Agents

Detoxification using mycotoxin-binding agents is effective for decreasing or preventing intoxication for feed at risk of mycotoxin contamination (51). Inorganic mineral adsorbents or binders including various clays and zeolites can be part of an integrated approach. Zeolites are silica-containing compounds that are effective for aflatoxin and cyclopiazonic acid but are less effective for T-2 toxin, DAS, or ochratoxin A (101). Several zeolitic ores and bentonite clay are active against aflatoxin and fumonisins (28, 93). Further processing of silicate-type binders may increase their efficacy for protection. Polyvinylpolypyrrolidone, a synthetic binder, is effective for aflatoxin. Cholestyramine is a bile acid sequestrant that binds fumonisins (71). Mycotoxin binders can interfere with other feed additives, including monensin and other antibiotics (18, 52, 53).

Organic compounds from fermentation productions, plant extracts, and essential oils of spices have efficacy in detoxification through binding activity and the reduction of oxidative stress (61, 62). Esterified glucomannan from *Saccharomyces cerevisiae* is protective against aflatoxin and ochratoxin, with moderate binding activity for fumonisins, zearalenone, and T-2 toxin (58, 59, 73). Algae and plant extracts demonstrate binding activity (30). Products may be combinations of enzyme detoxicants, binders, nutritional supplements (75), or essential oils of spices (20, 22, 35, 36, 67, 90).

Microbial feed additives such as *Lactobacillus* (19), *Bacillus subtilis* (27, 42), *Eubacterium* (8), *Nocardia corynebacterioides* (80), and mixed gut flora bacteria (92) detoxify through the metabolism and degradation of mycotoxins (8, 88). Ozone treatment and ammonization are effective in detoxifying feeds and grains for aflatoxin.

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32

Toxins and Poisons

Richard M. Fulton

Summary

Agents and Disease. There are many toxins and poisons that have caused decreased production parameters, clinical signs, debility, and death of poultry. The categories vary from substances used for the benefit and health of poultry, feed components, natural and man-made poisons which affect other species, to intentional as well as accidental poisons.

Diagnosis. Extensive investigation of affected flocks is typically required when toxins or poisons are suspected. A complete and thorough interview of personnel involved with care of poultry must be conducted. A visit to the poultry facility may reveal clues often overlooked in the interview process. Elimination of infectious disease is usually the first step of the investigation. Unfortunately, no single test will find all toxicants. The investigator must know what substance is causing the poisoning in order to select the test for that substance. In feed related problems, it is important to test feed samples from the batch of feed that was consumed when clinical signs occurred.

Interventions. Removal of the offending toxin or poison is of utmost importance. In suspected feed-related problems, removal of the feed, and replacement with fresh feed, may help to establish whether feed was the cause of the poisoning.

Introduction

Paracelsus recognized more than 400 years ago that it is "the dose that makes the poison." Although that may be obvious with known toxic materials, it is also true for more benign products such as growth promotants and chemotherapeutics usually considered safe. Deliberate or inadvertent overdosages may cause illness, and a misplaced decimal in water or feed medication concentrations frequently results in toxicity. A general feature of modern complex poultry rations, feed mill equipment, and feed delivery to poultry farms is that any component included in a ration may at some time be mistakenly included at a higher than desired rate. This may occur through human or mechanical error. Furthermore, when toxicoses do occur, they are added to the pre-existing background of complicating infectious agents, vaccine administrations, and environmental exposures. This usually leads to multifactoral mixed clinical presentations of toxic, infectious, environmental, and management diseases, rather than "pure" toxicoses. Additionally, some highly pathogenic infectious or environmental diseases of poultry present such rapidly elevating mortalities that acute toxicoses may be suspected incorrectly.

For example, sulfaquinoxaline poisoning occurs in meat-type chickens, even at recommended doses, because of high water intake in warm buildings, particularly in hot weather, or because of poor feed mixing. Disease may also be caused by toxic levels of some nutrients (e.g., excess dietary sodium causes significant losses in chickens and turkeys around the world). High levels of vitamins A and D are toxic. Some compounds have differential species or age toxicities, and others have increased toxicity in naïve animals with no previous adaptive exposure. Ionophore anticoccidials commonly exhibit such differential adaptive resistance to toxicity. Furthermore, waterfowl are sensitive to some drugs at a dose safe for chickens and turkeys. The immune system seems to be affected by many toxic agents. In addition to disease caused by poisons, the problem of residues in eggs and meat must also be considered. For information on drugs approved in the United States, withdrawal times, and drug and chemical residues, see Booth (33), the Green Book Online (www.fda.gov/animalveterinary/products/approvedanimaldrugproducts), poultrymeds.cvpservice.com, or the current Feed Additive Compendium. In rationalizing withdrawal times in egg-laying chickens, it must be remembered that compounds begin to be deposited in chicken egg yolk 10 days before that egg is laid.

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Poisonous substances are widely distributed in nature. Mycotoxins, covered in Chapter 31, are important to the poultry industries, but toxins are also produced by bacteria (botulinum toxin, methylmercury, toxic amines) or occur naturally (selenium, phytotoxins). Pesticides, herbicides, and other synthetic chemicals, metals such as lead, and industrial contaminants add to the list of toxic materials. Many chemicals and human drugs have been given to birds in feed and water to study their toxic effects. These experimental toxicities generally have not been included in this chapter, except as they may relate to potential naturally occurring or iatrogenic poisonings in poultry.

Poisons and toxins are not major causes of production loss or disease in poultry in most countries, although some, such as lead, pesticides, and botulism, are significant in wild birds. However, in 1985, Terzic and Curcic (326) reported that 40% of 2,065 poisoning cases seen at the Belgrade Veterinary Facility during a 17-year period were in poultry. In 2005, Sharpe and Livesey (295) reported that 1.4% of 876 poisoning cases of food safety concern that were seen at the Veterinary Laboratories Agency in England and Wales were in poultry, most of which were lead poisoning in waterfowl. In 2017, McFarland reported on poisoning of domestic animals in Germany from 2012 to 2015. At a testing facility in Munich, there was 1 detection of carbamates and 1 detection of anticoagulants in poultry which accounted for 1.2% of total toxicant detections (212). Poisoning occurs more frequently in free-range and backyard flocks and in village poultry where birds forage in neighboring gardens and fields or receive household waste and weeds cut from roadsides and fields. Some of these poisonings are malicious. Contaminated litter on the floor and in nest boxes is an added source of toxins in chickens not raised on wire. Because suspected toxicity cases are more likely to be submitted to a diagnostic laboratory than are other sick birds, statistics collected from that source may not be an accurate indication of the incidence of poisoning compared with other disease.

Toxicants covered in this chapter are presented by primary use. Levels of toxic substances that may cause depressed growth in broilers and turkeys or decreased egg production in layers are summarized in Table 32.1.

Antimicrobials, Anticoccidials, and Growth Promotants

Most reports of poisoning with chemotherapeutic agents involve inappropriate use or overdose of ionophore anticoccidials or growth promotants. Toxicity of a variety of chemotherapeutic agents in poultry and pigeons has been reviewed (273, 274).

Sulfonamides

Sulfonamides were used as the primary form of prevention and treatment for coccidiosis in poultry between the early 1940s and late 1950s. Sulfaquinoxaline and sulfamethazine were most widely used. The toxic level of sulfonamides is close to the therapeutic level in poultry, and even the therapeutic level has a detrimental effect on hemopoietic and immune systems. Previous low-level or continuous-preventive medication has a protective effect against subsequent higher doses (100).

Sulfonamides are difficult to mix evenly in feed, and they have low solubility in acidic water. These characteristics may cause some birds to receive a toxic dose even when appropriate treatment levels are added to bulk rations or water supplies. This is less likely at lower preventive levels. Both feed and water medication require accurate estimates of daily consumption if each chicken is to receive a therapeutic and nontoxic daily dose. Sulfa poisoning has occurred when no allowance was made for increased water and feed consumption of the modern broiler that eats to its physical capacity rather than to its metabolic need or, more frequently, for the effect of increased water consumption at high environmental temperatures or in hot broiler houses. For broilers, previous authors of this chapter recommended one-half of the therapeutic dose, and at temperatures greater than 27 °C (81 °F), one-third of the therapeutic dose for water medication. Repeat treatment is hazardous and should not be recommended without a postmortem examination of a subset of individuals to make sure that there is no evidence of sulfa toxicity. Even the newer so-called safe sulfas need to be used with care (64, 274). Under no circumstances should sulfas be given simultaneously in both the feed and water. Decreased solubility in acidic water may lead to delayed clearance of sulfas from water lines and result in detectable drug levels in meat and eggs beyond recommended withdrawal times.

Hemorrhagic syndrome, which occurred frequently when sulfas were in widespread use, is a manifestation of sulfa toxicity and occurs at and above therapeutic dose levels. In addition to blood dyscrasia, bone marrow depression, and thrombocytopenia, sulfonamides depress the lymphoid system and immune function in birds. Similar but more dramatic hematologic manifestations and diatheses are seen in domestic mammals given sulfa-containing poultry rations or water medications. Focal bacterial granulomas are often found in tissues and organs of chickens dying from sulfa poisoning. Epithelial necrosis and degeneration in the liver, kidney, and other organs may be caused by the direct effects of the drug, or hypoxia secondary to drug-induced anemia. When determining withdrawal times in chickens whose eggs reach the human food chain, deposition in the yolk 10 days prior to the production of an egg must be considered (31).

Table 32.1 Levels in feed (unless otherwise noted) of selected toxins documented to decrease growth rate in broilers and turkeys and reduce egg production in layers. Although some of these compounds are no longer available or in use, they are included for completeness. Reproduced with permission of Sherpa (266, 267).

Toxin	Broilers	Turkeys	Layers
Antimicrobials, Growth Promotants, and Protozoal Control Compounds			
Sulfadimethoxine (% in water)	NA[1]	NA	0.05
Sulfaquinoxaline (%)	NA	NA	0.10
Nicabazine (mg/kg)	NA	NA	70
Arsanilic acid (mg/kg)[2]	1000	400	NA
Nitarsone (mg/kg)[2]	300	600	NA
Roxarsone (mg/kg)[2]	90	550	NA
Nutrients and Other Feed- and Water-Related Toxicants			
Aluminum (%)	0.30	0.30	0.15
Arsenic (inorganic pentoxide) (mg/kg)	40	40	40
Boron (mg/kg)	435	435	870
Boric acid (mg/kg)	2500	2500	5000
Cadmium (mg/kg)	400	400	8–60
Copper (mg/kg)	500–1000	500–1000	1000
Fluoride (mg/kg)	1300	1300	1300
Iodine (mg/kg)	500	500	300
Iron (mg/kg)	200–2000	200–2000	NA
Lead (acetate) (mg/kg)	630	630	630
Mercury (mg/kg)	50	50	5
Molybdenum (mg/kg)	200	200	200
Potassium (%)	0.90	0.90	NA
Selenium (mg/kg)	5	5	80
Sodium (%)	0.80	0.80	0.80
Sodium chloride (%)	2.0	2.0	2.0
Tungsten (mg/kg)	1000	1000	1000
Vanadium (mg/kg)	6	6	20–30
Zinc (mg/kg)	NA	NA	20,000
Other			
Ammonia (ppm)	**50**	**25**	**75**

[1] NA, not available.
[2] NLA, no longer available.

Signs. Chickens and turkeys with sulfa toxicity are depressed, pale, and frequently underweight. In adults, there is a marked decrease in egg production and shell quality; brown eggs may be depigmented (71, 249). Secondary bacterial infections including septicemia and gangrenous dermatitis may follow sulfonamide toxicity (65).

Pathology

For descriptions of gross and microscopic pathology, see (65, 71, 100).

Hemorrhage in skin, muscles, and internal organs are the most consistent and extensive gross lesion of sulfonamide intoxication. Hemorrhage may be present in comb, eyelids, face, wattles, anterior chamber of the eye, and musculature of breast and thighs. Normal dark-red bone marrow in growing birds changes to pink in mild cases and yellow in severe cases. The entire length of the intestinal tract may be spotted with petechial and ecchymotic hemorrhages, and the cecal lumen may contain blood. Hemorrhage may be present in the proventriculus and beneath the ventriculus (gizzard) lining. There may be ulcers at the proventricular–ventricular junction. The liver is swollen, pale red, or icteric, and may be studded with petechiae or foci of necrosis. The spleen is commonly

enlarged, has hemorrhagic infarcts, and contains gray nodular areas. "Paintbrush" ecchymotic hemorrhages occur in the myocardium. Thymus and bursa of Fabricius are small.

Microscopically, areas of caseous necrosis surrounded by a mantle of giant cells occur in liver, spleen, lungs, and kidneys. Lymphocyte and heterophil infiltrates are present at the periphery of necrotic foci. Lymphoid hypoplasia around splenic sheaths, edema and fibroplasia of the capsule, and macrophages containing hemosiderin are common. Early changes in the liver are periportal mononuclear infiltration associated with bile duct hyperplasia. Hemosiderin deposits are present in necrotic areas, and thrombosis of portal vessels is present. An early change in kidneys is interstitial lymphocytic infiltrate, but this may be associated with concomitant infections. Degeneration and necrosis of tubular epithelium are associated with hyaline casts. Glomeruli are enlarged, and Bowman's capsule is dilated with hyaline material. Lungs are congested with interlobular and interstitial edema. Interstitial tissues contain mononuclear foci. There is degeneration and necrosis of lymphocytes and depletion of bursal follicles.

In femoral bone marrow, there is decreased intrasinusoidal erythropoiesis with thrombocytopenia and agranulocytosis, focal increase in extrasinusoidal lymphopoiesis, and, in some instances, myelopoiesis. There are also focal areas of hyalinization, necrosis, and fibroplasia. Hemosiderin deposits and extrasinusoidal edema are present.

Nitrofurans

Nitrofurans, although a very effective antibiotic, is no longer permitted in some countries and as such its toxicosis is considered historical. The reader is referred to previous editions of *Diseases of Poultry* for discussions on nitrofuran toxicosis.

Aminoglycoside Antibiotics

After subcutaneous injection, gentamicin (an aminoglycoside) causes depression in turkey poults, edema and hemorrhages at the injection site, and large, pale, and nephrotic kidneys (24, 283). Aminoglycosides and various other antibiotics used for egg inoculation have caused embryo mortality. Streptomycin and dihydrostreptomycin sulfate injected intramuscularly for sinusitis in turkey poults causes respiratory distress, paresis, and mild convulsions (273, 274).

Ionophore Antibiotics

Ionophores (ion carriers) facilitate movement of some monovalent cations, such as sodium and potassium, and divalent cations, such as calcium and magnesium, across cell membranes. They can have both anticoccidial and antibacterial activity, and the group is used extensively in poultry and ruminant feeds. Ionophores are coccidiocidal because of their ability to preferentially move ions, usually Na^+, into various stages of the parasite.

Toxic levels of ionophores cause potassium to leave and calcium to enter cells, particularly myocytes, resulting in cell death. Signs of toxicity are related to high extracellular potassium and high intracellular (intramitochondrial) calcium. For more specific information on metabolism and toxicity of monensin, see (33, 52, 84, 241). Ionophore toxicity varies with species and age; equidae are very susceptible, and adult poultry, particularly turkeys, are more susceptible than broilers (133, 154, 295). There is a synergistic effect with antibiotics in the same family of drugs (332) and increased toxicity with nonrelated antibiotics, other drugs (36, 41, 83, 186, 253, 259, 273), and low-protein rations (274). Dehydration because of diarrhea or periods of water and/or feed deprivation can precipitate toxic events (48, 128). Monensin, lasalocid, salinomycin, and narasin have been associated with toxicity in poultry, guinea fowl, quail, and other species (67, 128, 135, 207, 262, 290, 295, 344). Lethal toxicoses have been described in equidae and other mammals accidentally exposed to ionophore-containing poultry rations. Poultry have an adaptive resistance to dietary ionophores and an inverse age sensitivity, with adult naïve poultry being more susceptible than young previously exposed birds.

Signs. Signs vary from anorexia with depression, weakness, and reluctance to move to complete paralysis in which birds lie in sternal recumbency with neck and legs extended. Less severely affected birds may show posterior paralysis with legs extended. Dyspnea has occurred in affected adult turkeys (Figure 32.1). Signs are associated with muscle damage. Death may follow respiratory failure or be secondary to dehydration. Mortality is variable but may exceed 70% (105). In some cases of suspected ionophore toxicity in turkeys, morbidity may be low with only a few poults paralyzed. The term knockdown syndrome has been used for this condition (49). Poults with botulism may show similar signs. Reduced egg production (344) and fertility with weak chicks also have occurred (254).

Pathology. Subchronic monensin toxicity (338) resulted in opaque fibrin plaques on the epicardium, hemorrhage in coronary fat, and decreased liver weight. In acutely affected turkeys, pallor and atrophy of type I fibers of leg and back muscles have been observed associated with monensin use (25, 257, 337). However, clinical signs and gross lesions are often absent in breeders ingesting high levels of monensin (105).



Figure 32.1 Acute ionophore toxicity. Dyspnea and drooping wings suggest heat stress. (H.J. Barnes)

Microscopic changes in heart and skeletal muscle consist of scattered areas of hyalinization with muscle necrosis and myofiber degeneration and necrosis. Birds with respiratory signs often have lesions in tracheal muscles. Type I fibers appear to be selectively affected (135). Heterophils, macrophages, and occasionally lymphocytes may be present. Frequently, when exposed to low doses or interaction with other drugs occurs, affected areas are very cellular with large numbers of satellite or sarcolemmal nuclei, indicating regeneration is occurring (Figure 32.2). Ultrastructural changes have been described (332). Peripheral neuropathy characterized by edema, demyelination, and axonal degeneration accompanied by marked hypertrophy and hyperplasia of neurilemmal cells may be seen with lasalocid toxicity (128).

Differential Diagnosis. Because there is a marked individual, age, and species variation in susceptibility and the toxic effect may be potentiated by other drugs, normal levels of ionophore should not be dismissed if clinical signs and histologic changes indicate ionophore toxicity. High serum or plasma levels of muscle enzymes may be useful in differentiating ionophore toxicity from botulism (228). Ionophore toxicity also must be distinguished from vitamin E/selenium deficiency and *Cassia* (senna) ingestion, which may produce similar signs and lesions.

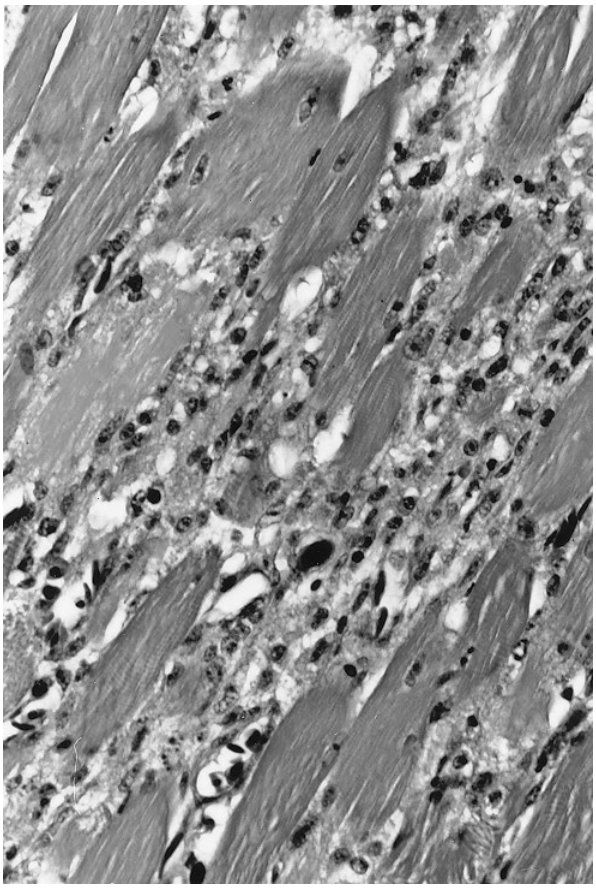


Figure 32.2 Muscle from a young turkey with knockdown. Minimal muscle necrosis and inflammation along with increased sarcolemmal or satellite cell nuclei indicate regeneration.

Other Anticoccidials

3,5-dinitro-o-toluamide (dinitrotolumide, dinitolmide, DNOT, Zoalene, Zoamix) can cause ataxia, torticollis, incoordination, and reduced growth (167, 249, 273). Nicarbazin (Nicarb) can make broiler chicks listless, dull, and ataxic; in older birds, reduced egg production, shell depigmentation, yolk mottling, and reduced hatchability (19, 166, 192) may occur. Nicarbazin depresses growth rate at 150 mg/kg feed. Even when used at recommended levels, nicarbazin increases metabolic rate and heat production (21, 273, 350). This makes older broilers more susceptible to heat stress and pulmonary hypertension syndrome. Generally, there

are no gross lesions, but there may be hepatic and renal epithelial degeneration (249, 274). Nitrophenide (Megasul) has caused nervous signs but with rapid recovery (249). Ducks, geese, and chukar partridges may have depressed growth and mortality from halofuginone (Stenorol) (22, 99), and reduced skin strength has been found in chickens (126, 211). Use of t-butylaminoethanol may result in reduced growth caused by choline deficiency.

Antiprotozoals

Organic arsenicals and imidazoles such as dimetridazole (Nitrazol, Emtryl), formally used for histomoniasis, have caused growth depression, drops in egg production, nervous signs (ataxia, incoordination, tremors), convulsions, and death in geese, ducks, pigeons, and turkeys (273, 274, 277). Waterfowl may be poisoned by doses safe for other poultry. Quinacrine HCl (Atabrine), used for *Haemoproteus* infections in pigeons, was fatal at a dose of approximately 50 mg/kg.

Organic Arsenical and Imidazole Feed Additives

Organic arsenicals are no longer approved for use in poultry feeds in the United States. As such, this classification is now of historical interest and the reader is referred to previous editions of *Diseases of Poultry*.

Anthelmintics

All anthelmintics are probably toxic if a sufficient overdose is given, but generally birds are more resistant than mammals to anthelmintics.

Benzimidazoles

Cambendazole, mebendazole, and fenbendazole are well tolerated by birds (280).

Imidazothiazoles

Levamisole and tetramisole are not quite as safe as benzimidazoles. The lethal dose – 50% (LD_{50}) of tetramisole for chickens – is 2.75 g/kg. Geese and captive birds are more susceptible (280), with a fatal dose for captive kiwis between 25 and 43 mg/kg (120); 300 mg/kg is toxic for geese and as little as 66 mg/kg of levamisole is toxic for some wild birds. Anthelmintic activity of *dl*-tetramisole resides in the l-isomer (levamisole), so the effective dose of levamisole is half that of tetramisole. This doubles the safety margin. Tetramisole is no longer available in most countries. Levamisole poisoning has occurred in geese being treated for *Amidostomum* infection (363). Levamisole was toxic for ducks parenterally at 40 and 80 mg/kg (132). Microscopic lesions in kiwis killed by levamisole were similar to those of mammals consisting of pulmonary congestion, edema, and bronchopneumonia and severe periacinar cytoplasmic vacuolation of hepatocytes (119).

Organophosphates

Organophosphorus compounds have caused poisoning in birds eating treated feed intended for horses (158, 203). The resin pellet form of dichlorvos (DDVP) is toxic because it is retained in the ventriculus. Colored breeds of chickens are more susceptible than white breeds to coumaphos, and naphthaphos has a narrow safety range for chickens, with 50 mg/kg being fatal (280).

Ivermectin

Ivermectin has a wide safety margin in birds. An oral or injectable dose of 0.1 mg/kg has been suggested (280). Ivermectin is effective against a wide range of parasites. Zeman (364) tried 1.8 mg/kg for *Dermanyssus gallinae*. This dose was more effective in chickens weighing more than 450 g. The toxic dose for chickens is 5.4 mg/kg, which causes 4-hour somnolence, 16.2 mg/kg, which causes 24-hour listlessness and ataxia, and 48.6 mg/kg, which results in death 5 hours postinjection. Canaries given 20–60 mg/bird intramuscularly showed temporary immobility.

Other Anthelmintics

Phenothiazine is relatively nontoxic for birds, and hygromycin B is safe at 8 g/900 kg feed (280).

Nutrients and Other Feed- and Water-Related Toxicants

Amino Acids

Interaction among some amino acids relates to growth, but only methionine is toxic to poultry. Methionine toxicity affects chickens and quail (175, 293) and has caused depressed growth and cervical paralysis in turkey poults (131). Mortality can occur at levels of 1.8% in feed. Methionine attenuates calcium-induced kidney damage (349). Ethinone (a methionine antagonist) toxicity in chicks can be relieved by methionine.

Antinutrients

A variety of feedstuffs and potential feed stuffs are poorly digestible, contain factors that inhibit digestion (protein inhibitors), depress growth, cause pasting of feces, or

increase the incidence of skeletal disorders. Antinutritional factors in some of these products (e.g., soybean and some other beans) can be destroyed by heat. The nutritional value of some feedstuffs (e.g., wheat, barley, and rye) can be improved by enzymes (37, 117, 156, 165). Antinutrients that can be found in plants include proteases, tannins, saponins, antivitamins, lectins, b-glucans, pentosans, polysaccharides, concanavalin A, hemagglutinins, vicine, convicine, alkaloids, and sinapines. Feedstuffs known to contain antinutrient factors are alfalfa (168, 331), amaranth (4), jackbeans (80, 195, 237), fababeans (240, 284), lima beans (236), narbon beans (90), soybeans (168, 197), jojoba (14), lupins (38, 255, 282), peas (39), vetch (283), barley, rye, and wheat (20, 37, 213), and sorghum (324).

Protein Supplements

Fish and Meat Meals

Gizzerosine, histamine, histidine, and other biogenic amines cause digestive disturbances, stunting, and osteoporosis (155, 321). Biogenic amines result from heating or bacterial spoilage of fish and animal byproducts. Toxic products get into poultry feed through fish or meat meal. Excess acid secretion in the proventriculus is stimulated by gizzerosine, causing gizzard erosion and hemorrhage (151, 219, 291). Broiler chickens may die from hypovolemic shock. Black ingesta and blood may run from the mouth (vomito negro), and the contents of the digestive tract are often melanic. Other biogenic amines reduce broiler feed efficiency (178).

Minerals

For information on trace mineral deficiency and toxicity (tissue levels, signs, etc.), see (266, 267). Information on poultry in these references is included for the following minerals: aluminum, arsenic, cadmium, calcium, chloride, chromium, cobalt, copper, fluoride, iodine, iron, lead, magnesium, manganese, mercury, molybdenum, nickel, phosphorus, potassium, selenium, sodium, tungsten, vanadium, and zinc. More detailed information on macro and trace mineral deficiencies and excesses are reported in Chapter 29, Nutritional Diseases.

Aluminum

Aluminum depressed growth rate in chicks due to decreased feed intake and decreased egg production in adults when 0.3% was added to the feed (32, 358). Aluminum also may interfere with phosphorus retention (88, 95, 160) and iron absorption resulting in anemia (134). Aluminum absorption after ingestion may be affected by the acidity of the ration (47).

Calcium

Excess absorbed calcium is excreted through kidneys; high levels cause ureter and kidney impaction, resulting in nephrosis. Very young birds are most susceptible. This condition may be produced in pullets by feed delivery mistakes in which layer ration is accidentally fed to pullets. High mortality from hyperuricemia with visceral urate deposits may result from kidney damage because of high dietary calcium. Lung changes with damage to parenchyma from calcium deposits also may occur in young chicks. It is possible that nephrosis and visceral urate deposits in young and dead-in-shell chicks may result from kidney obstruction by calcium. Excess unabsorbed calcium remaining in the intestine increases fecal water content of pullets and hens on high-calcium rations. If the source of calcium is dicalcium phosphate, the alkaline solution formed in the upper digestive tract may result in epithelial necrosis (246, 249, 341), particularly if the mineral has been "top-dressed" on feed and birds eat undiluted material.

Urolithiasis in pullet and layer flocks may be caused by high calcium and low phosphorus in pullet rations. The incidence also may be increased by infectious bronchitis virus infection (123).

Cobalt

Moderate levels (125 ppm) stimulate polycythemia and induce pulmonary hypertension. Higher levels (500 ppm) cause marked tibial dyschondroplasia as well as necrosis and fibrosis in the pancreas, liver, and skeletal, smooth, and cardiac muscles. All levels reduce feed intake and growth (76).

Copper

Copper sulfate is added to water for treatment of enteritis or yeast infection or to clean algae or scum from water lines and drinkers. Addition to feed is another method for treating enteritis and candidiasis. It also may be sprayed on litter to control *Aspergillus* or used as an antifungal preparation on wood. Birds occasionally are poisoned by eating copper sulfate crystals. Diets low in calcium may increase susceptibility to copper toxicity (190). Mortality in turkeys offered water containing copper sulfate may have resulted from dehydration caused by water refusal rather than from copper poisoning. Toxicity signs are depression and weakness with convulsions and terminal coma (249) or anemia (143, 241, 278). Gross lesions include necrosis of proventriculus and ventriculus (gizzard) epithelium with sloughing of koilin lining (120, 147). Broiler chicks receiving feed containing toxic levels of tribasic copper chloride demonstrated similar findings. Histologically, in addition to gastrointestinal lesions, there was hepatic degeneration and necrosis with golden-brown pigment in hepatocytes and Kupffer cells (206).

Fluoride

Growth, production, and egg quality were reduced by 700 and 1,000 mg sodium fluoride/kg feed (129). Leg

deformity has also been described. Laying birds can tolerate ingesting 4.453 mg fluoride/day for up to 74 weeks (56). In addition, poultry species vary in their susceptibility to fluoride and the lowest toxic levels for broilers is 500–600 mg/kg (299).

Iodine

Reduced egg production and weight and increased embryonic mortality in the first week and at pipping occurred when 350 ppm of iodine was added to the ration of turkey breeder hens (54). With experimentally induced iodine toxicity in chickens, researchers found that clinical signs were poor growth and a bizarre syndrome of chicks falling over, lying motionless, getting up, and then repeating falling over (17).

Magnesium

Excess magnesium causes bone abnormalities by replacing calcium and affecting phosphorus utilization (191).

Phosphorus

Excess phosphorus affects growth plate development of bones and increases tibial dyschondroplasia and leg deformities. Phosphate may also be caustic to moist oral and epithelial surfaces. White phosphorus induces mortality and hematologic abnormalities after oral ingestion (314, 315).

Potassium

Potassium in the form of fertilizer or potassium permanganate is toxic. The latter caused epithelial necrosis of the digestive tract (249).

Sodium (Sodium Chloride, Sodium Bicarbonate)

Excess ionic sodium, usually from sodium chloride in feed or water, causes significant economic losses in poultry in many countries. Most toxicity results from consuming saline water, not water deprivation. Sodium in feed can be toxic for young chicks and poults with or without water deprivation. In some cases of toxicity at apparently low salt levels, analysis may have been for chloride, with salt level calculated from chloride level. When Na^+ toxicity is suspected, both feed and water should be analyzed for Na^+, not estimated from chloride content. There may be sources of Na^+ in feed or water other than sodium chloride. Levels of Na^+ in feed and water are additive. Sources of sodium also may be naturally occurring in soil or water (214).

Young birds are much more sensitive to Na^+ toxicity than adults, probably because their kidneys are not yet fully developed (215). Water with Na^+ greater than 0.4% (4,000 ppm) is quite toxic and will cause high mortality within a few days. Lower levels may be toxic as well, depending on the amount of Na^+ in feed. Levels of Na^+ greater than 0.12% (1,200 ppm) are toxic for some chicks and poults and produce heart failure with edema and ascites. Feed with Na^+ greater than 0.85% is toxic for some chicks and poults. Much lower levels will cause heart failure and ascites even when water is available freely. Because steroids increase Na^+ and water retention (292), resulting in hypervolemia, hypertension, right ventricular failure, and ascites, stress may also contribute to Na^+ susceptibility. Birds have poor renal concentrating ability and difficulty reducing plasma osmolality by excretion of salt in excess of water. Some waterfowl have nasal salt glands, which allow them to excrete Na^+ if an excess is ingested.

Three forms of disease result from Na^+ toxicity in young birds. At high levels, birds develop acute, severe diarrhea and dehydration, lose weight, and die. There is often acute kidney damage, particularly with sodium bicarbonate (216), which may be ischemic because of increased red blood cell rigidity. Potassium may have a protective effect (309). At lower levels, loose droppings also occur, but birds gain weight, at least for 1–2 days, because of associated water retention. Depending on the Na^+ level, they may subsequently eat less and grow poorly, or continue to eat and grow well. Water retention, with hypervolemia and reduced red blood cell deformability (217), can lead to functional cardiac overload, causing marked right ventricular hypertrophy and dilation, valvular insufficiency, edema, and ascites in chicks (169, 170, 218). At intermediate levels of excess sodium, a variety of clinical signs and pathologic changes are seen, depending partly on how long birds survive with hypertension before heart failure occurs and how long they survive afterward. Many lesions described for Na^+ can be attributed to heart failure. The severity of ascites may be affected by other dietary, environmental, and water constituents (286, 305).

Signs. At low levels of excess Na^+, only watery droppings are seen until ascites occurs. At this stage, chicks and poults are dyspneic, depressed, and have a swollen abdomen. At high Na^+ levels, birds are obviously sick and depressed within a few hours, with thirst and diarrhea. They may have rough, dirty, wet feathers or down. Nervous signs may be present, and some birds may be prostrate. At intermediate levels, stunting of some birds may be prominent. Excess Na^+ may cause reduced egg production and increased mortality in adults (68).

Pathology. Chicks with ascites and edema frequently have excess fluid in lungs and hydropericardium. Young males may have cystic dilation of seminiferous tubules (280). There is cardiac hypertrophy, which in chickens is mainly right-sided. Poults have biventricular hypertrophy with dilatory cardiomyopathy. At levels of Na^+ causing dehydration, the following also may be seen: cyanosis, myocardial hemorrhage, nephrosis, and enteritis.

Microscopic lesions are frequently secondary to heart failure or dehydration. For a detailed description of histologic lesions, see (221). Glomerulosclerosis (292, 310) may be ischemic in origin. Ultrastructural changes in heart muscle (239) include glycogen accumulation, myofibrillar disarray, Z-band streaming, and disruption of intercalated discs.

Sulfate

The toxic concentration of sulfate is affected by age of the birds, source (water or feed), other salts, etc., and is not clearly defined. Magnesium sulfate may be more toxic than sodium sulfate (334). Diarrhea, reduced growth, and depressed egg production can occur.

Selenium

Some plants accumulate selenium (355), and the addition of acceptable treatment levels of selenium to rations already containing high normal selenium levels may produce toxicosis. This often manifests itself as embryo deformities of the eye, head, or beak in the progeny from such breeder flocks (234). Decreased growth and feed intake resulted when there was 4–8 ppm selenium in drinking water (46), but the toxicity of selenium varies with the form present (152). Selenium can accumulate in the food chain of aquatic birds causing emaciation, hepatitis, and ascites (127). Selenium often causes problems for aquatic birds in certain regions of the United States where selenium concentrations in aquatic plants, invertebrates, and fish increase. Chronic selenium toxicosis in mallards, in addition to weight loss, causes gross lesions of patches of liver necrosis (6). Mallard ducks have hepatocellular vacuolar degeneration progressing to centrolobular and panlobular necrosis. Nephrosis and apoptosis of exocrine pancreas cells were noted (127).

Zinc

Toxic levels of zinc (greater than 500 ppm) cause anorexia, depressed growth, reduced egg production, ventriculus (gizzard) and pancreatic lesions, and hematologic abnormalities (69, 73, 176, 199, 204, 308, 351). Individual birds may be poisoned by ingesting metallic zinc, such as coins or other objects, or galvanized wire from caging in the case of pet birds (275) and in wild waterfowl via contaminated mining areas (308).

Metals and Metalloids

Arsenic

Inorganic, aliphatic, and trivalent organic arsenicals are used as pesticides, weed and brush killers, and defoliants. Toxic effects include diarrhea, nervous signs, and cyanosis. There is inflammation of the digestive tract including crop, proventriculus, and ventriculus (gizzard); hepatosis; and nephrosis (241, 294). Most reports of arsenic toxicity in birds are experimental, except those associated with grasshopper bait (249). For information on organic arsenicals, see Antimicrobials, Anticoccidials, and Growth Promotants.

Cadmium

Toxic levels of cadmium found in industrial waste and sewage sludge cause decreased feed intake and decreased growth, induced kidney lesions, and reduced gonadal mass and function (157, 264, 266, 267, 348). Experimental cadmium toxicity in chicks, poults, and ducklings and free radical-induced lesions by cadmium, silver, and other minerals have been reported (26, 63, 333).

Chromium and Potassium Dichromate

Chromium from industrial waste or coated metal objects may cause depression, anorexia, and paralysis (157, 266, 267).

Lead

All species of birds are susceptible to lead poisoning. Lead is the only metallic poison causing significant disease in birds, and most toxicity occurs in wild species, especially waterfowl. Chickens are more resistant than waterfowl (266, 267). Birds as a group are at risk from metallic lead because the material is retained in the ventriculus (gizzard), ground down, and absorbed slowly. Experimental poisoning trials with chickens show an interaction with some nutrients (91, 188), production of immunotoxic effects with differential gender sensitivity (42), inhibition of avian bone healing (198), and immunosuppression (361).

Lead is widespread in the environment, and there are many possible sources for ingested lead when toxicity occurs. Wild water birds are at greatest risk from ingesting lead shot or contaminated sediments (153), which is the main hazard in North America (289) and elsewhere (142, 233). Lead weights from fishing lines are the most important source in England. Pigeons may also ingest lead shot (72). Birds that eat carrion may be poisoned by lead shot ingested with tissues. Backyard and free-range poultry may pick up lead from paint chips, lead batteries, or other lead objects. Chicks have been poisoned by eating contaminated grit (249). Cage birds may be poisoned from the same environmental sources as children and dogs, primarily paint chips, leaded windows, toys, and lead objects (360).

Signs. Most lead poisoning in birds is chronic. Clinical disease usually is seen as wasting, ataxia, lameness or paralysis, and anemia. In acute cases, anorexia, weakness, prostration, and anemia may be prominent. Green diarrhea may result from anorexia, or it may be a direct effect of lead on digestive and nervous systems.

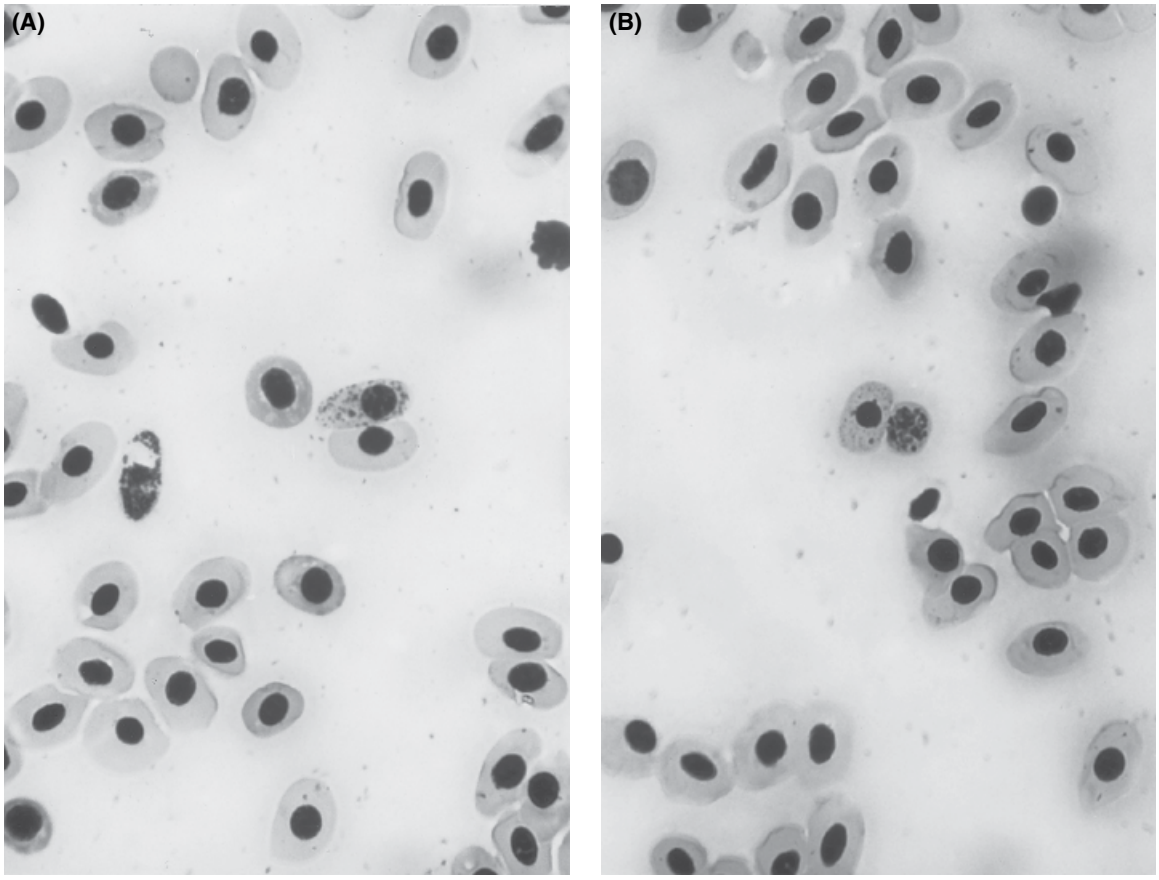


Figure 32.3 Duck with lead poisoning. (A) Immature erythrocytes and 2 cells showing basophilic stippling. (H.J. Barnes) (B) Basophilic stippling in an erythrocyte adjacent to an immature erythrocyte undergoing mitosis. (H.J. Barnes)

Hematology. Basophilic stippling and abnormal erythrocytes may occur in lead-poisoned birds but are not present in all affected birds (Figure 32.3) (249). Finding anemia with mitosis of erythrocytes and large numbers of immature cells may be more significant.

Pathology. Most lesions probably result from anorexia and debility. Emaciation may be prominent, but many ducks and geese that die from lead poisoning are in good body condition. The carcass may be pale with watery blood. Erosion and ulceration of the ventriculus (gizzard) lining can be extensive (Figure 32.4). Impaction of the proventriculus frequently is seen and is likely secondary to vagus nerve damage (Figure 32.5).

Microscopically, the most diagnostic lesions are demyelination of peripheral nerves and focal areas of vascular damage in the cerebellum (159), and acid-fast, intranuclear inclusion bodies in the kidney (Figure 32.6), liver, and spleen (200, 249, 278). Inclusions are composed of protein-bound lead and can be demonstrated by special staining or electron microscopy (Figure 32.7) (232). Nephrosis with degeneration and necrosis of tubular epithelial cells containing brown pigment has been described. Hemosiderosis is prominent in the spleen and other organs. Scattered myocardial necrosis associated with hyaline or fibrinoid necrosis of blood vessels



Figure 32.4 Ventriculus (gizzard) from duck with lead poisoning. Severe erosion, ulceration, and bile staining of koilin lining. Note 2 lead pellets retrieved from the gizzard. (H.J. Barnes)



Figure 32.5 Lead poisoning, showing distended proventriculus (arrow); there were 15 lead shots in the gizzard.

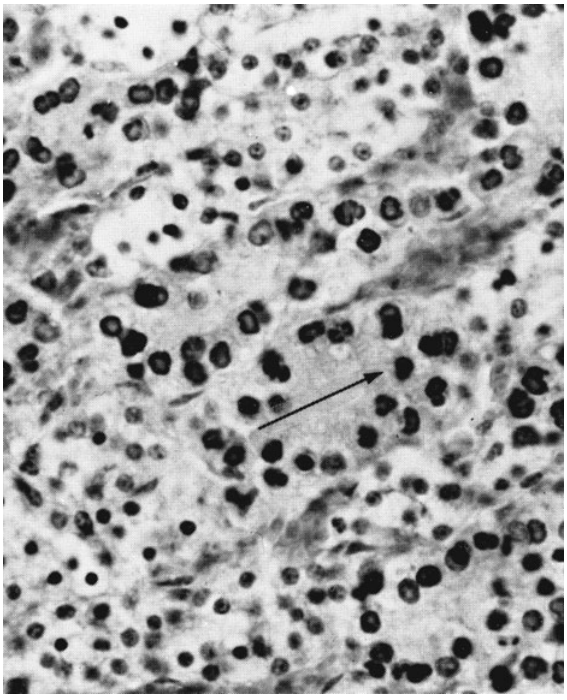
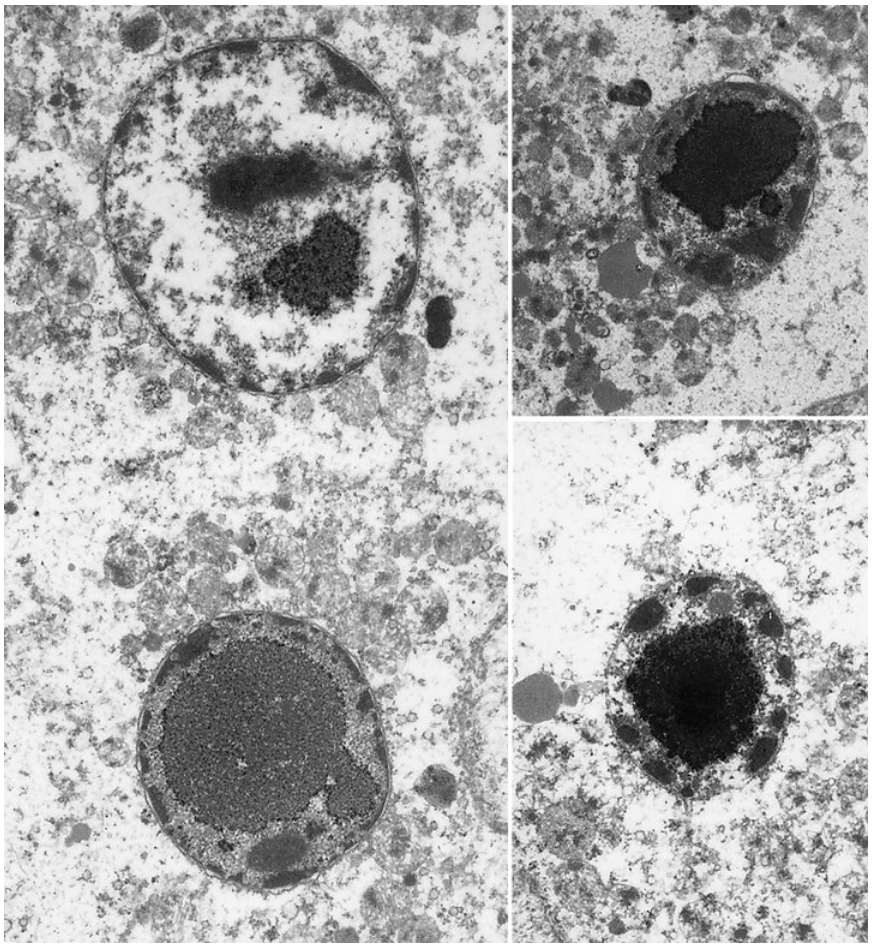


Figure 32.6 Lead poisoning. Acid-fast intranuclear inclusion bodies (arrow) in kidney of mallard duck. ×480. (L.N. Locke)

Figure 32.7 Proximal renal epithelium from a bird with lead poisoning. Nuclei contain irregular, variable electron-dense inclusion bodies typical of lead accumulation in kidney. Similar inclusions may be present in the liver. (H.L. Shivaprasad)



(173), arrested mitotic activity in proventricular epithelial cells, and degenerative changes in testes also may be found (209).

Diagnosis. The final diagnosis of lead poisoning is based on blood and tissue levels. In chickens, a blood lead level greater than 4 ppm, a liver lead level greater than 18 ppm wet weight, or a 20 ppm wet weight in kidney is considered diagnostic (266, 267). Lead levels in bone also can be determined. Acid-fast inclusions in kidney epithelial cells suggest lead poisoning but may be found in birds that could have ingested lead but died from some other cause. Peripheral nerve lesions, in conjunction with fibrinoid necrosis of blood vessels, which may be found throughout the body and not just in brain and heart, are useful in diagnosis, but similar changes are seen in methylmercury poisoning (280). In lead poisoning, however, lesions in the central nervous system are related only to vascular damage. In naturally occurring cases of lead poisoning in egg-laying chickens, egg yolk levels are highly correlated with blood levels whereas egg shell levels did not correlate. No lead was found within the albumen (329)

Mercury

Organic mercury, used previously as a seed protectant, is discussed later in this chapter along with fungicides. Most organic mercury in the environment today results from methylation by aquatic organisms and action of methogenic bacterial enzymes on elemental mercury from nature (decaying trees) or industry. Tons of mercury as bivalent inorganic mercury, elemental mercury, and phenyl mercury have been discharged into waterways around the world.

Methylmercury, a direct product of biotransformation, gets into small water organisms and enters the food chain when fish eat contaminated plants, insects, or animals (bioconcentration). Fish-eating birds, particularly ducks, may become poisoned from mercury in the food they eat (241). Mercury contamination of pheasants also has occurred. Experimental feeding of low levels of methylmercury resulted in decreased egg production, increased shell-less eggs, and reduced hatchability (241).

Residues in chickens given subclinical amounts of methylmercury were highest in liver, least in muscle, and intermediate in kidney. Eggs had 4 times as much mercury in albumin compared with yolk (241).

Inorganic mercury of medicinal or industrial waste origin may induce anorexia, enteritis, and nephrosis (241, 266, 267).

Tin

Tin from medicinal sources can cause depression, hunching up, and yellow diarrhea (303).

Uranium (Uranyl Nitrate)

Industrial uranium causes depression, anorexia, and nephrosis with severe lesions in collecting tubules, followed by hyperuricemia and visceral urate deposits in birds that survive (185).

Vanadium

Vanadium can contaminate phosphorus sources and cause reduced egg quality, growth, and hatchability (184, 266, 267). Also, there are many reports in the literature of experimental vanadium toxicity.

Vitamins

Vitamin A

Excess vitamin A reduces egg production (181) and growth rate and causes osteodystrophy and osteoporosis (323, 335).

Vitamin D_3 (Cholecalciferol)

Four percent mortality caused by kidney failure occurred in chicks when feed was top-dressed with vitamin D_3 powder. Nephrosis with focal mineralization was present throughout the kidneys. Mineralization was also present in the walls of arteries, particularly arteries in the proventriculus. Excess vitamin D_3 has resulted in increased incidence of leg abnormalities in broilers (61). Experimentally induced toxicity indicated that 25-hydroxycholecalciferol was 100 times as toxic as cholecalciferol (272). A variety of lesions were seen, but renal damage was most significant (223). Poultry, pigeons, and wild birds also may be poisoned by rodenticides in which the toxic agent is 25-hydroxycholecalciferol.

Vitamin B_6 (Pyridoxine)

Pyridoxine is toxic for pigeons at levels safe for poultry (90–100 mg/bird, i.e., approximately 200 mg/kg body weight given by injection) (251).

Other

Ethoxyquin

Ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinolone) is a commonly used antioxidant that may be toxic at high levels (6,500–12,500 mg/kg feed). Mortality is increased. Affected birds have pale, swollen kidneys, dark-brown, enlarged livers, and urates in joints. Proximal tubular necrosis in the kidney and accumulations of dark-brown pigment, interpreted to be ethoxyquin, in hepatocytes, bile ducts, and pulmonary blood capillaries are seen microscopically (196).

Lignosol

Calcium lignosulfonate, a pellet binder, may produce black, sticky cecal contents that adhere to the skin of

processed broilers, causing increased condemnation from contamination. It has no effect on body weight or feed conversion (265).

Nitrate and Nitrite

Nitrate is converted to nitrite by bacteria in the digestive tract and is much less toxic than nitrite. High levels of nitrate cause diarrhea, dyspnea, and death. Lower levels affect growth and egg production. Blood hemoglobin is changed to methemoglobin. The effect is greater in young birds, as it is in young mammals, with fetal hemoglobin (77). Most reports of toxicity are experimental, although there are some reports of toxic nitrate levels in leaves and stems of plants (354).

Pen- and Litter-Related Toxicants

Pen- and litter-related toxicants include products accidentally or intentionally incorporated into litter or applied to the pen that result in illness. Some are disinfectants and fumigants discussed later in this chapter. Except for boric acid, insecticides mixed into the litter (e.g., fire ant control products) or applied to walls, floor, or ceiling are covered later in this chapter. Copper sulfate, often used as a fungicide in litter, has been discussed with feed- and water-related toxicants previously. Toxic mixtures, such as copper-chrome-arsenic formulations, are used as preservatives in the timber industry (230). Occasionally, part of the building structure is toxic; geese have been poisoned from eating urea-formaldehyde foam insulation picked from the wall.

Boric and Orthoboric Acid

Boric acid is used in litter to control darkling beetles and may be consumed by broilers, which results in reduced growth and abnormal feathering (85, 281).

Iron

Ferrous sulfate heptahydrate added to litter to reduce ammonia formation was toxic to broilers (340). Affected chicks were depressed and lethargic. Those that died had severe ventriculus (gizzard) ulceration and liver degeneration. The LD_{50} of ferrous sulfate is 7,010 mg/kg body weight for a single dose. When added to the diet, 3% caused reduced growth and feed intake, and 1.5% had no effect (256).

Pentachlorophenol

Pentachlorophenol has been used as a pesticide in industry and agriculture, but its primary use is as a wood preservative. Logs may be treated before they leave the forest, or wood may be treated after cutting. Sawdust and shavings from treated wood frequently have been used as poultry litter; chickens can become contaminated from contact with these shavings. Because the product is used for many other purposes, pentachlorophenol may also contaminate broilers or table eggs in other ways.

Illness associated with pentachlorophenol has been caused by toxic impurities such as dioxins (see later discussion). Pure pentachlorophenol can reduce growth, cause kidney hypertrophy, and decrease humoral immune responses (263, 317). It has also been associated with a musty taste in eggs and broiler meat. Chlorophenols in litter are metabolized by bacteria and fungi to chloroanisoles. Anisoles have a musty or earthy odor even at very low concentrations, and they are responsible for the taste in eggs and meat from chickens in contact with contaminated litter (115). Reduced hatchability has also been associated with pentachlorophenol contamination (118).

Sulfur

Elemental sulfur may be sprinkled on dirt floors and vaporized by adding water and heating the building before litter is put down. Elemental sulfur is also used to control external mites in chickens (226). High mortality, ulcerative dermatitis primarily affecting moist areas of the body, irritation of respiratory mucous membranes, and conjunctivitis occurred in chicks placed in treated buildings (258). Lesions probably resulted from sulfur dioxide (from residual sulfur that had not vaporized) dissolving in moisture on the chick's body to form sulfurous acid (H_2SO_3).

Disinfectants and Fumigants

Fumigants are products producing toxic gases used to control rodents, insects, fungi, and bacteria. They can cause toxicity when inhaled or ingested. Phenolic disinfectants can be toxic when inhaled or absorbed through skin.

Phenolic Compounds and Coal-Tar Derivatives

Phenol, cresol, creolin, carbolineum, and creosote products cause damage to vascular endothelium, epithelia of respiratory and digestive tracts, and parenchymal organs, such as liver and kidney (249). Thymus and cloacal bursa are small, but this may result from stunting rather than being a direct effect on the immune system. Hydropericardium is prominent, but ascites and subcutaneous edema are also frequently

present if contact is severe. Mortality may be high. Diagnosis is based on a history of contact and by elimination of other causes of ascites and edema. Odor also may provide a useful clue. Cases of creolin toxicity still appear in the literature (194). Coal tar poisoning has been induced in ducks by feeding clay pigeons used in shooting sports (50).

Quaternary Ammonium (Cationic Detergents)

Use of sanitizers to clean poultry drinkers or treat water has resulted in reduced growth or production and, occasionally, severe lesions and death in young chicks (161). High levels of quaternary ammonia cause epithelial irritation of the mouth, pharynx, and upper respiratory tract, resulting in oral, ocular, and nasal discharges. Necrosis of epithelium leads to pseudomembranes in the mouth and epithelial thickening in the esophagus, crop, and proventriculus, with ulcers at the gizzard-proventricular junction (75, 249). Similar lesions have been reported in poults (208).

Chlorine

Low levels (37.5–150 mg/kg) may have a beneficial effect, but high levels (300–1,200 mg/kg) result in reduced growth and increased mortality (66).

Formaldehyde

Formaldehyde gas and formalin (a 37% solution of the gas in water, which is then 100% formalin) have been widely used for many years as antibacterial and antiviral agents in the poultry industry. Photophobia and respiratory signs from contact with high levels of formaldehyde are seen occasionally in newly hatched or recently delivered baby chicks and poults. Prolonged exposure to high levels of formaldehyde, which dissolves in liquids on mucous membranes to produce formalin, in the hatcher impairs cilial function and causes tracheal epithelial degeneration and sloughing (288). Air quality during subsequent grow-out, however, has a greater effect on productivity than does early formaldehyde exposure (287). Epithelial necrosis of eyes, mouth, and trachea with pseudomembranous plaques in the mouth and trachea also may be found. Edematous swellings under the lower beak (121), subcutaneous edema (28) during the acute phase, and ascites or edema occurred later in exposed poults.

Other Fumigants

It must be assumed that most or all chemicals used as fumigants are toxic to poultry (241). A few reports of deliberate or accidental poisoning of poultry by other fumigants appear in the literature (302, 347).

Fungicides

Fungicides are used as seed dressings (protectants), as wood preservatives, in paint and plastic, and on cereal crops, fruits, vegetables, and flowers. Previously, poisoning in poultry usually has resulted from the incorporation of treated seed into poultry feed.

Organic Mercurials

Mercurial fungicides, frequently ethyl or methyl mercuric chloride, that cause poisoning with central and peripheral nervous lesions in poultry, wild birds, animals, and humans consuming treated seed are no longer in use (145, 146, 278, 313). Signs of organic mercury poisoning may be nonspecific, or affected birds may show progressive paralysis or other neurologic signs. Specific gross lesions may be lacking, but microscopically, Wallerian degeneration of peripheral nerves and spinal cord and neuronal damage in the brain may be present. Vasculitis also may be obvious in some vessels, particularly in the brain.

Thiram

Arasan (active ingredient thiram, a dithiocarbamate) has caused poisoning in poultry, producing lameness and leg deformity in chicks and poults and soft-shelled eggs in layers (130, 249). It is also teratogenic (243). Thiram increases the incidence and severity of tibial dyschondroplasia (92). The LD_{50} is 485–932 mg/kg body weight in pheasants and 2,800 mg/kg body weight in mallard ducks (65).

Captan

Captan is an organic seed protectant. It is less toxic than Arasan. It depresses feed consumption, slows growth, and reduces egg production (249).

For descriptions of other organic synthetic fungicides, see (241). Pentachlorophenol, a widely used wood preservative, and copper sulfate, a litter treatment, were covered previously in this chapter.

Herbicides

Chlorates

Sodium and potassium chlorates used as herbicides and defoliants are moderately toxic for poultry. They act by converting hemoglobin to methemoglobin. The lethal dose for chickens is 5 g/kg (241).

Organic Synthetic Herbicides

Amitrate (3-amino 1,2,4-triazole) causes hypothyroidism and reduces weight gain in chickens (357). Phenoxy-herbicides, such as 2,4-D, cause kidney enlargement. Some herbicides are toxic for embryos (87). See (241) for additional information.

Dipyridyl Herbicides (Diquat and Paraquat)

Paraquat toxicity results from free radical-induced membrane damage caused by the inhibition of the glutathione peroxidase system. Selenium is protective. Experimental oral paraquat poisoning in turkeys produced diarrhea, listlessness, and anorexia with terminal convulsions. Gastroenteritis was present at necropsy (312). Turkeys are more resistant than mammals (143, 241), but exposure of mallard duck eggs produces cranial and pelvic deformities at hatch (285).

Insecticides

Insecticides may be referred to by either their common or registered name. The common name is not capitalized (e.g., carbaryl), but the trade name is (e.g., Sevin) (241). Organic insecticides (organophosphates, organochlorides, and carbamates) have been widely used, some on animals and birds as systemic larvicides and anthelmintics, as well as on buildings and pens. Wild birds have been poisoned by feeding on treated animals (35, 137). Many insecticides are quite toxic for animals as well as insects, arthropods, and helminths. Some more toxic products are used on crops, wood, and trees as soil insecticides, and as seed dressings. Extensive tables presenting general information on insecticides can be found in (241).

Organochloride Insecticides

The mode of action of organochloride (chlorinated hydrocarbon) insecticide toxicity is unknown. Generally, it acts to diffusely stimulate or depress the nervous system. Organochloride insecticides often remain longer in the environment than other insecticides. Some more persistent ones have been taken off the market or have restricted use. Because they are fat soluble, organochlorides tend to build up in the food chain and be present in yolks of eggs. There is considerable literature available on insecticide toxicity in wild birds, with emphasis on organochlorides (222).

Signs. Nervous signs varying from excitement with vocalization to tremors, ataxia, and convulsions are prominent. Prostration and death may occur without other signs. Other signs include salivation, vomiting, diarrhea, and depression. Lameness and leg deformity may also occur. There may be decreased egg production, a drop in hatchability, embryo mortality, loss of pigment on pigmented eggs, a change in shell texture (chalky), and eggshell thinning. Administration of atropine to acutely affected birds will not alleviate or modify signs of toxicity.

Pathology. Specific lesions do not occur in organochloride toxicity. Nonspecific changes such as congestion and hemorrhage may be present. Excess cerebrospinal fluid may be noted when the brains of affected birds are examined.

Chlordane

Chlordane causes chicks to develop ataxia and hyperexcitability. Hens have reduced body weight, decreased egg production, atrophy and cyanosis of the comb and wattles, and hydropericardium (249).

Dichlorodiphenyltrichloroethane (DDT) and Dichlorodiphenyltrichloroethane (DDE)

Dichlorodiphenyltrichloroethane (DDT) and dichlorodiphenyltrichloroethane (DDE) cause hens to develop tremors, decrease production, and lose weight, and there is eggshell thinning (249).

Dieldrin

Pigeons, gulls, and other birds exposed to dieldrin may show nervous signs (11, 249).

Heptachlor

Heptachlor may cause ataxia, salivation, prostration, and death (248, 339).

Lindane

Diarrhea, vomiting, anorexia, depression, convulsions, and sudden death have been associated with lindane poisoning (30, 249).

Mirex

Mirex has caused embryo mortality (3).

Toxaphene

Toxaphene may cause lameness, thin shells, and osteomalacia (245, 249).

Organophosphorus and Carbamate Insecticides

Organophosphorus and carbamate insecticides inhibit acetylcholinesterase, causing acetylcholine to accumulate, which results in the overstimulation of parasympathetic nerves and muscles (101). Atropine will often dramatically reverse clinical signs, but efficacy depends

on the type of organophosphorus compound ingested, the duration of the intoxication, and amount of time lapsed between exposure and treatment. Repeated treatments may be necessary. Some organophosphates and carbamates have delayed neurotoxic effects (see the following discussion). Chickens and other birds are more susceptible than mammals to this type of toxicity.

Signs. Chicks and poults may die quickly, showing few signs except dyspnea and paralysis, or they may exhibit lacrimation, salivation, diarrhea, tremors, depression, dullness, lethargy, cyanosis, ataxia, incoordination, and convulsions prior to death. Because of respiratory signs and salivation in early stages, respiratory infection may be suspected initially.

Pathology. Few gross lesions occur. There may be congestion with dark blood, and hemorrhages may be present in heart muscle, on serosal surfaces, and on mucosa of intestines. No specific microscopic changes have been identified.

Chlorpyrifos

Signs of chlorpyrifos toxicosis include salivation, lacrimation, gasping, frequent defecation, tremors, convulsions, and recumbency followed by death. The LD_{50} for broiler chickens has been cited as 10.79, 18.40, and 25–35 mg/kg (5, 220).

Diazinon

Diazinon is used to control fire ants and darkling beetles, but in birds, diazinon can cause incoordination, paralysis, respiratory signs, and death (150). It is also used to control pests in soil and grass, causing death in Canada geese (116, 316). The LD_{50} for diazinon in broiler chicks is 6.32 mg/kg (220).

Dichlorvos (DDVP)

Dichlorvos induces staggering, frothing from the mouth, paralysis, and convulsions (93) The LD_{50} in broiler chicks is 6.30 mg/kg (220).

Dimethoate

Toxic effects of dimethoate include reduced growth and egg production (297, 298).

Famphur

Mortality in raptors has resulted from famphur toxicity (148).

Fenthion

Laying hens exposed experimentally to fenthion had neurologic deficits followed by decreased egg production and reduced body weight (330).

Malathion

Malathion causes dullness, salivation, loose droppings, cyanosis, paralysis, and death. Lesions produced include injected subcutaneous vessels and dark, congested heart (40, 249). In geese, there may be flaccid paralysis.

Monocrotophos

Monocrotophos toxicity has been associated with salivation, mortality in quail, and weight loss and embryo abnormalities in chickens (304).

Parathion

Parathion induces lacrimation, salivation, dyspnea, tremors, and convulsions (249).

Carbamates

Various carbamates such as carbaryl, carbofuran, and others are toxic for pheasants, pigeons, turkey poults, chickens, and ducks (15, 94, 270). Signs include reduced growth, lameness, weakness, ataxia, and death. There may be tibial dyschondroplasia, retarded testicular development because of degeneration of seminiferous epithelium, nerve fiber degeneration, and congestion of organs and tissues.

Delayed Organophosphorus Neurotoxicity

Delayed neurotoxicity occurs several days to weeks after exposure, causing progressive degeneration of the peripheral nerves and spinal cord, which leads to weakness and paralysis. Acetyl cholinesterase is not affected. Delayed neurotoxicity may result from ingestion or absorption of: a variety of triaryl phosphates; chemicals found in phenylphosphorothioate insecticides such as leptophos, cyanofenphos, and their analogues; as well as a variety of industrial chemicals, including fire retardants and lubricants. Malathion and dimethoate also may cause delayed neurotoxicity. Mature chickens, pheasants, and mallard ducklings are highly susceptible (241). Chicks hatched after *in ovo* exposure also show clinical signs (102). There are many reports of delayed neurotoxicity in chickens from these products. Most describe experimentally induced lesions (1, 2, 89, 187, 202, 343). A previous author of this chapter saw turkeys in Ontario, Canada, with typical clinical signs of ataxia and paralysis and histologic lesions of delayed organophosphorus neurotoxicity in spinal cord and peripheral nerves. Clinical cases occurred in Europe after chickens ate scraps of synthetic leather containing tri-ortho-cresylphosphate (TOCP) (249).

Signs. Ataxia, falling sideways, inability to rise, lack of leg reflexes, and prostration may be evident. Birds appear bright and eat and drink if given access to food and water for several days.

Pathology. There are no gross lesions. Degeneration of axons and myelin in peripheral nerves and long tracts of the spinal cord are diagnostic. Axons may be swollen, and spheroids may be present in axon spaces. Digestion chambers containing macrophages and debris may be present in subacute cases.

Other Insecticides

Pyrethrum and Synthetic Pyrethroids

Pyrethrum and synthetic pyrethroids are not very toxic to animals or birds, and there are no reports of illness (55).

Rotenone

Rotenone (derris powder) is prepared from roots of *Derris* spp. Mature chickens are relatively resistant (lethal dose 1,000–3,000 mg/kg); young birds are more susceptible (241). Fish are very susceptible to rotenone.

Nicotine

Nicotine sulfate (Black Leaf 40) has been used to paint chicken roosts to control insects and arthropods, particularly northern fowl mite. It also has been used for internal parasites. In low doses, nicotine stimulates the nervous system through an acetylcholine-like activity. At toxic levels, neural transmission is blocked, causing death from respiratory paralysis (241).

Signs. Sudden death, occasionally preceded by depression and coma, is seen in affected birds.

Pathology. Because death is from respiratory failure, cyanosis and congestion may be marked. Hemorrhages may be present on the heart and in other tissues.

Rodenticides, Avicides, and Molluscacides

Rodenticides

Information on cholecalciferol and arsenic is presented in Nutrients and Other Feed- and Water-Related Toxicants.

Alpha-naphthyl Thiourea (ANTU)

Alpha-naphthyl thiourea causes depression, anorexia, weakness, prostration, and death. Lesions include pulmonary edema, hydropericardium, fatty change in liver, and myocardial degeneration.

Sodium Monofluoroacetate (Compound 1080)

Signs of sodium monofluoroacetate (Compound 1080) are reluctance to move, edema of wattles, dyspnea, cyanosis, and nervous signs. Lesions include dark, unclotted blood, pulmonary hemorrhage and edema, clotted blood in the trachea and air sacs, petechiation, enteritis, and hydropericardium (143, 241).

Strychnine

Toxic effects of strychnine are tonic spasms, respiratory failure, reproductive failure, and increased mortality of progeny from exposed hens (241, 249, 250, 359).

Warfarin, Brodifacoum, and Diphacinone

Warfarin, brodifacoum, and diphacinone are anticoagulant rodenticides sold under a variety of trade names and may be combined with sulfaquinoxaline to interfere with vitamin K synthesis. They inhibit epoxide reductase, which converts vitamin K to its active form. Toxicity causes anemia with fluttering, gasping, and hemorrhages in eyes, mouth, and other tissues (16, 143, 241, 300). Onset may be rapid, and death occurs within 72 hours of ingestion (225). With the long-acting anticoagulants, there is a cumulative effect, and if a small amount of anticoagulant is consumed repeatedly, there may be little or no product found in the digestive tract.

Phosphorus

Elemental yellow, red, and white phosphorus can induce depression, anorexia, diarrhea, ataxia, gastroenteritis, and death (241, 249, 314, 315).

Zinc Phosphide

Weakness, diarrhea, opisthotonos, and convulsions occur with zinc phosphide. There is enteritis, ascites, and hydropericardium (138, 300). In an accidental poisoning of broiler breeder chickens, birds were found dead following ingestion of zinc phosphide-treated oats used as rodent bait. No clinical signs were observed. Gross lesions consisted of hydropericardium and ascites. A petroleum-like odor, a common feature with this poison, from the crop contents was detected. Histologic lesions were those of congestion of many internal organs, along with severe pulmonary edema and congestion (328). Wild birds may also succumb to this poison if they are allowed access to zinc phosphide-treated grain (29, 261).

For birds, the toxic doses of several rodenticides (a-chloralase, crimidine, pyriminil, phosphorus, a-chlorohydrim) are given in *Clinical and Diagnostic Veterinary Toxicology* (241).

Avicides

Avitrol (4-aminopyridine or 4-AP)

Avitrol causes disorientation and vocalization (distress calls). Affected pigeons may be molested by normal pigeons. Generalized congestion is present at necropsy, and the characteristic small pellets usually can be found in the ventriculus (114, 227).

2-chloro-4-acetotoluidine (CAT) and 3-chloro-P-toluidine (CPT)

No clinical signs have been described from 2-chloro-4-acetotoluidine (CAT) and 3-chloro-P-toluidine (CPT), but kidney necrosis (from CAT) and liver and kidney necrosis (from CPT) occur (122).

Molluscacides

Metaldehyde

Nervous signs were prominent in ducklings following ingestion of metaldehyde (12).

Toxic Gases

Ammonia

Ammonia levels should be less than 25 ppm, but in poorly ventilated litter-type houses, ammonia may exceed 100 ppm (172). High levels of ammonia (50–75 ppm) reduce food consumption and growth rate (70). Egg production is also reduced. Ammonia dissolves in the liquid on mucous membranes and eyes to produce ammonium hydroxide, an irritating alkali causing keratoconjunctivitis. If levels greater than 100 ppm persist, corneal ulceration and blindness can occur. The condition is painful, and photophobia and stunting are marked. At levels of 75–100 ppm, changes in respiratory epithelium include loss of cilia (242) and increased numbers of mucus-secreting cells (10). Heart rate and breathing may be affected, and there may be hemorrhages in trachea and bronchi. For a review, see (49).

Carbon Monoxide

Carbon monoxide (CO) poisoning may occur in buildings in which defective or unventilated gas-catalytic or open-flame brooders or furnaces are in use, or where poultry are exposed to internal combustion-engine exhaust fumes. Affected chicks or poults show drowsiness, labored breathing, and incoordination. Spasms and convulsions may occur prior to death. At postmortem, blood is bright red. Sublethal levels cause stunting (249, 318). In suspected cases, CO should be measured at several locations in the pen with the ventilation system shut off. Carboxyhemoglobin can be measured in the blood of affected birds. A previous author of this chapter found levels of 70 ppm CO in pens where a repeated high incidence of ascites caused by pulmonary hypertension and right ventricular failure occurred. Toxic levels of CO for chickens are as follows: 600 ppm for 30 minutes causes distress; 2,000–3,600 ppm is lethal in 1.5–2 hours (241).

Aerial Endotoxin

Breakdown of bacteria in the litter and environment results in endotoxin in the air in broiler pens. Endotoxin from inhaled air can be found in the blood of people who work in broiler pens (82). Pulmonary hypertension develops in broiler chickens given intratracheal endotoxin and could be a contributing factor in ascites syndrome development (201).

Polytetrafluoroethylene (PTFE)

Fluorinated gases are released when polytetrafluoroethylene (PTFE), used as a nonstick coating (Teflon) on light bulbs, cookware, and ovens, is overheated. Pet birds that inhale the fumes die from lung edema and hemorrhage. Histologic examination reveals epithelial necrosis in tertiary bronchi or parabronchi and vascular damage in blood capillaries (319, 345). PTFE-coated heat lamp bulbs can cause mortality in exposed poultry (34, 307).

Other Toxic Gases

Levels of methane, carbon dioxide, hydrogen sulfide, methyl mercaptan, and dimethyl disulfide were found to be low in poultry and other livestock buildings in Finland (175), but toxic fumes from liquid manure pits in pig barns have killed humans and pigs in North America, and nitrogen dioxide formed in freshly filled silos has killed humans and animals in Canada and the United States. Toxic gases associated with livestock production, including poultry, have been reviewed (241). The effect of sulfur dioxide in chickens has been described (103).

Household and Commercial Products

Alcohol

Ethyl alcohol may be used to dissolve experimental chemicals or drugs given to poultry in feed or water. Clinical signs of intoxication include ataxia and reduced feed consumption. Fatty change in the liver and heart lesions may occur (9, 62). Wild birds frequently are intoxicated from ingesting fermented fruit (106). Pet birds may obtain alcohol inadvertently, or it may be given by the owner.

Antifreeze (Ethylene Glycol)

Ethylene glycol is toxic when ingested because it breaks down to oxalic acid, which combines with calcium to form calcium oxalate. Calcium oxalate crystals block renal tubules and cause tubular epithelial necrosis leading to hyperuricemia and urate nephrosis with visceral

urate deposits. Diagnosis usually is based on finding typical crystals and tubular changes on microscopic examination of kidney (268, 279, 280, 320). Liver necrosis is found in pigeons. Other forms of toxicity may occur in other species (241). Coccidia oocysts treated with ethylene oxide were toxic to chicks and caused kidney lesions similar to ethylene glycol (346). Clinical signs of accidental ingestion in a leghorn chicken consisted of sudden onset of depression, ataxia, convulsions, and death; pale kidneys were present at necropsy (362).

Carbon Tetrachloride

Carbon tetrachloride has been used previously as a household solvent and cleaner. It also has been used to treat tapeworms in chickens (280). It is toxic to animals and birds, interfering with fat metabolism and causing liver and kidney damage. Chickens are more resistant than rats, but low levels cause decreased growth.

Fertilizer

Lawn, garden, and farm fertilizers contain nitrogen, phosphorus, and potassium. The contents of the latter three, in that order, usually are given by numbers representing each element as a percentage of the total. These elements have been discussed previously. Fertilizer may be attractive to birds because it is frequently in the form of small, hard pellets. Some phosphate fertilizers contain very low levels of radioactive material.

Naphthalene

Mothballs frequently are recommended to keep pets and other animals away from gardens or out of attics. They also have been used in chicken nests for ectoparasite control. Mothballs are toxic and can cause poisoning in poultry and pet birds (182).

Urea

Urea is relatively nontoxic for birds. Because it is used in feed preparations for ruminants, the pellets occasionally are found in poultry feed.

Industry-Related Toxicants

Toxic Fat Syndrome, Chick Edema Disease, and Dioxin Toxicity

More than 30 years ago, the most toxic dioxin, 2,3,7, 8-tetrachl orodibenzodioxin (TCDD), and other dioxins in the same polychlorinated dibenzodioxin group, were found as contaminants in industrial fat (tallow from cattle hides) added to poultry feed. This material, which could be distilled from fat, was called chick edema factor until it was identified. It caused widespread disease in the broiler industry and in other poultry for several years. Occasional cases of dioxin toxicity (chick edema disease) occurred until about 1970. More recently, TCDD toxicity followed environmental contamination in Italy, where adult fowl died with lesions of chick edema disease (260). Dioxin was probably the material causing lesions in toxicosis caused by paint containing chlorinated hydrocarbon (210). In 1999, the Belgian polychlorinated biphenyl (PCB)/dioxin crisis occurred where they were accidentally added to recycled fat being used for animal feed. Although being fed to multiple farm animal species, poultry had the highest concentration of those elements (59).

Chickens are more susceptible than some mammals to the toxic effects of TCDD (241). In chickens, dioxin damages vascular endothelium, causing vascular leakage and extensive movement of fluid into body cavities and subcutaneous tissue. The epithelium of some parenchymal organs is damaged, and there is degeneration of heart and skeletal muscle.

Because right ventricular hypertrophy and dilation have been described (8), and many lesions in dioxin toxicity are similar to right-sided heart failure from other causes, the possibility of dioxin contributing to right ventricular failure should be considered.

Depending on the level of TCDD in feed, variable numbers of broilers in a flock will show severe signs of stunting, respiratory distress, weakness, ataxia, and edema. Mortality occasionally can be very high. For a description and review of the syndrome, see (249).

TCDD may be present as a contaminant in herbicides. It and other dioxins are produced by incineration (241) and by industry (96). Contaminated poultry carcasses potentially could serve as a route for human exposure because dioxins are retained in body fat after exposure.

Polybrominated Biphenyl (PBB) and Polychlorinated Biphenyl (PCB)

Polybrominated biphenyls (PBBs) or PCBs accidentally may be added to feed, get into feed as contaminants (as in oil or grease from equipment), or be present in the environment from industrial contamination and deliberate dumping. Both PBBs and PCBs are toxic to birds. At low levels, they reduce production, reproduction, hatchability, offspring viability, and thyroid hormone levels (112). Hepatocyte damage and lymphocyte depletion in the cloacal bursa also occur with low-level PBB toxicity (74). Exposure of young embryos magnifies these effects (110). Residues may be found in eggs and meat from birds without clinical signs. PBBs are concentrated in eggs at 1.5 times the dietary level (96). At high levels, lesions of

PCB toxicity are similar to dioxin toxicity. It is likely that in some cases, PCBs were contaminated with TCDD.

Crude Petroleum and Oils

Most information on toxicity of oil to birds deals with environmental contamination and the effect of oil spills on waterfowl. Ingested oil causes anorexia, weight loss, incoordination, tremors, and Heinz body anemia. Lesions include lipid pneumonia, enteritis, hepatosis with fatty infiltration, and nephrosis and degeneration of pancreas, spleen, and bursa (231). Immune responses are impaired. In herring gulls and puffins, lesions suggested a primary toxic hemolytic disease (193) with lymphoid depletion being secondary and stress related. Oil applied to chicken eggs caused embryo mortality and lesions in organs (58). Oils and oil products on the feathers and skin can be removed with detergents.

Biotoxins

Biotoxins are poisonous substances produced by living organisms including bacterial toxins such as botulinum toxin, which in birds is frequently associated with toxin-contaminated maggots, bacterial food poisoning, diseases such as necrotic and ulcerative enteritis, gangrenous dermatitis, and mycotoxins. It is possible that even methylmercury produced by bacteria should be classed as a biotoxin. Insect and snake venoms (189) are also biotoxins. Most of these conditions are of little importance or are discussed in other parts of the text. Botulism is increasing in frequency in housed turkeys and broilers. Birds are very susceptible to botulinum toxin and may show clinical signs following ingestion of very small amounts. The toxin apparently develops in dead birds left in the litter. Birds may pick up the toxin from eating decaying tissue or contaminated fly larvae, darkling beetles, or litter. An enzyme-linked immunosorbent assay (ELISA), which may be as sensitive as the mouse-inoculation test, has been developed to identify botulinum toxin (see Chapter 22). Only algae poisoning and rose chafer toxicity are mentioned here.

Algae

Several species of blue-green algae produce a toxin that, when concentrated by rapid algal growth (bloom) in warm bodies of fresh water and a constant light wind blowing the toxic material to the side of the lake, may poison animals and birds consuming it. Toxicity varies directly with concentration (162). Affected chickens may show nervous signs and paralysis before death. Ducks and turkeys also have been poisoned (157). Cyanosis, congestion, and a dilated, distended heart may be seen at necropsy (249). The liver is swollen with necrosis of hepatocytes. Diagnosis is based on identifying the toxin in the water (241).

Rose Chafers

Rose chafers (*Macrodactylus subspinosus*) are insects appearing in spring and early summer in eastern and central North America. Young chicks may be poisoned by 15–30 insects (249). Clinical signs include drowsiness, weakness, prostration, and convulsions (249).

Phytotoxins

All or parts of some plants are toxic, or if fed at low levels may only reduce growth rate. Antinutrients are discussed in the section Nutrients and Other Feed- and Water-Related Toxicants. For additional information on plants that are toxic to poultry and pet birds, see (13, 79, 81, 86, 111, 177, 306, 355).

Avocado (*Persea americana*)

The fruit contains a toxin causing muscle degeneration, hydropericardium, and subcutaneous edema (43, 139).

Black Locust (*Robinia pseudoacacia*)

The leaf of black locust contains a toxin causing listlessness, paralysis, and hemorrhagic enteritis.

Bladder Pod (*Sesbania [Glottidium] vesicaria*)

The bladder pod seed contains a toxin and consumption causes diarrhea, cyanosis, prostration, necrotic enteritis, and gizzard ulceration (98).

Cacao (*Theobroma cacao*, Theobromine Toxicity)

The bean waste from processing of cacao is toxic. In acute cases, nervous signs appear and are followed by convulsions and death, cyanosis, cloacal prolapse, and mottled kidneys. In chronic cases, anorexia and diarrhea occur (249).

Cassava (*Manihot* spp., Cyanide, Polyphenols)

The cassava root (tuber) is toxic with consumption causing sudden death and depressed growth (97, 247).

Carolina Jessamine (*Gelsemium sempervirens*)

The entire Carolina jessamine plant is toxic. Ingestion causes progressive muscle weakness, seizure activity, and death. Lesions reported include reduced muscle mass

and presence of identifiable leaves in the proventriculus. Histologically, there was neuronal degeneration and Purkinje cell loss and perimysial fibrosis (327).

Castor Bean (*Ricinus communis*)

The castor bean is toxic and consumption produces progressive paralysis with prostration (as with botulism), diarrhea, emaciation, swollen pale mottled liver, hemorrhagic catarrhal enteritis, petechiae, degeneration of lymphoid tissue and parenchymal cells of liver and kidney, and bile duct proliferation (164, 235, 249).

Coffee Senna, Sickle Pod (*Cassia occidentalis, C. obtusifolia, Senna occidentalis*)

The seeds of coffee senna are toxic. Consumption produces weakness, ataxia, paralysis, decreased egg production, diarrhea, and toxic myopathy in pectoralis and semitendinous muscles evident as pale coloration. Histologically, the lesions are edema, muscle degeneration, and necrosis (108, 244, 249, 310, 336). There is also primary axonal damage producing a neuropathy that adds to the paresis (44, 140). Lymphoid populations in the spleen and cloacal bursa also may be decreased (311). Experimental studies of *Senna occidentalis* seeds in laying hens revealed no clinical signs in treated groups although there was decreased egg production and leaking of yolk material around follicles. The vitelline membrane was dysplastic (124).

Corn Cockle (*Agrostemma githago*, Githagenin Toxicity)

The corn cockle seed is toxic with consumption causing: listlessness, rough feathers, decreased respiratory and heart rate; diarrhea; depressed growth; hydropericardium; and caseous necrosis of crop, pharyngeal mucosa, and mouth (149, 249).

Cotton Seed Meal (Gossypol Toxicity)

Toxicity from cotton seed meal results in cyanosis, inappetence, emaciation, reduced egg production and quality, enteritis, degeneration of liver and kidney, and reduced growth (249).

Coyotillo (*Karwinskia humboldtiana*)

The coyotillo fruit and seed are toxic, with consumption causing depressed growth, cyanosis, and paralysis.

Crotalaria spp. (Pyrrolizidine Alkaloids, Monocrotaline Toxicity)

The seed, leaf, and stem of *Crotalaria* are toxic with consumption causing dullness, inactivity, reduced feed consumption, stunting, bright yellow-green urates, subcutaneous edema, ascites, hydropericardium, lung edema, hepatitis, and bile duct hyperplasia (7, 51, 78, 249, 354).

Daubentonia (*Daubentonia longifolia, Sesbania drummondii, S. macrocarpa*)

The daubentonia seed is toxic with consumption causing weakness, listlessness, stunting, diarrhea, emaciation, proventriculitis with ulceration, enteritis, and liver and kidney degeneration (107, 109, 205, 296).

Death Camas (*Zygadenus* spp.)

The leaf, stem, and root of death camas are toxic with consumption causing weakness, salivation, diarrhea, and prostration (229).

Eucalyptus cladocalyx (Cyanide or Prussic Acid)

The *Eucalyptus cladocalyx* leaf is toxic with consumption causing acute death without premonitory signs.

Hemlock (*Conium maculatum*, *Conine Toxicity*)

The hemlock seed is toxic with consumption resulting in salivation, weakness, nervous signs, paralysis, diarrhea, reduced growth, hepatic congestion, and enteritis (113).

Jimsonweed (*Datura stramonium, D. ferox,* Scopolamine, Hyoscyamine)

The jimsonweed seed is toxic and consumption causes reduced growth (183).

Leucaena leucocephala (Mimosine Toxicity)

Mimosine is the toxin and is contained in the leaves. Consumption results in depressed growth and osteopathy (144, 171).

Lily of the Valley (*Convallaria majalis*)

Flowers, leaves, and stems of lily of the valley are toxic to geese, ducks, and chickens (249).

Milkweed (*Asclepias* spp. Asclepidin Toxicity)

Consumption of milkweed results in weakness and incoordination, convulsions, and prostration, which may lead to death or recovery (249).

Nightshade (*Solanum nigrum*, Belladonna Toxicity)

The immature nightshade fruit is toxic and consumption can cause dilated pupils, incoordination, and prostration (136).

Nitrate (Numerous Plant Species)

See the previous discussion on nitrates and nitrites.

Oak (*Quercus* spp., Tannin Toxicity)

The oak leaf is toxic. With consumption, there is severe diarrhea, anorexia, increased water consumption, enteritis, swollen kidneys, visceral gout, and diffuse necrosis of proximal renal tubules (180).

Oleander (*Nerium oleander*, Glycosides)

The entire oleander plant is toxic and consumption can result in listlessness, weakness, diarrhea, gastroenteritis, liver degeneration, and death (249, 322). Experimental toxicosis causes hypersalivation, vomiting, diarrhea, depression, and death. Gross lesions consisted of congestion and hemorrhage, whereas histologic lesions were those of myocardial necrosis, necrotizing hepatitis, and renal tubular necrosis with hemorrhage (238).

Onions, Green (*Allium ascalonicum*)

All parts of the green onion plant can be toxic. Excess consumption can cause sudden death with epicardial hemorrhage and pallor, hydropericardium, and hepatosplenomegaly. Microscopic lesions seen include hemosiderin in hepatocytes, Kupffer cells, and renal tubules. Onions are known to produce a Heinz body anemia in animals but were not found in geese (60).

Oxalate (Numerous Plant Species, Oxalic Acid)

The leaf and stem of many plants are toxic with consumption causing oxalate nephrosis (353). Also see the previous discussion on ethylene glycol.

Parsley, *Ammi majus*, Other Types of Plants (Photosensitization)

All parts of parsley can be toxic with production of dermatitis (unfeathered areas) and hepatitis (252, 301).

Pokeberry (*Phytolacca americana*)

The pokeberry fruit is toxic and can produce ataxia, leg deformity, and ascites (18).

Potato (*Solanum tuberosum*, Solanine Toxicity)

Green or spoiled potatoes, peelings, and sprouts can be toxic with production of incoordination and prostration (teratogenic) (136, 325).

Ragwort (*Senecio jacobea*, Pyrrolizidine Alkaloid)

All parts of the ragwort plant can be toxic with toxicity producing focal hepatic necrosis and portal fibrosis (52).

Rapeseed Meal (Erucic Acid/Glucosinolate Toxicity; Antinutrients Sinapine, Tannin, Phytic Acid); Canola at Low Glucosinolate Levels

The rapeseed seed can be toxic with production of abnormal odor and taste in eggs, hypothyroidism, depressed growth, anemia, sudden death, ruptured liver, hepatitis, ascites, hydropericardium, periacinar hepatic necrosis, and fatty change in skeletal and heart muscle (23, 27, 45, 57, 104, 125, 174, 271, 352).

Sweet Pea (*Lathyrus odoratus*, Lathyrism) and Related Species (*Lathyrus* spp.)

The sweet pea seed (pea) can be toxic, causing skeletal deformity, osteolathyrism (*L. odoratus*), or neurologic disease, neurolathyrism (*L. sativus*, Indian pea) (53, 224, 269).

Tannins (Numerous Plant Species)

Tannins are antinutrients that occur in a variety of plants. It may be important to determine tannin levels in some feedstuffs such as sorghum (163, 324, 342).

Tobacco (*Nicotiana tabacum*; Nicotine Sulfate Toxicity)

The tobacco leaf and stem can be toxic, producing stunting and reduced production (teratogenic) (249).

Velvetweed (Malvaceae Family, Cyclopenoid Fatty Acids)

The velvetweed seed can be toxic with consumption causing pasty and rubbery yolk in eggs (179).

Vetch (*Vicia* spp., Cyanogenic Glycoside)

The vetch seed (pea) can be toxic, producing excitability, respiratory distress, and convulsions (141, 276).

Yellow Jessamine (*Gelsemium sempervirens*)

The whole yellow jessamine plant can be toxic with consumption depressing growth (249, 356).

Yew (*Taxus* spp., Taxine Toxicity)

All parts of the yew are toxic with consumption producing labored breathing, incoordination, collapse and cyanosis (168).

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Section VII

Other Diseases

Chapter 33 Emerging Diseases and Diseases of Complex or Unknown Etiology 1385

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Emerging Diseases and Diseases of Complex or Unknown Etiology

Introduction

Yehia M. Saif and David E. Swayne

The emergence of new diseases and the re-emergence of recognized diseases are familiar events in the annals of poultry medicine. Some of these emerging diseases could have been present earlier but were not recognized because of low prevalence, mild signs and lesions, lack of diagnostic techniques, or misdiagnosis. In other situations, genetic changes in infectious agents could have rendered them more virulent or pathogenic. Similarly, genetic changes in the bird could have altered its susceptibility and resistance to disease. In addition, changes in environmental conditions or management could result in conditions that are favorable for an infectious agent to express pathogenic properties. Because of the global activities of the poultry industry, resulting in the continual movement of personnel, live birds, eggs, and poultry products across political borders, it is difficult to contain an emerging or re-emerging disease to a country or a continent. Hence, it is necessary to maintain a vigilant attitude toward poultry health and to sustain capable diagnostic facilities.

There are disease conditions that have multifactorial etiologies including combinations of infectious agents and, at times, infectious agents plus nutritional or environmental factors. Examples abound of seemingly harmless microbes that do not cause disease in healthy individuals but can express pathogenic potential following an insult, although it might be mild, to the host. *Escherichia coli* is a prime example of such a microbe, earning it a designation of a universal secondary infection in poultry. In the commercial poultry environment, viruses and bacteria, including some that have the potential of causing disease, are common. Live vaccine viruses, some of which are very mild pathogens, may also be present.

In addition, flocks that are immunocompromised because of infectious or noninfectious agents could present unusual disease syndromes, increased susceptibility to disease, or lack of responsiveness to vaccination. The combinations of etiologies of disease could result in additive or synergistic effects. The pathogenesis of the multiple etiologies is not completely understood, but some mechanisms have been suggested or shown to occur.

The upper respiratory tract and the gastrointestinal tract are bombarded continually by a variety of microbes; yet, disease is not necessarily a common event. Natural and acquired defense mechanisms function efficiently to eliminate infections, inhibit replication, or prevent colonization of tissues by microbes. The mucociliary apparatus of the respiratory tract is a highly efficient system for the elimination of microbes and particulate matter. Some viral infections result in the deciliation of parts of the respiratory tract and lysis of infected cells, resulting in accumulation of cellular products and debris, creating an environment favorable for bacterial multiplication and attachment to cells, which are important events in the pathogenesis of bacterial infections. In the gastrointestinal tract, similar events initiated by viruses have been described, including villous atrophy and consequent increased bacterial replication and adherence to cells. It is a common finding in respiratory and enteric diseases of poultry to encounter a variety of infectious agents. Because of the possible complex etiology of respiratory and enteric disease, it is important to understand the role of the different agents in the disease process. Such understanding should be helpful in designing logical control or prevention strategies.

This chapter was first introduced in the 10th edition of *Diseases of Poultry* with the realization that there will be continuous change in its content. Some of the diseases recognized as emerging at a point of time become either established entities or fail to have a sustained impact. In either case, the outcome will be the removal of these diseases from this chapter. For this edition, Hypoglycemia-Spiking Mortality Syndrome of Broiler Chickens, and Proventriculitis and Proventricular

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Dilatation of Broiler Chickens have been eliminated because of little new information; the reader is referred to the 13th edition for details

In this chapter, information is presented on the complexity of respiratory and enteric diseases. In addition, information is presented on 4 emerging conditions: White Chick Syndrome, Focal Duodenal Necrosis in Table Egg Layers, Wooden Breast and Other Muscle Abnormalities, and Idiopathic Egg Production Drops in Brown Layers. The etiology of these conditions has not been definitely established although possible infectious causes are speculated.

Multicausal Respiratory Diseases

Mary J. Pantin-Jackwood and Erica Spackman

Summary

Agents, Infection, and Disease. Respiratory diseases are a common cause of economic losses in poultry production worldwide. Although much is known about the individual agents responsible for respiratory diseases in poultry, uncomplicated infections with single agents are the exception in the field. Under commercial conditions, complicated infections involving multiple etiologies including viruses, mycoplasmas, bacteria, immunosuppressive agents, and unfavorable environmental or management conditions, are more commonly observed than simple infections. Vaccination with live attenuated agents is a common practice in commercial poultry operations, and respiratory reactions induced by routine vaccination programs play a role in the development of complicated respiratory disease. Additional research on the pathogenesis of respiratory diseases is needed to understand the complex interactions among host, pathogens, and environment more fully.

Interventions. Good management, proper vaccination, and biosecurity help minimize respiratory disease caused by interactions of multiple agents.

Interactions Among Respiratory Pathogens

Coinfection of poultry with more than 1 respiratory disease agent is common and often results in more severe clinical signs when compared with single agent infections. Live agent vaccines are routinely used in commercial poultry to control respiratory disease, so infections with more than 1 respiratory agent are unavoidable. Coinfections present a complex clinical picture and not much is known about the interaction between coinfecting pathogens. In most cases, coinfections result in exacerbation of clinical signs; however, in some cases infection with 1 pathogen can interfere with infection with a second pathogen, thus ameliorating clinical signs. The timing of exposure to the infectious agents also affects the outcome of complicated infections. Although some of the mechanisms of these interactions are known, including damage to the respiratory epithelial integrity and reduction of the mucociliary transport, much more research needs to be done to understand interactions between infectious agents more fully to inform control programs for respiratory diseases.

Coinfection of poultry with different respiratory viruses commonly occurs in commercial settings (97, 101), and different viral coinfections have been investigated under laboratory conditions. In many cases, the disease caused by a virus in the laboratory is different than what is observed in the field. For example, low pathogenicity avian influenza virus (LPAIV) causes subclinical to mild respiratory disease in the laboratory, but in commercial poultry is frequently associated with moderate to severe clinical signs indicating a role of coinfection with other pathogens or concomitant stressors on the severity of the disease (reviewed in [116]) (102). For example, the Mexican lineage H5N2 LPAIV and the H9N2 LPAIVs from Asia, the Middle East, and Africa, can cause significant and economically important disease in chickens, with tracheal plugs and bronchial casts commonly reported (78, 79). However, when using LPAIV strains from these outbreaks, these lesions have not been reproduced experimentally with LPAIV alone.

Infectious bronchitis virus (IBV) live vaccines have been shown to increase the severity of H9N2 LPAIV infections in commercial chicken operations (48), and experimentally, coinfection of chickens with IBV and H9N2 LPAIV induced severe clinical signs with bronchial cast formation and more severe histological lesions than either of the viruses given alone (38, 41, 45). These studies indicate that coinfection of LPAIV with other respiratory viruses can increase the severity of the disease in the field. Interactions between LPAIV and other infectious agents, including mycoplasmas, bacteria, and chlamydia, have also been associated with increased respiratory disease in both chickens and turkeys (8, 12, 15, 53, 83, 89, 102, 106, 107).

Natural coinfections of avian influenza virus with avian avulavirus 1 (commonly termed Newcastle disease virus [NDV] and used hereafter) occur commonly in

poultry in countries in which these viruses are endemic (9, 85, 97). Field observations indicate that the impact of virulent NDV is more severe in countries with concomitant circulation of LPAIV (10). Experimental exposure of chickens to H9N2 LPAIV affected subsequent infection with a virulent NDV, showing that the H9N2 virus increased the susceptibility to NDV, exacerbating the clinical outcome whereas delaying the onset of the disease and time of death in a dose-dependent manner (10). In turkeys, nonvirulent NDV interacted with avian metapneumovirus (aMPV) to cause more severe disease compared with turkeys infected only with aMPV or NDV (115).

Interference among respiratory viruses has also been reported. Interference is a phenomenon in viral infections where a cell is temporarily resistant to infection with other viruses soon after infection with certain viruses. The mechanisms of viral interference are varied and include direct competition for viral receptors on the cell surfaces, intracellular competition for replication host machinery, and induction of innate immune responses that block further viral infections. Research has shown that IBV can interfere with the replication of NDV and aMPV, including live vaccines (18, 33, 40, 90, 111). NDV vaccination delayed the replication of live aMPV vaccine and also reduced the humoral antibody responses to the vaccine (31). The serological response to aMPV codelivered with IBV and NDV vaccines was lower than when the aMPV was given alone (7, 109). Coinfection of chickens and turkeys with NDV vaccine strain and LPAIV affected the replication dynamics of the viruses but did not alter clinical signs (21). However, virulent strains of NDV interfered with the replication of highly pathogenic avian influenza virus (HPAIV) in chickens, with increased survival observed in coinfected birds. This effect depended on the strain and titer of the viruses, and the timing of the infections (19, 20).

Another good example of multiple respiratory infections is those involving mycoplasmas; this subject has been reviewed previously (11, 47). Interactions with IBV or NDV are known to increase the severity of *Mycoplasma gallisepticum* (1, 81, 112, 128) and *M. synoviae* infections (43, 44, 52, 56, 105, 125). However, in 1 study, concurrent IBV vaccination had a negligible effect on the pathology induced by *M. gallisepticum* (61). Experimental coinfection with LPAIV significantly enhanced the pathogenesis of *M. gallisepticum* (106, 107). Similarly, experimental coinfection of turkeys with aMPV and *M. gallisepticum* increased clinical signs and reduced weight gain (75). Synergistic effects between *Avibacterium paragallinarum* and *M. gallisepticum* are also well known (1, 49, 59, 76). Three-way interactions between vaccine virus (NDV and/or IBV), mycoplasma (*M. gallisepticum* or *M. synoviae*), and *Escherichia coli* resulted in more severe respiratory disease than any 2 alone (74, 105). *M. gallinarum*, ordinarily considered to be nonpathogenic, induced airsacculitis in broilers when given in combination with NDV/IBV vaccine virus (57). Interactions with various agents have also been described for *M. meleagridis* in turkeys. Enhanced airsacculitis was observed in poults challenged with *M. meleagridis* and *E. coli* (99). Combined infections with *M. meleagridis* and *M. iowae* caused more severe airsacculitis than either agent alone (93). Pathogenicity also increased when IBV was combined with *M. imitans* in chickens (30); *M. imitans* also shows a similar effect when combined with *Ornithobacterium rhinotracheale* (ORT) in turkeys (32).

Interactions between respiratory viruses and bacteria are also common. For example, the pathogenicity of NDV in broilers was enhanced when occurring in combination with ORT (114). ORT has been isolated in coinfections from chickens with H9N2 LPAIV (83). Turkeys exposed to the LaSota vaccine strain of NDV had a decreased tracheal mucus transport rate and reduced tracheal clearance of *E. coli* (28), which could exacerbate disease. Coinfection of H9N2 LPAIV with either *Staphylococcus aureus* or *Haemophilus paragallinarum* enhanced the replication of the virus in chickens (53). Fibrinonecrotic casts in the tracheal bifurcation were reported in turkeys infected with H7N1 LPAIV in Italy in association with the presence of bacterial pathogens including *E. coli*, *Reimerella anatipestifer*, and *Pasteurella multocida* (12).

In other studies, exposure to *E. coli* or IBV alone resulted in few or no clinical signs or mortality, but challenge with various strains of IBV along with *E. coli* resulted in significantly increased clinical signs and mortality in chickens (86, 103, 131). An increase in susceptibility to *E. coli* infection was observed after previous infection of chickens with mild or virulent IBV strains, with lesions in the airsacs more pronounced and of longer duration in the coinfected groups (67). Lesions in the airsacs persisted longer after an infection with *E. coli* was preceded by inoculation with either the virulent IBV M41 strain or the H120 IBV vaccine strains 5 days earlier, in contrast to broilers inoculated with *E. coli* only (25).

Many studies have focused on the effects of bacteria on aMPV infection with various degrees of success at reproducing the clinical signs seen in the field (17, 18, 32, 51, 64, 75, 121). *E. coli* can worsen the effects of aMPV infection in both chickens and turkeys (88, 119, 120). Poults that received aMPV, followed 3 days later with *E. coli*, *Bordetella avium*, ORT, or a mixture of the 3, developed more severe clinical signs than poults inoculated with aMPV alone or bacteria alone (46). Similar findings have been reported in experiments with an aMPV in combination with *B. avium* and a *Pasteurella*-like organism (17). Dual infection in turkey poults with aMPV and *E. coli* resulted in increased morbidity, higher incidence of

lesions, and isolation of higher quantities of *E. coli* from inoculated poults compared with groups exposed to a single agent (119). Infection of broiler chicks with aMPV and *E. coli* demonstrated a synergistic effect by increasing the severity of clinical signs and lesions (2). A dual aMPV infection in turkeys with *E. coli* resulted in increased morbidity rates and gross lesions compared with single agent infections (115). When aMPV was administered to turkeys 5 days prior to aerosol challenge with ORT, airsacculitis scores increased (123). Combined aMPV/ORT infections in turkeys resulted in overt clinical signs and longer persistence of ORT in the respiratory tract in comparison with the groups given a single agent (65). In chickens, ORT infections, and/or the resulting clinical signs, were shown to be aggravated by administration of NDV, IBV, and aMPV (123). aMPV also had a mild effect on infection with *R. anatipestifer* in turkeys (98).

Interactions between different bacteria have also been reported in respiratory disease in poultry. Turkeys infected with *B. avium* had less ability to clear *E. coli* from the trachea and lungs than birds free of *B. avium* (117, 118). *B. avium* also adversely affected vaccinal immunity of turkeys to *P. multocida* (94). Coinfection of *A. paragallinarum* (causative agent of infectious coryza) and ORT were found in 2 outbreaks of infectious coryza in chickens (71). In experimental studies, chickens inoculated with both *A. paragallinarum* and ORT had more severe clinical signs than chickens inoculated with *A. paragallinarum* alone. ORT has also been isolated in coinfections from chickens with *Streptococcus zooepidemicus* (82), and *M. synoviae* (96). Clinical signs of infectious coryza were reproduced in chickens challenged with *A. paragallinarum* alone or together with *Gallibacterium anatis*, but were more severe in the coinfected birds (84).

Chlamydia psittaci, an obligate intracellular bacteria, has also been shown to interact with respiratory pathogens. A survey conducted in broilers indicated *C. psittaci* always preceded ORT clinical infection (23). Experimentally, aMPV infection during the acute phase of a *C. psittaci* infection aggravated the severity of clinical signs, gross lesions, aMPV excretion and histological tracheal lesions in turkeys (63). *C. psittaci* and *E. coli* coinfection also exacerbated clinical disease in turkeys (124). Coinfection of *C. psittaci* with H9N2 LPAIV, ORT, and *Aspergillus fumigatus* contributed to severe pneumonia, airsacculitis, and high mortality in chickens (16).

In summary, numerous, often synergistic interactions have been observed and demonstrated between almost all of the common viral and bacterial respiratory pathogens of chickens and turkeys. These interactions are affected by agent, dose, and timing of exposure to the agents; details of these factors are not well understood for most combinations of agents. Most practically, this demonstrates the importance of controlling all respiratory diseases because even infections with relatively mild agents may result in a substantial impact on production if combined with other agents under the right circumstances.

Immunosuppression and Respiratory Disease

Disruption of normal innate and adaptive immune system function in poultry can be caused by numerous infectious agents as well as environmental factors (for review see [42]). Mycotoxins in feed or in the environment can also cause immunosuppression similar to that of infectious agents (29). Immunosuppression can result in increased susceptibility to infection with viral and bacterial disease agents, increased severity of disease, and decreased vaccine efficacy, all of which are relevant to the pathogenesis of respiratory disease in poultry.

Increased incidence of respiratory disease (by lowering resistance), as well as increased duration and severity of disease, have been shown to result from infection with many viruses especially those which specifically target cells of the immune system. In chickens, infectious bursal disease virus (IBDV) (4, 34, 35, 87, 95, 129, 130) and Marek's disease virus (27, 55, 80) are the best characterized, although other agents may also have an immunosuppressive effect (15, 39, 62, 91). In turkeys, hemorrhagic enteritis virus (HEV) is among the best described immunosuppressive agent that can exacerbate respiratory disease, especially colibacillosis (60, 73, 77, 104, 122). Codelivery of live vaccines for NDV and HEV in turkeys also enhanced the pathologic response of the host (92). Immunosuppressive effects have also been reported with aMPV in turkeys (14, 113).

Perhaps the best described effect of immunosuppression on viral respiratory diseases in chickens is the reduction and even elimination of vaccine efficacy by IBDV. The negative effects of IBDV on NDV vaccination have been the best characterized (35, 70, 73, 95, 130), but the negative effects are also seen with HPAIV, IBV and infectious laryngotracheitis virus (ILTV) as well (87, 95). Data are lacking on whether and to what extent immunosuppression from chicken infectious anemia virus, Marek's disease virus, avian reoviruses and mycotoxins affect vaccination, but it is reasonable to assume that it is possible.

The Impact of Environmental Factors

Because of the ubiquity of infectious agents and because no management environment is perfect, exposure to a respiratory disease agent will always occur in conjunction with other stressors in natural settings. Although it is evident that environmental factors play a significant role in interacting with infectious agents in the induction

of respiratory disease in poultry, there are relatively few published studies investigating such interactions. The role of management and environmental conditions have not been described as well as infectious diseases because they are more difficult to reproduce under laboratory conditions and are highly variable in the field.

Poor environmental and management conditions (high animal density, inadequate ventilation, high ammonia levels, too high or low relative humidity and temperature) interact with respiratory pathogens and stress the host, thus exacerbating disease. Thermal stresses affect the host immune response and can cause a transitory immunosuppression which increases susceptibility to infection and can increase the severity of disease (54, 66). Respiratory disease and airsacculitis condemnations are well known to increase during the winter months, but there have been few studies on the effects of temperature on susceptibility to respiratory disease. Chickens challenged with *M. synoviae* and IBV had more extensive air sac lesions when housed at temperatures of 7 °C–10 °C than when housed at 29 °C–24 °C or 31 °C–32 °C (132).

Several factors including litter type, bird activity, stocking density, manure handling, frequency of manure removal, ventilation rate, and the pH, humidity, and surface area of the stored manure, can affect the ammonia content of the air in poultry houses (22). Chickens and turkeys continuously exposed to 20 ppm of ammonia showed gross or histologic signs of damage in the respiratory tract after 6 weeks of exposure, and exposed chickens were more susceptible to infection with NDV (5). Turkeys exposed to 10 or 40 ppm of ammonia had deterioration of their normal mucociliary apparatus, excessive mucus production, matted cilia, and deciliation in tracheal tissue (72) and exhibited impaired clearance of *E. coli* from air sac, liver, and lung (73). Chickens exposed to 70 or 100 ppm of ammonia for 4 days exhibited increased thickness of atrial walls and shrinking air capillaries in their lungs (3). Ammonia levels of 25 or 50 ppm resulted in reduced body weights and increased airsacculitis in chickens challenged with IBV (58). The severity and duration of *M. gallisepticum* infection were exacerbated by exposure to ammonia (50). A reduction in respiratory tract lesions was observed among broilers raised on litter treated to reduce ammonia levels (110).

Atmospheric dust has also been shown to have a detrimental effect on the response to respiratory infections. Atmospheric dust significantly increased the severity and incidence of air sac lesions in turkeys with high or low rates of infection with *M. meleagridis* (6). Hatchery fumigation of day-old chicks may also damage the tracheal epithelium (24, 100), possibly increasing susceptibility to early respiratory disease.

A common observation is that increasing down-time between broiler flocks has a beneficial effect on reducing respiratory and other diseases. In a field study, larger flock sizes were associated with increased early respiratory disease, and increased down-time decreased early respiratory disease (108). Nutrition likely has a role as well, because certain nutrients can counteract the immunosuppressive effects of heat stress (13).

In summary, good air quality with low ammonia, low dust levels, and appropriate levels of humidity is essential to respiratory health and maximizing resistance to infection with respiratory disease agents. The impact of other management factors is less well defined and requires more investigation.

Virus Vaccination Reactions

Protection of chickens and turkeys against viral respiratory diseases is dependent upon the widespread use of attenuated live respiratory virus vaccines. The live respiratory virus vaccines that have been most widely used are NDV and IBV vaccines. These viral vaccines replicate in the respiratory tract of the bird and although are attenuated still cause some degree of cell damage. The clinical manifestation of this viral replication, and its resultant pathology, is called the "vaccination reaction." If the vaccination reaction appears clinically to be unusually severe or prolonged, it is often referred to as a "rolling" vaccination reaction or, more simply, as a severe vaccination reaction (36).

Severe or prolonged vaccination reactions following the use of live NDV or IBV vaccines are a common occurrence in the commercial poultry industry. Most typically, flocks that undergo a severe vaccination reaction develop respiratory colibacillosis. The pathogenesis of this complex disease interaction follows the same pattern described for the interaction of virulent wild-type (i.e., non-vaccine strain) respiratory viruses with *E. coli* (68, 69, 103, 131). Most poultry health specialists agree that respiratory disease that results from the interaction of viral respiratory vaccine viruses with *E. coli* is the most common respiratory disease of commercial poultry (36).

Several different sets of circumstances can culminate in a severe respiratory vaccination reaction. As mentioned previously, immunosuppression has been demonstrated to enhance the ability of pathogens to induce disease. Likewise, immunosuppression can impede a bird's ability to limit replication of a respiratory virus vaccine, allowing a severe vaccination reaction to occur. Vaccinating birds with respiratory virus vaccines in environments contaminated with other pathogens can produce a severe vaccination reaction. The most noted examples are *M. gallisepticum and M. synoviae* (11). Newly hatched chicks that have hatched in an environment heavily contaminated with *E. coli* also may develop

severe respiratory reactions when vaccinated at a young age with live NDV or IBV vaccines (36).

Importantly, some live NDV, IBV, and ILTV vaccine viruses may become more virulent if allowed to spread from bird to bird (26, 37, 43), which biologically is serial-passaging of the virus in the host. This vaccine "back-passage" can occur in commercial poultry houses if only a portion of the birds are provided with an immunizing dose of the vaccine and the remaining birds in the house become infected by spread of the vaccine virus. For example, improper application of vaccines in the drinking water can prevent all birds in a house from receiving an immunizing dose of vaccine, thus providing an opportunity for spread of the vaccine virus with a concomitant increase in virulence of the virus. This type of vaccine reaction appears to be both prolonged in duration and of increased intensity as compared with a successful vaccine application. Similarly, spray application with a very fine spray can allow access of a vaccine virus to the deep respiratory tissues and result in excessive viral replication in lungs and air sacs in addition to eliciting a stronger immune response (126). Aerosol vaccination also resulted in more severe airsacculitis after challenge with *M. synoviae* (127).

Environmental factors can influence the intensity of a vaccination reaction. As discussed previously, ammonia and dust can interact with respiratory pathogens to enhance the severity of disease. This interaction is similar with respiratory vaccine viruses (58). Improper application of viral respiratory vaccines can enhance the severity of vaccination reactions.

The most critical point is that live vaccines must be applied correctly to assure that they do not cause the same disease and/or production losses through interactions with other agents as the wild-type counter parts they are used to prevent.

Multicausal Enteric Diseases

Timothy J. Johnson and J. Michael Day

Summary

Continued efforts in characterizing the various factors will be needed to identify strategies for better control of enteric diseases in poultry. Control of enteric diseases requires an integrated approach, incorporating both drug and management components (5). Good management practices can help reduce or eliminate exposure of young birds to enteric viruses, but the ubiquity and genetic variability of the many enteric viruses make it difficult or impractical to keep commercial flocks free of infection. Other factors including nutrition, stress reduction and control of other enteric pathogens (bacteria, protozoa, parasites) are important in preventing or mitigating disease. Future strategies may rely on drugs and biologics that modulate the immune and neurologic component of the disease in addition to those treatment regimens directed toward specific pathogens. Currently, there are no effective commercial vaccines available for control of most enteric diseases, and there are a variety of alternative products promoting "gut health." At this point, more data is needed to identify which of these product types are capable of impacting gut health and preventing or reducing enteric disease in the bird.

Introduction

The intestinal tract is a highly complex system that serves numerous functions and integrates with other body systems and their functions. The intestinal tract is one of the most important body organs because it provides mechanisms by which the body can utilize nutrients from its environment while simultaneously safeguarding the host.

The pathogenesis of enteric diseases is complex. Combinations of viruses, bacteria, other infectious agents, and even noninfectious factors may contribute to the establishment of enteric disease. The complex nature of the microbial populations in the intestine, referred to as the microbiome, makes it difficult to sort out the role of specific pathogens causing enteric disease (5). Furthermore, interactions between the intestinal tract and other body systems can affect the severity of the disease and modulate the disease process. The observation that multiple infectious agents, environmental factors, and even host factors may be involved in eliciting an enteric condition has prompted the use of the term multifactorial disease.

Enteric diseases cause great economic losses to the poultry industry resulting from depressed weight gain, impaired feed efficiency, and decreased flock uniformity. Enteric syndromes have been described both in young turkeys and chickens and have been attributed to a variety of causes. These diseases likely result from infection by a mixture of pathogenic agents coupled with or exacerbated by nutritional imbalances, poor management, environmental conditions, and stress (24, 29, 52). Age, immune status of the birds, and their genetic background can also affect the clinical presentation of the disease. Consequently, the spectrum and intensity of lesions associated with enteric diseases can vary from case to

case. In turkeys, a classical multifactorial enteric disease of young poults is coined poult enteritis mortality syndrome (PEMS) (5). Highlighting the complexity of such enteric conditions, poult enteritis complex (PEC) is another term commonly used that encompasses not only PEMS but several other infectious intestinal diseases of young turkeys including syndromes such as infectious enteritis (21, 68), malabsorption syndrome (42–45), stunting syndrome (1, 2), runting and stunting syndrome (11, 35), poult enteritis syndrome (31, 32), and others (5). The most important clinical features associated with PEC include enteritis, growth depression (stunting), impaired feed utilization, and secondary nutritional deficiencies. Many other clinical signs might be present in turkey flocks affected by PEC and are well described by Barnes et al. (5). In the more severe forms of PEC, retarded development (runting), immune dysfunction, and mortality have been reported (5, 48). Gross and microscopic lesions of enteritis are present in all forms but tend to be nonspecific.

Pathogenesis

The principal mechanisms by which infectious agents cause enteric disease have been reviewed by Moon (36), and these concepts still apply. Agents that produce diarrhea are not restricted to only 1 mechanism, but often a combination of mechanisms. The first mechanism is hypermotility and is defined as the increase in intensity, frequency, or rate of intestinal peristalsis that leads to the accelerated transit of ingesta or intestinal contents through the intestines. An alteration in intestinal permeability is another mechanism of diarrhea. When alterations occur in intestinal permeability that allows the net secretion to exceed absorption, the result is increased fluid within the lumen of the intestinal tract, resulting in diarrhea. Hypersecretion is a third mechanism defined as the net intestinal efflux of fluid and electrolytes into the intestinal lumen that occurs despite changes in permeability, absorptive capacity, or osmotic gradients. The mature intestinal epithelial cells, which line the distal portion of the villi, are responsible for the absorptive capacity of the intestine, whereas the immature crypt cells are responsible for secretion. Therefore, alterations in the balance of these cells can contribute to diarrhea. For example, loss of mature cells alters the absorptive capacity and often stimulates the formation of new immature cells to replace the lost cells, thereby altering the ratio of mature to immature cells in favor of the secreting immature cells. The net result is secretory diarrhea. Certain bacterial toxins stimulate secretion of the crypt cells beyond the absorptive capacity of the mature intestinal epithelial cells that result in diarrhea. The mechanism by which bacterial toxins produce secretory diarrhea has also been observed with some enteric viruses (25). Malabsorption is the fourth mechanism identified to cause enteric disease. Malabsorption is the process by which the absorptive capacity of the intestines is altered. This may be caused by impaired absorptive capacity such as loss of mature epithelial cells. However, malabsorption is often a sequela of maldigestion because the undigested feedstuffs cannot be absorbed, even though the absorptive capacity of the intestines is not impaired. In cases of malabsorption or maldigestion, nutrients within the intestinal lumen may contribute to the diarrhea by creating an osmotic effect.

The involvement of immune cells, and/or their products, in inducing diarrhea and enteric disease is well established. It has been reported that cytokines such as interleukins IL-1 and IL-3 contribute to hypersecretion in chickens (9). Various cytokines and biochemical mediators have been implicated in inflammatory processes leading to enteric disease and diarrhea (53). A neuroimmunophysiologic model has been suggested for all forms of infectious diarrhea (46). In this model, cytokines liberated from intestinal epithelial cells affect various other cells including immune cells, nerve cells, intestinal mesenchymal cells, etc. The end results include crypt hyperplasia, hypersecretion, malabsorption, and alterations in permeability, with the final sequela being diarrhea. It was also pointed out that this is a host defense mechanism, and although there may be points to intervene and counter the process, one must be careful not to further jeopardize the host by overzealous intervention strategies. Several studies have demonstrated the role of the immune response in eliciting enteric disease in broiler chickens and poults (51, 59, 63, 71). There is evidence to indicate that a variety of immune cells, including numerous types of T cells and heterophils are involved (59, 71). Additionally, the ability to genetically select broiler chickens that are less susceptible to MAS may be closely linked to the genetic expression of immune cells and their products (59, 70).

Etiology

Rarely is a single etiologic agent the cause of an enteric disease syndrome or the sole cause of the poor performance often observed in commercial poultry flocks. Although a single agent may be implicated – via specific molecular diagnostic assays, for instance – often several agents can in fact be identified in the poultry gut, and management factors on the farm can contribute significantly to disease severity (5). Further, experimental infections with single agents such as laboratory-isolated enteric viruses often fail to reproduce the full enteric syndromes as observed in the field, suggesting that other agents are likely involved as well. Several enteric viruses

have been identified and studied in detail following their implication in the poultry enteric disease syndromes (see Chapter 12), but little is known about the contribution of concomitant viral infections and possibly the full enteric viral community to the onset and severity of enteric disease. Enteric viruses are commonly the cause of most primary insults to the gastrointestinal tract of young birds, allowing other agents such as bacteria to colonize the intestinal epithelium and cause further intestinal damage (11). To complicate investigations further, many of these enteric viruses identified as having an association with enteric disease in poultry have also been identified in the gut of otherwise clinically healthy birds (23, 30, 39, 40, 58). The role(s) that each of the enteric viruses play in the enteric diseases and malabsorption syndromes remains unclear, and the prevalence and distribution of enteric viruses in commercial poultry is not well understood, particularly for recently described viruses.

In determining the etiology of enteric diseases, intestinal samples or cloacal swabs have often been examined for the presence of viruses via virus isolation, electron microscopy, and specific molecular assays. Many viruses, alone or in combination, have historically been associated with poult enteritis in turkeys, including turkey coronavirus (TCoV), turkey astrovirus (TAstV), avian nephritis virus (ANV), avian orthoreovirus, avian rotavirus, adenovirus, parvovirus, picornavirus, torovirus, and uncharacterized "small round viruses" (SRVs). In chickens, several enteric viruses – also either alone or in combination – have been detected in cases of enteric disease. They include ANV, chicken astrovirus (CAstV), enterovirus, infectious bronchitis virus, avian orthoreovirus, avian rotavirus, parvovirus, and uncharacterized entero-like viruses (ELVs). The implication of viruses as causative agents in the poultry enteric syndromes is supported by the observation that intestinal homogenates that have been filtered to remove bacteria can still cause decreases in weight gain under experimental conditions (31). In fact, poultry enteric disease is readily reproduced by inoculating young birds with fecal material or intestinal contents collected from affected birds; further, enteric disease can also be reproduced by placing experimental birds in contact with the litter from an affected flock (11, 31, 52, 54, 57, 60, 61). A challenge model using treated gut homogenates to reproduce runting-stunting syndrome (RSS) in broiler chickens has been developed, and has provided very good evidence of the viral etiology of RSS (33, 54).

The historical inability to define the contribution of a single etiologic agent as the cause of recognized enteric disease syndromes has led to the investigation of the entire virus community in the poultry gut; an approach known as viral metagenomics. The study of the viral community in the poultry gastrointestinal tract has benefitted greatly from the "next-generation" of nucleic acid sequencing technologies. This approach does not require viral cultivation and treats the complex enteric viral community (the enteric virome) as a single genome during the sequencing portion of the protocol. The nonbiased nature of the high-throughput nucleic acid sequencing protocols allows investigators to apply downstream bioinformatics techniques to identify individual viruses as well as their relative quantity in a particular sample.

Recent and ongoing investigations in the United States and abroad have utilized these high-throughput sequencing techniques and downstream bioinformatics analyses to investigate the complex virome of the poultry gut, resulting in descriptions of the novel chicken and turkey parvoviruses (18, 73), novel turkey and chicken picobirnaviruses (16, 19, 65), and avian enteric picornaviruses (6–8, 16, 20, 22, 26, 34, 72). This high-throughput approach has been used to determine the virus community present in the turkey gut, and a similar approach has been used to identify and characterize novel parvoviruses in turkeys and chickens. The common theme of these metagenomic analyses is that none of them required *a priori* knowledge of the viral population in question, and in many cases viral sequences new to science were discovered. The turkey gut viral metagenome alone revealed viral sequences from the dsRNA viruses (Reoviridae and novel picobirnaviruses), and the ssRNA viruses (Caliciviridae, Leviviridae, Picornavirales, and Astroviridae); viral sequences from the Myoviridae family of phages, the family Tymoviridae, and from the dependoviruses (family Parvoviridae) were also detected. As described above and elsewhere in this volume, the avian enteric reoviruses and astroviruses have been implicated for decades as possible pathogens in the poultry gut. The majority of the assigned viral sequences from the turkey gut showed similarity to database sequences from the Picornavirales order and other picorna-like viruses (16). Similarly, comprehensive metagenomic analyses of the viral metagenome in several flocks of broiler chickens revealed a number of viral taxa that were present in the turkey gut viral metagenome, with the addition of the viral families Retroviridae, Siphoviridae, Birnaviridae, Adenoviridae, and Coronaviridae (17). Further, the use of comparative metagenomics to characterize the intestinal virome in 2 turkey flocks that originated from the same source hatchery – 1 reared under high biosecurity as a research flock and 1 raised on a commercial farm – revealed numerous picornavirus sequences in the commercial setting that were not present in the biosecure flock; the commercial flock did not perform as well as the biosecure flock, and in fact showed signs consistent with poult enteritis complex, including a poor feed conversion (20). A comparative metagenomic analysis of RSS-affected and unaffected broiler chickens in the United Kingdom

revealed enteric viruses historically associated with RSS such as CAstV, ANV, chicken parvovirus, and chicken calicivirus, and also revealed viruses such as chicken *Megrivirus* and *Sicinivirus 1*, both members of the Picornaviridae (22). Although the role(s) the avian enteric picornaviruses play in enteric disease, gut health, and performance problems in general still need to be characterized fully, it is evident that these novel viruses are common in poultry worldwide – with many genera often simultaneously infecting a single flock or bird (6, 38, 69).

Bacterial and fungal populations are critically important towards the establishment of a healthy gut, and these populations play a direct role in susceptibility to disease. Study of the microbiome in poultry has mainly focused on bacterial populations using 16S rRNA-based amplicon sequencing for community profiling. To date, the bacterial populations comprising the normal microbiota of chickens has been clearly defined, with emphasis on the ceca and to a lesser extent the ileum (15, 28, 37, 47, 66). Commercial turkeys have also been profiled, albeit less extensively, to define their core gut microbiome (13, 14, 66, 67). Common themes from these studies have emerged. First, there is great variability between birds related to bacterial community composition. However, despite this variability, there is a consistent and reproducible succession of bacterial species as chickens or turkeys age. This predictable succession of bacterial species is observed irrespective of flock, system, bird type, or even geographical region. Thus, it is apparent that there is a poultry-adapted microbiome that is most dependent on bird age. The variability in the bird microbiome is likely attributed to differences in bird immune status, environmental challenges, and other unknown parameters.

Some bacterial species have been positively associated with performance in multiple studies, such as *Lactobacillus aviarius*, a poultry-adapted lactobacillus (12, 13, 27, 62) and *Candidatus* Arthromitus, a segmented filamentous bacteria (10, 13, 49). These species thus serve as useful biomarkers of microbiome health and succession in the poultry gut. It is also clear that pathogen colonization has a significant impact on the bacterial microbiome of the avian host. Most studies to date have focused on the impacts of foodborne pathogens, such as *Campylobacter jejuni* (3, 47). Other factors also have the ability to modulate the poultry gut microbiome. For example, bacterial vaccines such as that towards *Salmonella* Typhimurium can have an impact on the avian microbiome (4, 41). Perhaps more importantly, there appears to be a predisposing microbiome component in susceptibility to pathogen challenge, as demonstrated by the pioneering work of Nurmi with the prevention of *Salmonella* colonization through use of undefined cultures from adult birds (50, 56), and more recent work of others (55, 64). This suggests that modulation of the microbiome towards reduced pathogen susceptibility would be desirable. Alternative products, such as prebiotics/probiotics and essential oils, are currently widely used in poultry production. However, we know little about their efficacy or their mechanisms of action on the poultry gut. Thus, although these products are not themselves new, renewed interest in their use is coupled by renewed interest in understanding how they work.

As these lines of investigation continue, comparative metagenomic studies will benefit from analyses that also include multiomics approaches analyzing the viral, bacterial, and fungal communities in tandem. For example, an initial analysis of the intestinal virus community from specific pathogen free (SPF) chickens ("sentinels") placed in contact with existing flocks on commercial farms revealed colonization of the sentinel gut by viruses that were not present in precontact birds or were present in proportionally lower numbers, including – notably – the enteric picornaviruses (17). Analysis of the sentinel gut bacterial community in the same birds revealed an altered community in the postcontact birds, notably by members of the *Lachnospiracea/Clostridium* and *Lactobacillus* groups of bacteria. Correlational analysis defining enteric viral/bacterial dynamics in the chicken gut, along with host gene expression, is an approach that will allow investigators to form a more holistic picture of the effects that early viral infections can have on the bacterial community in the gut, or vice versa.

White Chick Syndrome

David French

Summary

Agent, Infection, and Disease. White chick syndrome, purported to be caused by chicken astrovirus (CAstV), has been reported from many countries around the world. Susceptible breeders exposed to the virus while in production may show few signs. They may experience a mild to moderate drop in production. Hatchability drops initially with mortality occurring in the middle to late stages of embryonic development. Chicks that hatch are weak and undersized with short shanks, swollen abdomen, and white plumage.

Diagnosis. Tentative diagnosis is based on signs described above. Definitive diagnosis requires virus isolation and identification, and recreation of lesions in embryos.

Studies looking at reverse transcription polymerase chain reaction (RT-PCR) and sequence analysis have been used to date and will continue to aide our understanding of this virus and the pathogenesis of this syndrome.

Intervention. A vaccine would be helpful but none are currently available. Some are using litter to spread the virus to pullet houses and assure seroconversion before those birds come into production. Moving litter creates a biosecurity risk but is the only option for some in the absence of a commercial vaccine.

Introduction

Poor hatch with weaker chicks and a distinctive white plumage have been reported for several years. The condition appears sporadically and lasts for a limited period, before returning to normal. Both infectious and noninfectious etiologies have been explored, but investigation of this condition usually points to a common breeder flock source, indicating the possibility, at least, of a vertically transmitted infectious cause. An association of this white chick syndrome with chicken astrovirus (CAstV) has been made (7).

Etiology

Astroviruses are small (28–30 nm), round, nonenveloped, single-stranded RNA viruses that get their name from a characteristic star-like shape that can be observed by negative-staining electron microscopy (3). This characteristic shape is only evident about 10% of the time, and if relatively few virus particles are present it can make diagnosis difficult, if that diagnosis is based on morphology alone. Astroviruses are very stable in the environment and resistant to inactivation by heat, low pH, and most disinfectants (6).

Astroviruses are commonly found in a wide range of species and typically associated with enteritis. Chicken astroviruses (CAstV) have been found in normal healthy birds as well as birds that are not performing well. They have been noted in cases associated with enteritis and in cases where enteritis was not a problem (4). They have also been identified in association with avian nephritis virus (ANV), with kidney damage, diarrhea, and decreased growth rate (1, 2, 3, 5). Recognition and investigation of white chick syndrome is complicated by the fact that it is entirely possible to have infection with different types of chicken astrovirus at the same time, some may be significant and others considered normal (2).

Pathobiology and Epizootiology

Progeny from susceptible breeder flocks between 25 and 45 weeks of age have been the most likely to demonstrate effects of white chick syndrome. Impact on the breeders is so mild that there may not be any signs at all in the adult birds. Drops in egg production for breeder flocks producing white chicks have been reported to range from 0% to 20%. Usually production drops 5%–10% and lasts for about 2 weeks before returning to normal. Once a breeder flock has had this condition, it does not have a problem with it again. Fertility is not affected, but eggs laid during that 2-week period when breeders may be mildly affected, do not hatch well. Hatchability drops between 4% and 50% of normal have been reported. This often happens at a time when birds are at peak production and peak fertility, and the presence of a large number of unhatched eggs in each hatching tray is alarming. Most of the hatch loss appears to happen between 8 and 10 days of embryonic development. As the condition progresses, embryonic mortality may shift toward later mortality, and weak and dead chicks in the hatcher tray. Like egg production, the hatchability tends to return to normal after about 2 weeks. Broiler chicks that do hatch are usually weak and tend to have poor livability for the first few days. Many of the chicks that hatch have a characteristic pale plumage. Affected chicks, and embryos that do not hatch, may have bright green or mottled livers, and subcutaneous edema. Histopathology may note hepatocellular vacuolar degeneration and glycogen accumulation, with heterophilic and lymphocytic interstitial nephritis.

Diagnosis

Tentative diagnosis can be made on the basis of signs and lesions, particularly the drop in production and hatchability with mid to late embryonic death, and weak chicks with pale feathers. Definitive diagnosis of white chick syndrome involves taking isolates from the affected birds or embryos and putting them back into SPF embryos to recreate the lesions. The best organs for virus isolation appear to be liver, pancreas, or spleen (5). Some researchers are looking at the utilization of tools including reverse transcription polymerase chain reaction (RT-PCR) and sequence analysis to help differentiate the white chick astrovirus from other similar viruses that may be found in the same flocks (1, 2, 4).

It is relatively easy to make the case for association of this condition in the progeny with a given breeder flock source. Laboratory trials have been designed to mimic both vertical and horizontal transmission. Vertical transmission appears to allow the virus to be isolated for up to 21 days in the progeny following initial exposure.

Horizontal transmission has resulted in virus isolation from the contact birds for up to 24 days following the initial exposure (5). Understanding a little more about how this virus is shed and its relative stability in the environment, raises questions about the pathogenesis of this syndrome. It is likely that this condition occurs when pullets remain naïve in the pullet house and become exposed to live virus challenge as they are coming into production. However, this has yet to be documented to the satisfaction of the scientific community.

Intervention Strategies

There is no treatment currently available for white chick syndrome. Egg production typically returns to normal in about 2 weeks. Hatchability returns to normal in the same time frame. The white chick condition will disappear as the ensuing hatchlings look stronger and flock livability returns to normal. Because of the sporadic nature of this syndrome, and relatively short duration of the problem, concern for prevention and control are moderate when compared with more serious threats. If economics so warrant, this syndrome could be a good candidate for a vaccine in the pullet stage that would prevent breeders from problems associated with vertical transmission (8).

In the absence of a vaccine, some companies use serology and transfer litter from known positive flocks to naïve pullet flocks. Biosecurity risks are a concern when moving litter. Pullet flocks should respond to the exposure to positive litter in about 2 weeks, and they will not likely repeat the viral challenge if they follow the pattern noted in natural infection.

Focal Duodenal Necrosis in Table Egg Layers

Eric Gingerich

Summary

Agent, Infection, and Disease. Focal duodenal necrosis is an intestinal, bacterial disease of egg-type chickens older than 12 weeks of age. The causative agent is purported to be *Clostridium* spp. but has yet to be determined by fulfilling Koch's postulate. The disease is characterized by reduced egg weights and variable loss of egg production. This disease has been seen in North America and Europe.

Diagnosis. Diagnosis is by visualizing the gross lesions of grey, 1–10 mm diameter foci on the duodenal surface. Histopathology can aid in verifying the lesions.

Interventions. Antibiotics have shown the best results in treatment. For prevention, bacitracin has been used continuously with or without a probiotic. For antibiotic-free production, botanical compounds, probiotics, and prebiotics have been utilized successfully.

Introduction

Definitions and Synonyms

Focal duodenal necrosis (FDN) is defined as finding typical duodenal lesions in egg-type chickens over the age of 12 weeks. The disease has also been called "gray gut", "focal necrotic duodenitis (FND)" or "multifocal ulcerative duodenitis (MUD)."

Economic Significance

Losses from this disease include lower egg weights than expected and, in some flocks, loss in egg production of 1%–10%. The incidence of FDN can vary from 0% to 40% of flocks at a multiple house location.

Public Health Significance

This disease has no public health significance.

History

Focal duodenal necrosis was first described by Dr. Patricia Dunn at the diagnostic laboratory at Pennsylvania State University in a cage-free layer flock in 1997. Since then, FDN has been observed by poultry health workers in most of the states in the United States and in Europe. Pullets as young as 12 weeks of age and all ages of layers have been observed with FDN. FDN occurs equally in conventional and organic-fed layers.

Etiology

Unfortunately, the definitive etiology of FDN is unknown. The cause of FDN is felt to be bacterial, with *Clostridium colinum* being the leading candidate, and accompanied by the absence of certain beneficial bacteria. The purported

etiology is supported by cooperative studies between a biotech company and a Pennsylvania egg producer using polymerase chain reaction techniques on duodenal samples from both affected and normal flocks (1). This is also borne out because of the fact that treatment with antibiotics that are effective against Gram positive bacteria are effective against FDN (i.e., bacitracin, tylosin, oxytetracycline, etc).

One cannot truly say that *C. colinum* is the cause of FDN as Koch's postulate has not been fulfilled (i.e., the disease has not yet been reproduced by administration of the organism). This fact has greatly inhibited research as to the effect of the disease and how to control it. An attempt to reproduce the disease by administering *C. perfringens* daily for 7 days reproduced very mild lesions but not the full blown syndrome (2). Originally, the disease was felt to be associated with the use of various feed ingredients such as bakery byproducts, animal byproducts, or certain calcium carbonate products. This was disproved by finding FDN at the same incidence rate in operations that were not using these ingredients. The finding of tapeworms was also felt to be a factor originally but flocks both with and without tapeworms appear to be equally affected.

There are apparently different pathogenicities associated with the causative agent because lesions are sometimes seen with no associated clinical signs.

Pathobiology and Epizootiology

Incidence and Distribution

The incidence of this disease is difficult to assess because active surveillance is needed to know if the disease is present or not. Many producers do not actively assess their flocks on a regular basis to know the incidence. FDN has been found in all major egg producing states of the United States. It also has been diagnosed in several countries in Europe.

Natural and Experimental Hosts

The egg-type chicken is the only known bird affected by this disease.

Transmission, Carriers, and Vectors

Because this disease is felt be caused by a clostridial agent, the agent is then present ubiquitously. The infection route is likely oral. It has been theorized that darkling beetles or flies could transmit the agent onto the feed or transmit the agent on their bodies when eaten by a chicken.

Incubation Period

The incubation period is not known as the causative agent is not known.

Clinical Signs

The disease is characteristically associated with a lack of attaining target egg weights and/or egg production goals. Egg weights tend to lag behind normal by one-half to two pounds per 30 dozen case (0.6–1.3 g/egg) in young flocks. Egg production may not reach a normal peak by 2%–3% or may drop by 1% –10%. Some of the birds may show pale combs.

Pathology

Lesions are only found in the duodenal loop and consist of single to multiple, dark, irregularly shaped, 5–15 mm diameter areas of the mucosa that can be seen from the serosal surface (Figure 33.1). Upon opening the duodenum, the grey areas can be visualized on the surface (Figure 33.2). A rotten-egg smell (hydrogen sulfide) is noticed emanating from the surface lesion. Histologically, a heterophilic infiltration of the duodenal villus tips is observed with numerous bacteria visualized on the surface (Figure 33.3).



Figure 33.1 Multiple gray areas of focal duodenal necrosis viewed through serosa of a layer chicken. (E. Gingerich)



Figure 33.2 Typical erosive gray areas seen on the mucosal surface of a layer chicken with focal duodenal necrosis. (E Gingerich)

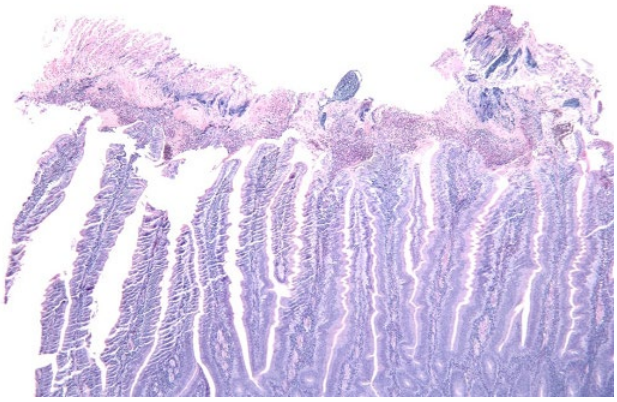


Figure 33.3 Superficial necrosis of epithelial cells at the surface of the duodenum and intralesional bacteria with focal duodenal necrosis. (E. Gingerich)

Immunity

Apparently, immunity is short-lived because flocks treated will have a reoccurrence of lesions within 6–8 weeks after the initial outbreak.

Diagnosis

Diagnosis is by necropsy of freshly euthanized birds and finding typical lesions in the duodenum. Necropsy of birds that have died naturally is not fruitful because the duodenal decomposition eliminates the lesion.

Intervention Strategies

Management Procedures

Preventative measures include the addition of an antibacterial compound in the feed with or without a probiotic. Because FDN usually affects egg weight gain early in production, an antibiotic may be fed until a target egg weight is reached before the medication is ceased. A reoccurrence of FDN is normally seen 6–8 weeks after the antibiotic is withdrawn. A decision is made at that time whether or not to treat depending on the effect on production parameters. Bacitracin (25 g/ton) is the most commonly used product for prevention with or without a probiotic. Probiotics, prebiotics, fermentation metabolites, or botanical products, alone or in combination, have not yet been thoroughly tested for their effect on FDN. Organic or antibiotic-free egg producers must, however, rely on these types of products for prevention.

Vaccination

No vaccine is available for this disease

Treatment

If the lesions are associated with clinical signs of poor egg weights or loss in egg production, an antibiotic such as tetracycline is administered for 5–7 days, followed by bacitracin plus a probiotic or prebiotic in the feed for 2 weeks. For antibiotic-free flocks, copper sulfate or botanical treatments such as oregano and others have been used with some effect.

Wooden Breast and Other Muscle Abnormalities

S.F. Bilgili

Summary

As with all tissues, the muscular system of chickens is vulnerable to abnormalities and pathologies. White striping and wooden breast are the terms used to describe the 2 recent breast myopathies in broilers. The etiologies of these 2 myopathies have yet to be defined, but the histological changes indicate a similar pathogenesis. When severe, both myopathies affect the organoleptic quality and consumer acceptability of whole muscle products.

Introduction

Meat-type chickens (i.e., broilers) have been bred for economically important traits, such as health and disease resistance, growth efficiency, and lean muscle (primarily breast) deposition. Commercially, broilers are marketed during the growth phase where a high rate of cellular protein synthesis and accretion rates lead to the most efficient growth of the breast muscles. The muscle growth is optimized when the complex regulatory control mechanisms assure a positive protein accretion rate by reducing protein turnover and a successful tissue regenerative capacity.

Myopathies

At times, the rate of muscle development can overwhelm the regulatory and homeostatic systems, resulting in cellular dysfunction and abnormalities (10, 17). Recently, 2 myopathic conditions of unknown etiology have been described (6, 14) in broiler superficial pectoral muscles: white striping (WS), a myopathy characterized by streaks of fat, with



Figure 33.4 White striping myopathy with characteristic streaks of fat deposited parallel to the direction of muscle fibers. (S. Bilgili)

varying severity, deposited parallel to the direction of muscle fibers (Figure 33.4), and wooden breast (WB), a diffuse hardening of the muscle with surface petechial hemorrhaging, viscous surface transudate, and thickened loose connective tissue in severe forms (Figure 33.5). Severely affected breast fillets exhibit both WS and WB conditions. Both myopathies have been observed in all commercial breeds/strains of chickens with varying prevalence and under a wide range of slaughter weights, management, and production systems (4, 13, 16). WS and WB are only revealed following slaughter and carcass portioning, because no growth problems and/or health issues are detected in affected flocks on antemortem inspection. No infectious and/or pathogenic agents have been associated with myopathies. Histologically, affected muscles show changes typical of focal or diffuse ischemia, including varying severities of muscle fiber fragmentation, swelling, and degeneration, as well as connective tissue, fat, and inflammatory cell infiltration (15). Severe forms of WS and WB can exhibit changes in organoleptic and sensory quality (i.e., color, texture, and composition), nutritional value (lower protein and higher fat), and impaired protein functionality (low water holding capacity and high cooking loss) as compared with normal fillets (12, 16).

Breast muscles of broilers are made up of white muscle fibers (glycolytic and anaerobic) that are extremely



Figure 33.5 Wooden breast evident as a diffuse hardening of the muscle with surface petechial haemorrhaging. (S. Bilgili)

susceptible to ischemic stress. It is likely that WS and WB result from incomplete regenerative processes that follow the inflammatory response triggered by the loss of cellular homeostasis in the muscle (10, 17). Limitation in posthatch satellite cell proliferation rate in the muscle may contribute to the inadequacy of regenerative capacity (5, 18). Gene expression and proteomic analysis of muscles affected by WS and WB indicate localized hypoxia and oxidative stress (1, 11) in addition to up- and downregulation of various genes related to protein synthesis, glycolytic pathways, and inflammation (3, 7, 19).

The genetic basis (i.e., heritability) of WS and WB myopathies was estimated to be low (2). However, breeding efforts have been put in place to reduce their prevalence. In the meantime, growth modulation efforts have been promising in reducing the severity of WS and WB (8, 9).

Idiopathic Egg Production Drops in Brown Layers

Kelli Jones, George Boggan, and Milos Markis

Summary

Background. Egg production drops of 10%–30% have been reported in brown layer chickens with a history of floor rearing in older turkey, breeder, or broiler facilities. Drops typically occur between 23 and 35 weeks of age, and may last up to 20 weeks before returning to an acceptable rate of egg production.

Clinical Presentation. Common complaints include delays in the onset of production, low peak production, short clutching, reduced feed consumption, increased water consumption, poor body size uniformity, mild mucoid enteritis, and small pale combs.

Interventions. Agents identified from affected flocks vary and include spirochetes, reovirus, astrovirus, adenovirus, and coronavirus, but attempts to reproduce the condition with these agents have been unsuccessful. By far the best intervention has been complete cleaning and disinfection of affected barns between flocks, including total manure removal. This has resulted in an immediate positive effect for the subsequent flock indicating that exposure to contaminated feces may have a role in egg production drops.

Introduction and History

Significant egg production drops (10%–30%) in brown egg layer chicken flocks raised on the floor in old turkey, broiler, and broiler breeder houses have been reported in the United States since the early summer of 2014. Clinical presentation can vary but flocks typically experience production drops between 23 and 35 weeks of age. In general, every house on the affected multiage farm experiences a drop in egg production at some point. The drops can be transient or persistent in an affected flock and can last between 10 and 20 weeks. At the same time, there could be unaffected sister flocks in lay elsewhere that were reared on the same pullet farm.

Incidence and Distribution

In the United States, idiopathic egg production drops in brown layer chickens have been reported primarily in the Northeastern, Mid-Atlantic and Southeastern regions. There is a strong association between the affected farms and the Amish communities in the Northeastern and Mid-Atlantic affected states, where it is common practice to share workers and equipment among farms. Multiple brown layer type breeds have been involved and different layer housing types. Affected flocks in the Northeastern and Mid-Atlantic United States are predominantly cage-free egg production facilities, whereas, affected flocks in the Southeastern United States have mostly been deep-pit cage systems. Reports in white layer chickens in the Southeastern region of the United States have not been consistent.

Etiology

The specific etiology of idiopathic egg production drops in brown layer chickens has not been determined. Agents that have been identified in affected flocks include spirochetes (*Brachyspira pilosicoli*, *B. innocens*, and *B. intermedia*), reoviruses including recently described viral arthritis variants, astrovirus ("white chick syndrome" [6, 22]), adenovirus, and coronavirus (8). Attempts to confirm a specific etiological agent responsible for these reported drops in egg production have been unsuccessful, because several challenge studies have failed to reproduce the condition.

Egg production is a commodity in chickens, therefore any stress factor that hens are exposed to can express itself as an egg production drop. Experimental administration of the stress hormone corticosterone via drinking water results in decreased body weights of pullets and hens, delay in the onset of egg production, and decreased egg production (18). Factors such as pullet quality, bird density, feed and feeding program, water quality, disease outbreaks, and light can affect egg production.

Avian influenza virus, both low and highly pathogenic, and Newcastle disease virus infections can cease egg production in laying hens within several days postinfection (7, 10–13, 21, 22, 26). Drops in production during avian influenza outbreaks can be as high as 40%, depending on the timing of eradication. Infectious bronchitis virus infections can cause egg shell deformities and drops in egg production (2, 3, 14). Egg drop syndrome, although exotic to the United States, is caused by adenovirus infection and is characterized by the loss of egg shell pigmentation, production of up to 40% soft-shell or shell-less eggs, and a 20% drop in egg production (15). More recently, astrovirus in broiler breeder hens has been identified as the etiology of "white chick syndrome," which is characterized by transient egg production drops of about 10%, followed by a drop in hatchability and hatch of white chicks (6, 22).

Egg drops can be observed in layer flocks presenting focal duodenal necrosis, *Escherichia coli* infections (salpingitis, peritonitis), and spirochete infections (1, 4, 5, 17, 20, 23). Spirochete species (*Brachyspira pilosicoli* in particular) has been associated with egg production drops and dirty eggs in chickens. Egg production drops are observed in field cases of intestinal dilatation syndrome, which is exclusively observed in floor-raised brown hens (25). Roundworms (*Ascaridia galli*) can cause drops in egg production, which are related to the loss of appetite and weight (9).

The type of light spectrum that the hens are exposed to has been associated with delayed onset of lay and peak in production, where red light is the most preferable and blue light the least preferable for commercial production practices (16). Feed source, quality and concentration of ingredients, as well as water quality have been associated with decreases in egg production and egg quality. Aflatoxin in feed can cause egg production drops as high as 18% (19). When dealing with egg production drops in commercial laying flocks, all of the above factors have to be considered and often more than one can be involved.

Pathogenesis and Epidemiology

This condition was first described in 2016 (8). Common clinical signs include delays in the onset of production, low peak production, reduced feed consumption and increased water intake prior to egg production drops, poor body size uniformity, and small pale combs. Typically, affected birds are difficult to identify in a flock because outwardly they appear "healthy." For the most part, affected birds "short clutch" but can remain in partial egg production. This leads to an overall drop in egg production of a flock. It has also been reported that the worst performing flocks tend to have the poorest body weight uniformity, which raises the question of feed and feeding involvement. Although there does not appear to be a feed type or feed mill relationship associated with this problem, it has been noted that mash-type feed is the predominant feed used in affected flocks. Selective feeding or feed sorting by the hens is a potential concern for the mash feed, and there have been cases of unaffected sister flocks that were fed only pelleted feed.

Treatment, Prevention, and Control

The most impactful intervention thus far has been cleaning and disinfection (C&D) of affected houses, including complete removal of manure. Water sanitation, including chlorine dioxide treatment to address biofilm, has also been implemented at some affected farms in conjunction with C&D. After thorough C&D efforts, a subsequent flock would show minimal egg production drops. Unfortunately, over the life of the flock, the house becomes recontaminated and the subsequent flocks in the barns would experience egg production drops if old manure was not removed.

In addition, delaying the age at transfer to the hen house by even a few weeks appeared to have some positive effects. Many companies have added vectored infectious bursal disease virus (IBDV) vaccine to their programs in an effort to strengthen the immune system of pullets. Additional, but seemingly unsuccessful, efforts to reduce effects on egg production included the use of autogenous variant reovirus vaccines, exposure of pullets to the feces from affected flocks, and changing feed mill sources.

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