ANIMAL INFLUENZA

SECOND EDITION

Edited by **DAVID E. SWAYNE**

WILEY Blackwell

Animal Influenza

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Second Edition

EDITED BY

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This edition first published 2017 © 2017 by John Wiley & Sons, Inc
The following chapters remain with the US Government: 1, 2, 4, 5, 6, 8, 10, 12, 13, 14, 15, 18, 19, 24

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Editorial offices: 1606 Golden Aspen Drive, Suites 103 and 104, Ames, Iowa 50010, USA
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9600 Garsington Road, Oxford, OX4 2DQ, UK

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Library of Congress Cataloging-in-Publication Data

Names: Swayne, David E., editor.
Title: Animal influenza / [edited by] David E. Swayne.
Other titles: Avian influenza (Swayne)
Description: 2nd edition. | Ames, Iowa : John Wiley and Sons, Inc., 2016. | Preceded by Avian influenza / edited by David E. Swayne. 2008. | Includes bibliographical references and index.
Identifiers: LCCN 2016015390 (print) | LCCN 2016034176 (ebook) | ISBN 9781118907467 (cloth) | ISBN 9781118924327 (pdf) | ISBN 9781118924334 (epub)
Subjects: LCSH: Avian influenza. | Influenza. | Mammals–Virus diseases. | Influenza A virus. | MESH: Influenza in Birds–prevention & control | Influenza, Human–prevention & control | Influenza A virus–pathogenicity | Disease Outbreaks–prevention & control
Classification: LCC SF995.6.I6 A92 2016 (print) | LCC SF995.6.I6 (ebook) | NLM SF 995.6.I6 | DDC 636.5/0896203–dc23 LC record available at https://lccn.loc.gov/2016015390

A catalogue record for this book is available from the British Library.

Wiley also publishes its books in a variety of electronic formats. Some content that appears in print may not be available in electronic books.

Set in 8.5/12pt, MeridienLTStd by SPi Global, Chennai, India.

1 2017

This book is dedicated to the veterinarians and veterinary scientists whose long and distinguished careers focused on studying influenza for the benefit of animal health, and whose discoveries have laid the scientific foundation for all who have followed in the concept of "One World, One Health" for influenza. This book is dedicated to Dennis J. Alexander, Charles W. Beard, Bernard (Barney) C. Easterday, David A. Halvorson, Yoshihiro (Yoshi) Kawaoka, Hans D. Klenk, Rudolph Rott, Werner Schäfer, Richard Slemons, David Stallknecht, and Robert G. Webster.

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Foreword

Over the last two decades, influenza has arguably become the most important disease of poultry, attracting attention from researchers and grant-awarding institutions around the world. Current journals are filled with new findings on influenza. In this respect, this disease resembles Marek's disease (MD) in the 1960s, a challenging time when MD threatened the very existence of the poultry industry. It is fitting, therefore, that a volume dedicated to the study of influenza should be made available to the increasing number of workers in this field. Such a volume will serve to distill current knowledge and present it with an appropriate historical perspective.

As the successor to the earlier text on avian influenza, the present volume has continued its focus on avian species, but with an important expansion to encompass influenza in several important mammalian species. The long history of avian influenza (AI), unlike many other diseases, is reflected in a series of distinct outbreaks or epizootics, each of which is not only unique but also a rich source of information. Each provides lessons by which knowledge is expanded and strategies for control can be improved, justifying a systematic and detailed analysis. The focus is understandably on epizootics caused by high-pathogenicity AI (HPAI) viral strains, and such epizootics have numbered more than 37 since 1959. One of them, involving H5N1 HPAI originating in South-East Asia, has spanned more than a decade and involved multiple animal species and many countries worldwide. It is outbreaks such as this that command the attention of veterinarians, virologists, epidemiologists, and public health specialists, as well as the poultry and animal industries and the general public.

Meanwhile, influenza in swine, horses, and dogs has attracted new interest as certain influenza viruses from both avian and mammalian species have shown a propensity to infect humans. The expansion of this volume to encompass a wider range of host species meshes well with the "One World, One Health" initiative, which recognizes the synergy embodied in a multidisciplinary and multifaceted approach to the study of disease.

This text brings together in a comprehensive manner the knowledge and experience accumulated during more than a century of research and observation of influenza in animal species. The list of authors is impressive and distinctly international. The emphasis on avian influenza is retained and updated, and the nine new chapters on influenza in mammalian species are complemented by the five introductory chapters that deal with both mammalian and avian species. Thus this edition is in some respects totally new, and in other ways represents a logical continuation and updating of the information on avian influenza that was so aptly detailed in the previous edition. Like its predecessor, this book will surely become the major reference source in its field.

The publication of this volume has been sponsored by the American Association of Avian Pathologists, an organization that has long supported the publication of information relevant to poultry medicine. This book continues the tradition of excellent, science-based educational publications produced by this organization.

Like the previous edition, this volume was conceived and edited by Dr. David E. Swayne, who has devoted more than a quarter of a century to the study of avian influenza, and leads the Southeastern Poultry Research Laboratory, Agricultural Research Service, US Department of Agriculture, in Athens, Georgia, which has been a strong contributor to knowledge in this field. Dr. Swayne is a world authority on avian influenza pathobiology and vaccination in poultry, and his work has facilitated the application of vaccines and diagnostic tests used worldwide. He has not only amassed critical knowledge but also translated this into international policy to improve food safety and to protect the USA from HPAI infection. Drawing

on his considerable experience as a pathologist, researcher, international consultant, research leader, and editor, he has personally authored three of the present chapters and contributed to two others as a co-author.

This book will be valued by veterinarians, researchers, and regulatory officials who deal with influenza in avian and mammalian species, and will also assist public health officials in understanding the animal health aspects of this important and complex disease, which will surely pose a continuing threat to animal agriculture and human health.

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Preface

Avian Influenza (2008), the predecessor of the current textbook *Animal Influenza* (2016), was published as a first edition with the intent of periodic updating through successive editions. The impetus for *Avian Influenza* was the emergence of the H5N1 Goose/Guangdong (Gs/GD) lineage of high-pathogenicity avian influenza (HPAI) from the late 1990s to the mid-2000s, which not only caused infections and deaths among poultry in over 60 countries of Asia, Europe, and Africa, but also resulted in infections and deaths among wild birds and numerous mammalian species, including humans. Such an HPAI epizootic involving a large number of animals and dispersion over a wide geographic area had not been seen since the 1920s and 1930s, when fowl plague was endemic or was causing epizootics among poultry in Europe, Asia, Africa, and North and South America. Between 1959 and 2008, 28 epizootics of H5 or H7 HPAI had been reported, with the Gs/GD-lineage H5N1 being larger than all the other 27 epizootics combined, and it was justified in having the designation of a veterinary or agricultural panzootic.

Since the first edition of *Avian Influenza* was published in 2008, H5 or H7 HPAI has caused 13 additional epizootics, and the Gs/GD-lineage H5 HPAI has continued, cumulatively affecting over 70 countries, including outbreaks in North America during 2014–2015, and resulting in deaths or culling of over 500 million poultry. The continuing significance of HPAI has necessitated an update on avian influenza. However, the emergence of a human H1N1 pandemic in 2009, caused by a reassortant influenza A virus with gene segments most closely related to human seasonal influenza, swine influenza, and avian influenza viruses, as well as the identification of cross-transmission of human and swine influenza viruses between humans and pigs in the USA, the emergence of H3N8 influenza in dogs, and the emergence of equine influenza in Australia, has solidified the idea of expanding the *Avian Influenza* text, and its renaming as *Animal Influenza,* for a second edition. Specifically, the first five chapters were broadened from avian influenza alone to more generic animal influenza information. Chapters 6 to 15 were updated with information specific to avian influenza, and Chapters 16 to 24 were added to provide new information about swine influenza, equine influenza, canine influenza, and influenza in other mammalian species.

Both *Avian Influenza* (2008) and *Animal Influenza* (2016) were commissioned by the American Association of Avian Pathologists (AAAP), a non-profit educational foundation whose mission is to promote research and apply such new knowledge to solving avian health problems, which includes providing educational resources to avian veterinarians and health professionals around the world. The authors and editor of this book have received no financial compensation from the sale of this book, but we do acknowledge the valuable professional satisfaction of helping colleagues around the world and advancing the discipline of poultry medicine. All profits have been used to further the educational programs of the AAAP, including donations of educational materials to developing countries.

As editor, I wish to extend special thanks to Anita J. Swayne, my wife, whose patience and encouragement made possible the long journey of this book from idea to reality. I also thank the Board of Directors of the AAAP for commissioning this text, and several colleagues for providing anonymous critiques and reviews of some chapters to ensure accuracy. The highly skilled and professional assistance of John Wiley & Sons, especially of Nancy Turner, Melissa Wahl, Catriona Cooper, and Susan Engelken, over the past three years is much appreciated. Finally, I personally thank Dr. Richard D. Slemons, Dr. Charles W. Beard, and Dr. Max Brugh for introducing me to the exciting world of influenza research, and for their continual career guidance and mentoring, which has made the past

29 years of researching influenza viruses and the diseases that they cause a daily, fun adventure.

Any mention of trade names or commercial products in this book is solely for the purpose of providing specific information, and does not imply recommendation or endorsement by the authors. The content of individual chapters is based upon the scientific literature and the knowledge and experience of the individual authors, and is not the official position of the United States Department of Agriculture or other employers of the individual authors.

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SECTION I

Common aspects of animal influenza

1 Influenza A virus

David L. Suarez

Introduction

Influenza A viruses (IAVs) are important veterinary and human health pathogens that are present worldwide. The category of viruses has a diverse host range, including a large number of avian and mammalian species. The ecology and epidemiology of influenza A viruses are very complex, involving various free-living, captive-raised, and domestic bird hosts as well as various wild and domesticated mammalian hosts within diverse environments, including humans, pigs, horses, dogs, bats, and sporadic infections in miscellaneous mammalian hosts (Figure 1.1). The other key characteristic of the virus is the genetic and antigenic variation that occurs through the combination of a high mutation rate and a segmented genome that provides an ability to rapidly change and adapt to new hosts. In the right conditions, an IAV can adapt to a new host such that it replicates and transmits efficiently to become endemic in a particular species. In general, this adaptation process produces a viral lineage that has some level of host specificity, so that it becomes more difficult to infect other species. For example, a virus that becomes endemic in horses becomes less able to infect other species such as swine or humans. The species barrier can be less clear in avian species, as a chicken-adapted virus will typically also infect other gallinaceous species, but other classes of birds, such as ducks or pigeons, may be resistant to infection. The IAV can cause a wide range of clinical disease that generally relates to the pathogenesis of the virus, whether it infects just on mucosal surfaces or causes systemic infection. The control of IAVs in animals has used a variety of tools, including vaccines, quarantines, and even culling of infected animals. The goal of eradication of the virus from a host population can in some situations be achieved, but often at a high cost. In many countries, IAVs are endemic and control efforts are used primarily to mitigate economic losses. Because the primordial reservoir for IAVs is wild birds, the ultimate goal of complete eradication is not feasible, and the potential for introduction of new and unique viruses from the wild bird reservoir is a constant threat.

Etiology

Classification

Type A influenza virus (IAV) belongs to the Orthomyxoviridae family of segmented negative-sense RNA viruses that are divided into six different genera accepted by the International Committee on Viral Taxonomy, including influenza types A, B, C, Isavirus, Thogotovirus, and Quaranfilvirus [130]. Two additional segmented RNA viruses have been proposed as potential new genera, including a potential type D virus associated with respiratory disease in swine and cattle, and a virus associated with cyclic mortality events in eiders in North America, named the Wellfleet Bay virus [4, 23]. The IAVs are the most widespread and important members of the group, infecting many different avian and mammalian species. Type B and C influenza viruses are human pathogens that rarely infect other species, although infection of swine and seals has been reported [100]. The Isavirus group includes the important fish pathogen infectious anemia virus [61], the Thogotoviruses are tick-borne arboviruses that have been isolated from both humans and livestock [71], and the Quaranfilviruses are tick-associated viruses that have been detected in humans and birds [117]. The remainder of this chapter will be focused mostly on IAVs of

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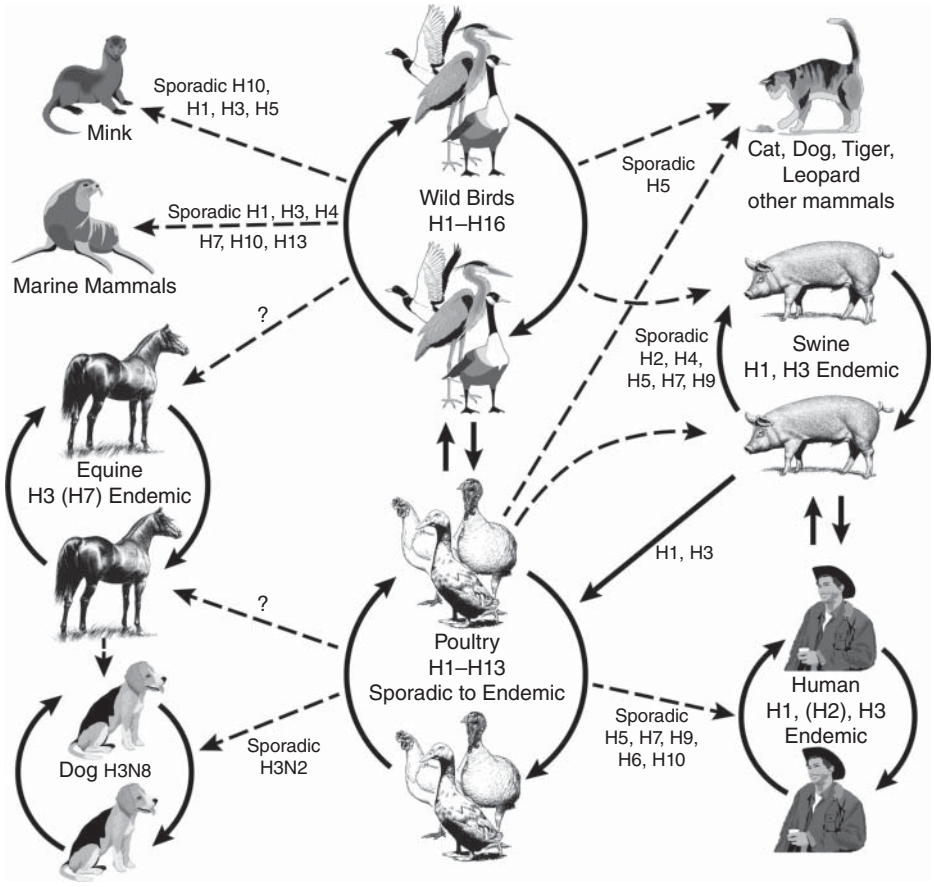

Figure 1.1 Diagrammatic representation of the source and movement of influenza A viruses or their genes within avian and mammalian ecological and epidemiological situations (updated from [160]). H = hemagglutinin subtype, ( ) = subtype previously common but no longer circulating. Source: K. Carter, University of Georgia, and D. Swayne, USDA/ARS.

birds and mammals, but with brief coverage of influenza B viruses contained in human influenza vaccines.

Composition

All IAVs have 8 different gene segments that encode at least 10 different viral proteins. The structural proteins in the mature virion can be divided into the surface proteins that include the hemagglutinin (HA), neuraminidase (NA), and membrane ion channel (M2) proteins. The internal proteins include the nucleoprotein (NP), the matrix protein (M1), and the polymerase complex comprised of the polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase acidic protein (PA) [103]. Two additional proteins produced by IAV are the non-structural proteins, namely non-structural protein 1 (NS1) and non-structural protein 2 (NS2), which is also known as the nuclear export protein (NEP) [97]. The NS1 protein is considered to be a true non-structural protein that is not found in the virus particle, but is produced in large amounts in the host cell [14, 172]. The NS2 protein is primarily found in host cells, but some protein can be found in the virion [130]. Several additional accessory proteins have been described that result from transcription from alternative open reading frames, although the function of many of them is poorly understood [177]. The PB1-F2 protein, an 87-amino-acid protein that is transcribed from a different reading frame from the PB1 protein, is a potential virulence factor thought

to be involved in apoptosis in host cells, but it is not found in all IAVs [21]. The PA-X protein, a product of a ribosomal frame shift, has been shown to modulate the mouse immune response [51]. The role and importance of these accessory proteins are still being studied, and their importance to the pathogenesis of the virus is unknown.

The HA protein is categorized into 18 different subtypes, originally based on the hemagglutination inhibition (HI) assay, but now confirmed by gene sequencing and analysis (Table 1.1). The different subtypes are not uniformly distributed among the various bird and mammal species, but the greatest diversity of IAVs occurs in the class Aves, principally in two orders of wild birds, namely the Anseriformes and Charadriiformes. The subtype distribution is more limited in mammals, with restriction of a few HA subtypes to endemic or sporadic infections of mammals.

Morphology

The IAVs can be morphologically extremely variable, ranging from spherical particles with a diameter of 80–120 nm to filamentous forms that can be several micrometers in length. The filamentous forms seem to predominate in clinical isolates, but after passage in cell culture or embryonating chicken eggs the virus often changes morphology to the spherical forms, at least for human viruses [15, 130]. The morphology appears to be primarily controlled by the matrix 1 protein, and two

Table 1.1 Hemagglutinin subtype distribution[a] of influenza A viruses between different birds (class: Aves) and mammals (class: Mammalia).

HA subtype	Host of origin						
	Mammalia				Aves		
	Humans	Swine	Equine	Bats	Anseriformes (e.g. dabbling ducks)	Charadriiformes and Procellariiformes (e.g. shorebirds, gulls, seabirds)	Galliformes (domestic poultry)
H1	++[a]	++			+	+	++[e]
H2	(++)[b]	±			+	+	+
H3	++	++	++		++	++	++[e]
H4		±			++	+	+
H5[c]	±	±			+	+	++[b]
H6	±				++	+	+
H7[c]	±	±	(++)[b]		+	+	++[b]
H8					±		±
H9	±	±			+	++	++
H10	±				+	+	+
H11					+	++	+
H12					+	+	±
H13					+	++	+
H14[d]					±		
H15[d]					±	±	
H16[d]						+	
H17				+			
H18				+			

[a] ± = sporadic, + = multiple reports, ++ = most common.

[b] ( ) = Previously common but now not reported.

[c] Both LP and HP viruses.

[d] Rare subtypes.

[e] Primarily swine influenza virus infections of domestic turkeys.

Modified from Swayne, D. E. and M. Pantin-Jackwood. 2008. Pathobiology of avian influenza virus infections in birds and mammals. In: *Avian Influenza*, D. E. Swayne, ed. Blackwell Publishing: Ames, IA. 87–122.

specific amino acids have been identified as being important [15]. The overall structure of the virus includes a lipid membrane derived from the host cell that has three viral integral membrane proteins, namely the hemagglutinin, neuraminidase, and matrix 2 proteins. The hemagglutinin protein exists as a trimer that appears as spikes on the lipid membrane, and is the most abundant surface protein [25]. The neuraminidase protein exists as tetramers and forms more of a globular structure extending from the lipid membrane. The M2 protein is a small protein that functions as an ion channel that is important for triggering viral uncoating. The M1 protein appears to be the primary bridge between the lipid membrane and the viral core of nucleoprotein, viral RNA, and the polymerase complex.

Propagation

Influenza A viruses are easily propagated in the laboratory, and this has allowed them to be widely studied. Avian, human, swine, and equine IAV were all originally propagated in embryonating chicken eggs, and this method is still commonly used both for diagnostic purposes and for virus propagation, especially for vaccine production. Recently there has been more emphasis, particularly for the mammalian IAV, on growing influenza viruses in cell culture, both in primary and continuous cell lines, for both routine diagnostics and vaccine production [36, 101, 195]. Common cell lines for virus isolation and propagation are chicken embryo fibroblast cells, chicken embryo kidney cells, Madin–Darby canine kidney cells, Vero cells, and others. For avian influenza (AI) viruses (AIVs), the isolation and characterization of viruses is most commonly performed in 9- to 11-day-old embryonating chicken eggs by inoculation of the allantoic cavity. Embryonating chicken eggs provide the added advantage of allowing replication for both low-pathogenicity avian influenza (LPAI) and high-pathogenicity avian influenza (HPAI) viruses [41]. Primary chicken embryo cell cultures are also used, but for LPAI virus (LPAIV), trypsin must be added to the media for efficient virus replication and plaque formation. Alternatively, the use of some cell culture systems, such as primary chicken kidney cells, allows replication and plaque formation of LPAIV without additional trypsin, presumably because it produces a trypsin-like protease as seen with mammalian kidney cell cultures [62]. Recently, however, the use of chicken eggs has been found to be inadequate for the isolation of some IAVs from humans, swine, and turkeys. As early as 1996, human H3N2 variants were isolated in cell culture that no longer grew well in chicken eggs without adaptation [195]. For these viruses, isolation in mammalian cell culture was more reliable for primary isolation [167], although in one case the use of the egg yolk sac route of inoculation instead of allantoic sac inoculation resulted in a virus isolation [155]. The same viruses that no longer replicate well in chicken eggs also no longer efficiently hemagglutinate chicken red blood cells, which has necessitated the use of alternative red blood cells (RBCs), such as turkey or guinea pig RBCs [90, 155].

Nomenclature

The nomenclature for describing IAVs has been standardized to provide a consistent and informative nomenclature for all IAVs. The features used to name all new IAVs include the following: (1) antigenic type (A, B, C, or D); (2) the host animal from which the virus was isolated, but for human isolates this may be omitted and is simply implied; (3) the geographic origin of the isolate, which can be a city, state, province, or country designation; (4) the unique laboratory or other reference identification number for each isolate; (5) the year of isolation; and (6) the hemagglutinin and neuraminidase subtypes, which are often included in parentheses at the end. For example, an influenza virus isolated from turkeys in Missouri would be A/turkey/Missouri/24093/1999 (H1N2).

Virus life cycle

The initial step in IAV infection is the attachment of the viral hemagglutinin protein to the host cell receptor sialic acid, which initiates endocytosis. Sialic acid is a general term for the terminal sugars found in N- and O-linked glycoproteins that can be made of many derivatives of neuraminic acid. Sialic acid molecules are often classified in terms of how they are linked to the underlying sugars at the α-2 carbon. The most common linkages are the α-2,3 and α-2,6 linkage [158]. These different sialic

acid linkages result in different conformations of the host receptor protein that affects virus binding. The hemagglutinin protein, based on the amino acid structure, will bind different types of sialic acid with different affinity that can determine whether the virus can initiate the infection process. The virus needs to bind strongly enough with the host protein to initiate endocytosis, and typically has strong specificity for either the α-2,3 or α-2,6 linkage. Different animal species will have different patterns and levels of expression of α-2,3 and α-2,6 sialic acid, that may vary between different tissues in the same animal. The α-2,3 sialic acid is predominantly expressed in avian species, and the α-2,6 sialic acid is expressed in humans. The differences in affinity of the hemagglutinin are thought to be one factor that contributes to the species barrier that IAV usually maintains. Although evidence suggests an important role for sialic-acid-binding preferences, some species, including humans, quail, and swine, express both types of sialic acid, although with different tissue distributions and avidities [169, 180]. This receptor distribution can directly affect pathogenesis, as has been proposed for H5N1 infection in humans, where pneumonia is commonly seen and not an upper respiratory tract infection. The pathology appears to correlate with the expression of α-2,3 sialic acid in alveolar type II pneumocytes in the lung [131]. An additional factor is that the specificity of the hemagglutinin for either type of sialic acid is not absolute, and some viruses can bind both α-2,3 and α-2,6 sialic acid [194]. In experimental studies in humans and animals, replication can often occur with many viruses if the subjects are given a large enough challenge dose [11, 46].

The hemagglutinin receptor specificity for sialic acid is not absolute, and can change with as little as two amino acid substitutions at positions 226 and 228 (H3 amino acid numbering) [26, 179]. *In vivo* studies have documented a number of cases of selection of amino acid changes reflecting the host or isolation system in which the virus is being passaged [106, 147].

Pigs have previously been suggested to be a major mixing vessel for human influenza and AIV because they express high levels of both α-2,3 and α-2,6 sialic acid in their respiratory epithelium. The theory was that pigs could be simultaneously infected with human IAV and AIV, and reassortment could occur between the two viruses, resulting in a new virus that could result in a pandemic strain [125, 183]. The pig as a mixing vessel has some support from field data, and complex reassortant viruses have been isolated from pigs [56, 176]. The 2009 pandemic H1N1 IAV is likely to have been a reassortant virus between two different swine viruses, but the identity of the host and where the reassortment occurred are unknown [138]. However, the outbreaks in humans with AI-like viruses (H5N1, H9N2, H7N7, H7N3, and H7N9), although not resulting in a pandemic virus, show that exposure to infected poultry and not exposure to pigs was the main risk factor for infection [66, 108, 153, 175, 192].

Once viral attachment has occurred the IAV is endocytosed, and when the endosome becomes acidified that triggers the fusion domain of the hemagglutinin protein to become active, and the viral RNA is released into the cytoplasm [146]. The M2 protein plays a key role in the triggering process, as it is an integral membrane protein that allows H^+ ions to enter in the virion, causing a conformational change of the HA at the lower pH to allow the fusion domain to become active [115]. The adamantane class of antiviral drugs act by blocking the function of the M2 protein, which prevents the fusion of the hemagglutinin within the endosome [43, 157]. The fusion of the viral membrane and the endosomal membrane, mediated by the fusion domain of the hemagglutinin protein, allows the release of the viral RNA–polymerase complex into the cytoplasm, where it is then actively transported to the nucleus because of nuclear localization signals in the nucleoprotein [96].

The negative-sense viral RNA is copied into positive-sense mRNA by the polymerase complex, which includes the three polymerase proteins and the nucleoprotein, in the nucleus. The virus also uses host proteins to initiate mRNA synthesis, including RNA polymerase II. The mRNA requires a 5′ capped primer that is stolen from host mRNA by the PB2 protein in a process known as cap snatching [67]. The positive-sense viral mRNA then migrates from the nucleus to begin viral protein translation in the cytoplasm using the host cellular machinery. The positive-sense RNA also serves as a template to produce the negative-sense viral RNA that will be packaged into the virion.

Two viral proteins, the M1 and NEP, are crucial for trafficking of viral proteins to and from the nucleus. The M1 protein also plays a critical role in the assembly and structure of the virion [15]. The viral assembly process includes the three integral membrane proteins, hemagglutinin, neuraminidase, and small amounts of the M2 protein, entering the endoplasmic reticulum, where they are folded and glycosylated before eventually moving to the apical plasma membrane [9]. The M1 protein is believed to be critical in bridging the surface integral membrane proteins and the ribonucleoprotein complex and each of the eight viral gene segments before the virion is complete. All eight viral gene segments have highly conserved regions, 13 and 12 nucleotides long, on the 5′ and 3′ end of each segment respectively, that are important packaging signals. RNA packaging appears to be an inefficient process, and many viral particles do not package all eight gene segments, creating a high proportion of defective viral particles. It has been estimated that more than 90% of viral particles are non-infectious [29, 31]. The packaging process may also allow multiple gene segments, particularly of the smaller genes, to be included in the virion. This multiple packaging may even affect the phenotype of the virus, since it has been hypothesized that when multiple copies of the NS gene are packaged per virion, an increased resistance to interferon production will occur [127].

The efficient budding of the viral particle from the cellular membrane requires, among other things, the enzymatic activity of the neuraminidase protein to remove sialic acid from the surface glycoproteins, specifically the hemagglutinin protein. This prevents self-binding of the protein and the aggregation of the virus at the cell surface [89, 129]. In experimental studies, viruses that have reduced neuraminidase activity will aggregate on the cell surface because of particles attaching to each other, which can greatly reduce the effective titer of the virus [8]. The loss of neuraminidase activity is not just a theoretical exercise, because one of the markers of AIV adaptation to poultry is the presence of stalk deletions of the neuraminidase protein [88]. These stalk deletions result in a marked decrease in neuraminidase activity. Although the neuraminidase active site is not affected by the stalk deletion, the shorter stalk is thought to reduce flexibility of the protein, which reduces its ability to attach to the sialic acid substrate. The IAV can at least partially compensate for this reduced neuraminidase activity by making changes in the hemagglutinin protein that reduce the affinity of binding to sialic acid, typically by the addition of extra glycosylation sites near the receptor binding sites [91]. We currently do not understand the selective advantage of neuraminidase stalk deletions in poultry.

For LPAIV, the released viral particles are not infectious until the hemagglutinin protein is cleaved into HA1 and HA2 subunits by trypsin or trypsin-like proteases. The role of HA cleavage will be discussed in more detail in the pathogenesis section.

Virus genetics

Ecology in wild birds

The natural host and reservoir for all type A influenza viruses occur in wild birds, primarily in waterfowl, gulls, and shorebirds [58, 133]. In the natural host the virus appears to be evolving slowly, with most internal genes being highly conserved at the amino acid level [149]. The surface glycoproteins, HA and NA, are much more variable in amino acid sequence, demonstrating the greater diversity of these genes. For both proteins, multiple antigenic subtypes have been characterized, where antibody to one subtype will neutralize, with high specificity, only viruses of that subtype. For the HA protein, 16 subtypes of AIV have been characterized (Figure 1.2), and 9 subtypes have been characterized for the NA protein. At the amino acid level the difference between subtypes is as little 20%, but the most divergent subtypes are up to 63% different. About 25% of the amino acids are conserved among all 16 HA subtypes [95]. Similar comparisons are found for the NA subtypes, with amino acid differences of between 31% and 61%.

In comparing the nucleotide sequence of most of the gene segments from wild bird AIV, including within an HA and NA subtype, a clear separation is found to occur among viruses isolated from Europe, Asia, Africa, and Australia (Eurasian lineage) and those isolated from the Americas (American lineage) [149]. At the amino acid level for the more conserved internal proteins, the distinctions between American and Eurasian lineages

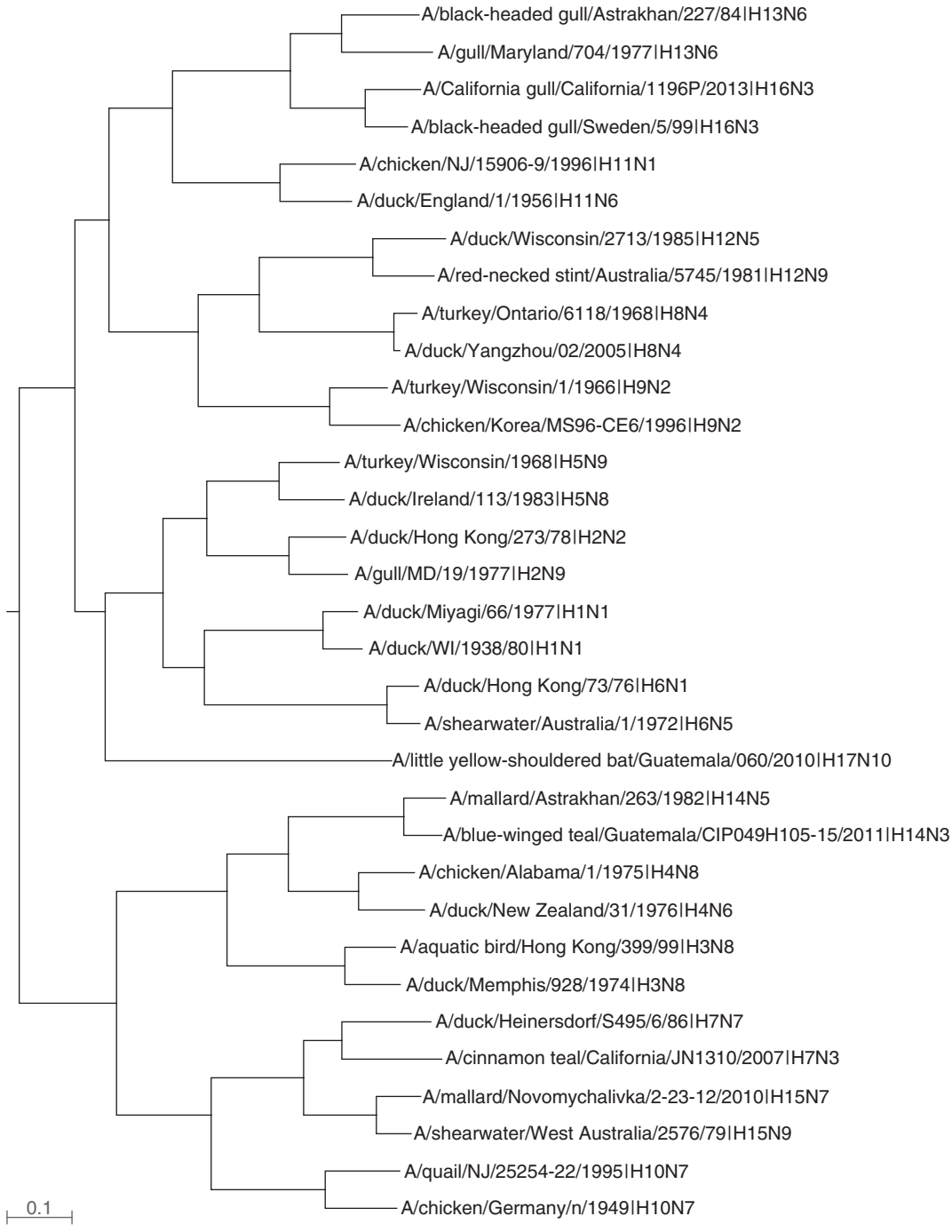

Figure 1.2 Phylogenetic tree of 17 hemagglutinin subtypes. The complete amino acid sequence of representative isolates for all 16 avian HA subtypes and the H17 bat subtype are included, with a representative North American and Eurasian isolate where available. The tree was midpoint rooted using the Influenza Research Database PhyML program, version 3.0 [144].

are lost. The HA and NA genes having greater nucleotide sequence diversity still separate at the amino acid level into clear Eurasian and American lineages for most hemagglutinin subtypes. For the H7 subtype a further division of lineages can be observed between the North American and South American lineages and between the Australian viruses and European and Asian viruses [154]. This distinction of the H7 subtype may reflect the availability of sequences, particularly from South America, where few AIV sequences are available. The differentiation of the wild bird isolates into distinct Old World and New World lineages suggests that infrequent transfer of AIV genes is occurring between these two geographic regions. However, the recent outbreak of Eurasian H5N8 HPAI in North America in 2014 does show that viruses can on occasion move long distances [52].

As more sequence information becomes available from wild bird and poultry isolates, the general rule of American versus Eurasian lineage appears to have more exceptions. For example, the H2 subtype influenza viruses appear to follow the rule of American and Eurasian lineages for poultry and duck isolates, but the North American origin shorebird and gull viruses are more closely related to Eurasian isolates than to other North American H2 isolates [84, 124]. Although the H2 shorebird and gull viruses are more similar to Eurasian viruses, they do cluster as a unique sublineage. A similar Eurasian-like gull and shorebird sublineage also exists for H6 influenza viruses from North America, but the internal genes, including the matrix and non-structural genes, have the anticipated American origin sequence [143]. Therefore these data probably represent a unique subpopulation of the hemagglutinin gene circulating in North America, and not evidence of recent movement of Eurasian-lineage viral genes into the Americas.

The complete host range of AIV in wild birds is not known, but based on sampling studies, two orders of wild birds are most consistently infected, the Anseriformes and the Charadriiformes (Table 1.1). The Anseriformes include ducks, geese, and swans, but the incidence of infection appears to be highest in dabbling ducks, including mallards, pintails, and teal. The incidence of infection appears to be seasonal, with the highest isolation rate being in juvenile birds in the fall of the year [145]. A lower incidence of infection occurs in the Charadriiformes, which include shorebirds and gulls. Wild bird AIV seems to pass easily between different bird species, and it is not currently possible to predict the species from which the virus was isolated based on the nucleotide sequence. The one possible exception to this rule is that most H13 and H16 viruses are from gulls, and gulls also seem to have a predominant gull lineage for at least some of the internal genes (Figure 1.3) [40, 152]. The ecology of AIV in wild birds is discussed in detail in Chapter 8.

Bat origin influenza

Recently, two unique IAVs have been identified in several species of bats, including yellow-shouldered and flat-faced bats, by molecular detection and sequencing from clinical samples from Central and South America. The bat isolates have not been obtained in eggs or cell culture. The viral sequences show enough similarities to IAV to remain in those genera, but these viruses also have enough unique differences for them to be unlikely to reassort with the traditional type A viruses. The viruses belong to two new subtypes, H17N10 and H18N11 [170, 171]. The internal genes are compatible with human influenza HA and NA genes in a reverse genetics system, but the HA and NA genes have enough structural differences for it to be likely that the HA protein uses a completely different receptor from other type A influenza viruses, and the NA gene has no measurable neuraminidase activity and also probably has a different function [197, 199]. It is not surprising that an influenza-like virus has been detected in bats, as the high density of bats within colonies should favor transmission of the virus, but it is currently not known whether these viruses cause any clinical disease and how widespread the virus may be in bat populations.

Epidemiology in man-made systems

AIVs are unusual in that they can infect and replicate in a wide variety of host species, including chickens, turkeys, swine, horses, humans, and a wide variety of other avian and mammalian species. However, the amount of virus required to infect the host can vary greatly depending on the level of host adaptation, which provides at least some level of species barrier [141, 173]. The virus as it becomes adapted to the new host typically

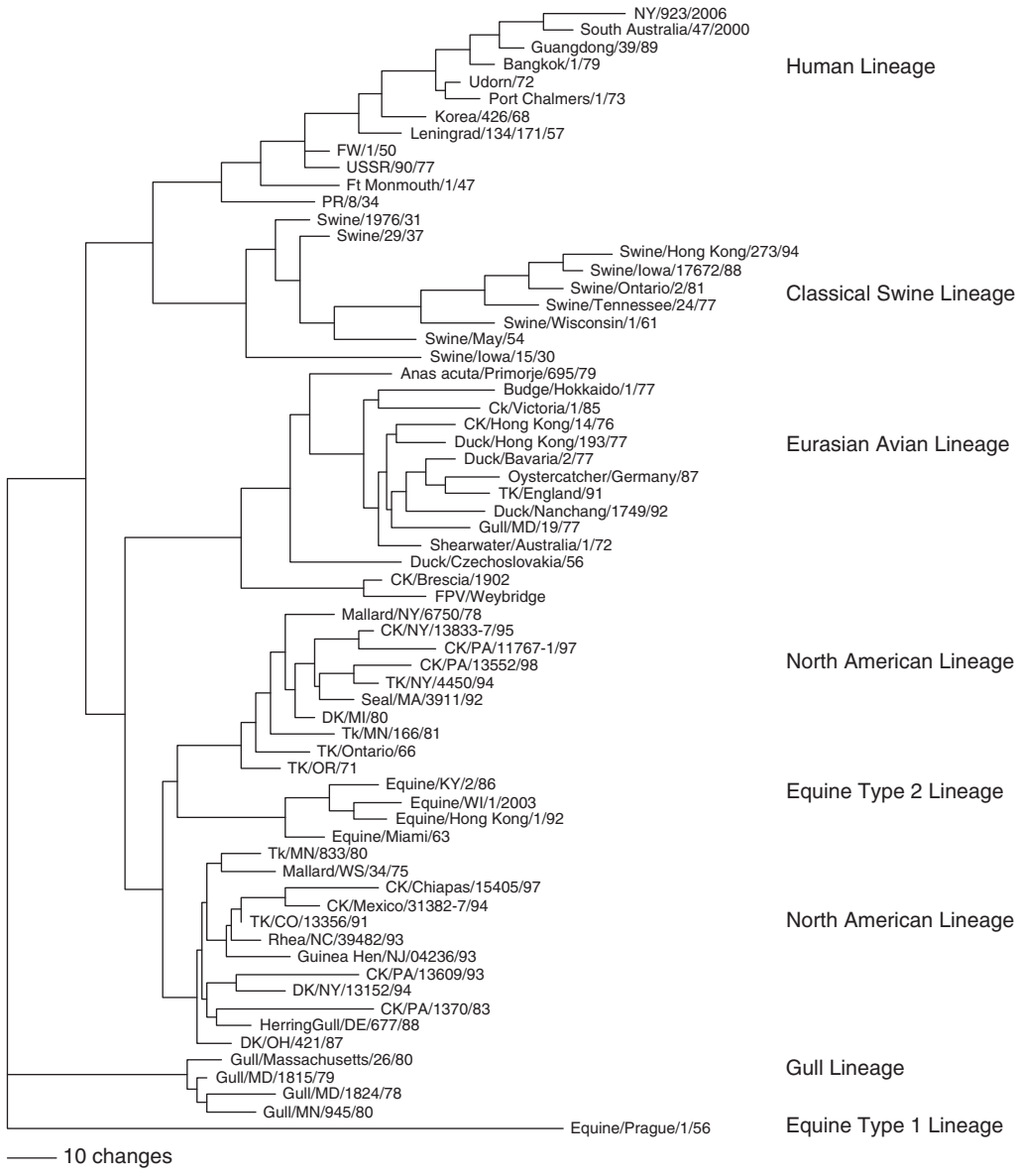

Figure 1.3 Phylogenetic tree of the matrix gene. The tree is based on the complete nucleotide sequence of representative isolates for major groups of type A influenza viruses. The tree is rooted to equine/Prague/1/56, which is the most divergent type A influenza virus. CK = chickens, DK = ducks, TK = turkeys. Standard two-letter abbreviations are used for states from isolates from the USA.

becomes less able to replicate in the original host species, such as wild birds. If the virus is allowed to circulate long enough in the new host, it becomes a human-, chicken-, or swine-adapted virus, and this results in the creation of unique phylogenetic lineages [16, 40]. Influenza viruses in a new host change at a high and predictable level that is the result of the high error rate of the virus and host selection pressures [17, 40, 126, 150]. For species under immune pressure from natural infection and/or vaccination, the changes in the HA and NA genes can occur at an even faster rate [35, 76]. The changes in both genes are concentrated in specific antigenic sites. For example, the human H3 protein has five antigenic sites that are binding sites for neutralizing antibody [182, 184]. Even with our

current level of understanding, we cannot predict the changes that allow species adaptation or allow the virus to evade the host immune response. However, the number of specific amino acid sites linked to species adaptation continues to grow. Although all eight genes probably play important roles, the HA and PB2 genes are prominent for changes thought to be important for adaptation from avian to mammalian hosts [93].

IAVs have become endemic in a number of species, including humans, swine, horses, and poultry, and once a strain of influenza circulates in a particular species for an extended period of time (months to years), the virus becomes increasingly species specific. Thus human IAVs do not usually infect swine, equine IAVs do not infect turkeys, and poultry IAVs do not infect humans. However, this general rule of host-adapted influenza viruses staying within a single species or related species does have many exceptions. For example, classical swine H1N1 IAV from North America routinely crosses the species barrier from swine to turkeys, causing costly disease outbreaks [45]. The sporadic infection of humans with some AIVs (H5N1, H7N7, H7N3, H7N9, and H9N2) from poultry has been observed, and therefore AIVs do present a public health threat as a zoonotic pathogen, although the risk is considered to be low [66, 108, 156, 175, 192]. Few experimental challenge studies of humans have been performed with AIVs, but in general the viruses replicated poorly and caused little to no clinical disease [11]. It is not understood whether all HA and NA subtypes of AIV have the same ability to infect humans or other species. Currently only a limited number of subtypes have become endemic in humans (H1, H2, H3, N1, and N2) [190].

The movement of AIV from wild birds to domestic bird species is not uncommon, but rarely results in viruses becoming endemic in poultry. Several routes of exposure of wild bird viruses to poultry have been documented or suspected of being the origins of outbreaks. Direct exposure to wild birds is the most likely method, with some of the best documented cases of exposure being in commercial turkeys in Minnesota, where multiple outbreaks of AI were observed yearly in the 1980s and early 1990s [42]. AIVs of many different HA and NA subtypes were isolated from turkeys in different outbreaks, and usually at times when wild ducks were migrating to or from their summer breeding grounds. During the migratory wild duck season, turkeys were raised outside and the wild birds could fly over or actually land in the turkey pens. During the 1990s the management system was changed so that the turkeys were reared in confinement for their entire lives, and the incidence of AIV was greatly decreased [164]. Limiting exposure of poultry to wild birds through confinement rearing and other biosecurity measures provides an opportunity to reduce the risk of AIV introduction from wild birds.

Another source of introduction of AIV to poultry is the live poultry marketing (LPM) system, which is found in many countries around the world, including the USA. LPMs typically offer a variety of birds that can be slaughtered and used for human food consumption. For many developing countries where refrigeration is not available, LPMs provide a way to maintain freshness until the product is sold. For other countries, such as the USA or Hong Kong, the LPM system caters to consumer preferences at a premium price for specific selection of a food bird compared with the purchase of a chilled or frozen bird from a supermarket. However, this marketing system provides an ideal environment for introducing and maintaining AIV in the poultry population [70, 150]. A common scenario is when domestic waterfowl, primarily ducks, are raised on ponds where exposure to wild ducks and other birds is common [10]. This creates a high risk of infection for domestic ducks, which can be transported to the LPM system where there is close contact with other poultry, including chickens, quail, and other gallinaceous birds. A constant supply of AIV-naive poultry continues to enter the LPM system, and provides the opportunity for viruses to become adapted to chickens and other avian species. Once AIV becomes entrenched in the LPM system, it provides an ongoing source of infection back to commercial poultry. One example is the H7N2 AIV that began circulating in the north-east USA in 1994 and was associated with at least five different outbreaks in industrialized poultry in seven states before it was eradicated [142]. The concern for LPMs in the introduction of AIV has resulted in Hong Kong banning the selling of live ducks and geese in the markets, a comprehensive surveillance program, and stricter sanitary requirements [70]. Quail have also been implicated as a highly susceptible species that may play an important transition role for viruses in the

market [85, 106]. These biosecurity and management changes have been effective in reducing the incidence of infected poultry in the markets.

An additional risk of introduction to farms is through the birds' drinking water. Typically this occurs when surface water sources, such as lakes or rivers, are used for drinking water or other purposes. If the drinking water is not properly purified, AIV from wild birds can be introduced to the poultry flock. The use of raw drinking water was suggested to be the source of AI outbreaks in the USA, Australia, and Chile [47, 132, 154].

At least one other common source of transmission of IAV for turkeys is exposure to pigs infected with swine influenza virus (SIV). Turkeys are susceptible to SIV, and having a turkey farm and swine farm in close proximity is a risk factor for the introduction of SIV. Infections with both classical H1N1 SIV and the more recent reassortant H1N2 and H3N2 SIV, and pH1N1 viruses in turkeys have been reported [45, 105, 155, 191]. Swine influenza has a unique and complex history that has some similarities to the disease in poultry, but also some important differences. SIV genes are also thought to be of wild bird AIV origin, but the detection of AIV genes in swine IAV either *in toto* or as a reassortment with endemic SIV is relatively rare.

The circulating strains of SIV in North America and Europe were quite distinct before the human pandemic H1N1 (pH1N1) virus that emerged in 2009. The pH1N1 virus was able to infect not only humans, but also swine, turkeys, ferrets, and sporadic cases in other species [105, 178]. The origin of swine influenza in North America is associated with the H1N1 Spanish flu pandemic in 1918. The virus diverged from the human isolate and was relatively stable for almost 80 years, and is considered to be "classical swine influenza." In 1998, new SIV emerged in the USA that had a unique internal gene cassette that consisted of swine, human, and avian IAV genes and human influenza-like H3 and N2 genes [181, 198]. The triple reassortment internal gene (TRIG) cassette allowed for multiple reassortment viruses of different HA and NA subtypes. The TRIG cassette included multiple genes that formed the basis of the human pandemic H1N1 virus in 2009 [138]. Since 1999, multiple antigenic variants and multiple reassortment events with human viruses have created an ever changing collection of viruses in North America [5].

Classical SIV circulated in Europe for many years, but it was replaced by avian-origin IAV in 1979 [68]. The avian-like swine virus reassorted with human H3N2 viruses in 1984 to establish a stable lineage. Many additional reassortant viruses of different origins were detected, with H1N2 viruses being commonly observed [72]. The human pH1N1 added to the picture in 2009, and currently avian-like H1N1, human-like H3N2 and H1N2 with different internal gene cassettes, and pH1N1 genes are circulating in the European swine population [178].

Although the surveillance in North American and European swine was far from comprehensive for SIV, enough representative isolates are available to document the major variants of the virus. Surveillance in Asia was sporadic in nature, but it did document a variety of viruses circulating in swine, including classical SIV, European avian-like SIV, human influenza viruses, and additional H1N1, H3N2, and H1N2 viruses not found in Europe and North America. The high density of swine and the importation of pigs to the region provided a unique mixing site for viruses from around the world [178]. Swine surveillance was almost non-existent in Australia, Africa, and South America before 2009, when the human pandemic H1N1 emerged. Studies have documented swine being infected with the pH1N1 virus on all three continents, and for Australia they were the first detections, as the continent had previously been free of SIV [28, 94]. Multiple subtypes of virus were identified in Argentina, including unique human-influenza-origin viruses as well as pH1N1 [110].

The emergence of pH1N1 provided a new impetus to increase surveillance of swine, because the pH1N1 had clear origins in SIV, but exactly when and where this viral lineage emerged is still unknown. The emergence of new viruses in swine indicates that viral genes can come from a variety of sources, including avian and human ones. The restricted movement of swine has allowed unique lineages of virus to develop in Europe and North America, although there is overlap of viruses in Asia. Evidence of infection of swine with avian-origin IAV, either from wild birds or from poultry, continues to be reported, and to pose a threat of introduction of novel viruses with both veterinary and human health implications [44, 55, 83].

Equine and canine influenza

Only two lineages of equine influenza viruses (EIVs) have been reported to be endemic in the horse population. The original subtype detected was H7N7 virus that was first isolated in 1956. The H7N7 EIV lineage based on the sequence divergence from other influenza viruses had been present in the horse population for an extended period of time [189]. The introduction of H3N8 in 1963 resulted in the likely extinction of the H7N7 lineage. The H3N8 lineage infected horses worldwide, probably as the result of frequent international movement of horses for racing and other equestrian sporting events. More similar to human influenza, which also has a worldwide distribution, the H3N8 virus has continued to evolve into unique sublineages, although there are only a limited number of these, presumably because the most fit virus outcompetes the less fit viruses. Currently two clades from the Florida sublineage are the dominant strains [24, 39]. In one of the clearest examples of influenza viruses jumping the species barrier, the H3N8 Florida clade 1 EIV jumped into dogs, probably in Florida, which resulted in the establishment of a unique canine influenza lineage of virus [24]. A recent study has shown that the canine-adapted virus has greatly reduced virulence in horses [119]. A second unique event was also reported, with H3N8 jumping from horses to dogs in Australia during the equine epidemic in that country in 2007 [63].

Clinical disease in poultry

Field presentation

Influenza infections in poultry, primarily chickens and turkeys, can be asymptomatic, but often cause production losses and a range of clinical disease from mild to severe in affected flocks. The virus can be generally divided into viruses that cause mucosal infections in the respiratory and/or enteric tract, and those viruses that also cause systemic infections. The viruses that cause mucosal infections are usually referred to as LPAIV, and typically these viruses do not cause high mortality in affected flocks. The viruses that cause systemic infections usually cause high mortality and are referred to HPAIV (they were historically known as fowl plague viruses) [64].

The LPAIV can cause asymptomatic infections, but typically the most common symptoms are mild to severe respiratory disease. A decrease in feed or water consumption is another common indication of flock infection when careful records of consumption are kept. For layer flocks or breeder flocks, drops in egg production can also be observed. The drops in egg production can be severe, with the flocks never returning to full production, as is commonly seen in turkey breeders infected with swine-like influenza viruses [45, 92]. In large flocks, small increases in daily mortality can be observed as the virus spreads through the flock. The LPAIV infection at least contributes to this increased mortality, because diagnostic testing of the daily mortality is considered to be a sensitive way to identify LPAIV infection [3, 151]. In some situations, infection with LPAIV may result in high mortality, generally in association with concurrent or secondary pathogens and/or poor environmental conditions [7]. On rare occasions, LPAIV may cause specific lesions in internal organs, either through direct infection or by other indirect causes [200].

The disease and lesions caused by AIV infections in domestic ducks will be discussed in more detail in Chapter 14, and in the chapter on pathobiology of avian influenza virus infections in birds and mammals in the previous edition of this book [160]. Elsewhere in the present volume, disease and lesions of IAV infections in humans (Chapter 5), pigs (Chapter 16), horses (Chapter 20), dogs (Chapter 22), miscellaneous mammals (Chapter 23), and laboratory mammalian models (Chapter 24) are presented.

Molecular and biological features of low- and high-pathogenicity avian influenza viruses

The LPAIVs can be of many different hemagglutinin and neuraminidase subtypes. The HPAIVs, for unknown reasons, have been restricted to the H5 and H7 subtypes, but most H5 and H7 influenza viruses are of low pathogenicity. It is only rare that these LPAIVs mutate into the HPAIV. It is generally believed that HPAIVs arise from H5 and H7 LPAIVs that have been allowed to circulate in poultry for extended periods of time. For example, LPAIV circulated for several months to years in poultry flocks in the H5 outbreaks in Pennsylvania in 1983

and Mexico in 1994, and the H7 outbreak in Italy in 1999, before the viruses mutated to become HPAI [49, 59, 196]. The selection pressures for viruses to change from LPAIV to HPAIV are not currently known, but the replication of virus in gallinaceous birds, including chickens, turkeys, and quail, is considered a critical part of the process. HPAIVs are not believed to be normally present in the wild bird host reservoir [121]. However, on four separate occasions HPAI has been detected in wild birds. The first outbreak was in terns in South Africa in 1961, which was not associated with a poultry source [12]. Three widespread outbreaks of H5 HPAI in wild birds have been reported in the last 10 years that were all associated with poultry outbreaks. The initial spillover event in 2005 of a clade 2.2 H5N1 virus resulted in mortality events in multiple wild bird species. The virus moved through wild birds to eventually reach most of Europe and several countries in Africa. This lineage of virus did not persist permanently in wild birds [82]. The second spillover event was a clade 2.3.2.1 H5N1 virus first detected in 2007 [139]. The virus was detected primarily in East Asia, but spread to Eastern Europe and Southern Asian countries in 2010 and 2011, and became established in poultry populations in Bangladesh. Isolates from wild birds were often from dead or sick birds, but were not associated with large mortality events in wild birds that characterized the initial introduction of the clade 2.2 viruses. Experimental testing showed continued high virulence in chickens, but variable mortality in different duck species [22, 33, 53, 99]. It is unclear whether this lineage is persisting in wild birds.

The third wild bird epornitic was detected in late 2013 and has spread from East Asia to Europe and North America. This virus includes multiple reassortants, with N8 being predominant, but N2 and N1 reassortants have also been detected. The hemagglutinin gene is classified as clade 2.3.4.4. This virus has also not been associated with mass mortality events in wild birds, and appears to have less virulence in chickens than previously characterized H5N1 viruses [32, 140].

Cellular pathobiology and hemagglutinin cleavage

The primary virulence characteristic that separates the LPAIVs and the HPAIVs in chickens and other gallinaceous birds is the ability of the hemagglutinin protein of HPAIVs to be cleaved by the ubiquitous proteases found within most cells in the host. Influenza viruses must have the HA protein, which is produced as a single polypeptide, cleaved into the HA1 and HA2 subunits before it can become infectious. This cleavage is necessary for the fusion domain to be activated during the uncoating step of virus replication. Normally trypsin or trypsin-like proteases (plasmin, blood clotting factor-like proteases, tryptase Clara, bacterial proteases) cleave the hemagglutinin protein by recognizing a single arginine in the extracellular environment [41, 62, 65, 73]. The distribution of LPAIVs in the host is believed to be highly influenced by the local availability of these trypsin-like proteases in the respiratory and enteric tracts [65]. Other proteases can also cleave influenza, and in chick embryos it is believed to be a prothrombin-like enzyme similar to blood clotting factor X [41]. However, when multiple basic amino acids (lysine and arginine) are present at the HA cleavage site, particularly by the insertion of multiple basic amino acids, the cleavage site becomes accessible to furin or other ubiquitous proteases that are found in most cells of the body [148]. The HPAIVs' HA protein is cleaved during the assembly stage of virus replication, and therefore is infectious when it is released from the cell [146, 148]. This allows the HPAIV to greatly expand its ability to replicate in a number of different cell types, including a range of cell types in the brain, heart, skeletal muscle, and pancreas. The damage to critical organs or to endothelial cells lining the blood vessels can cause a variety of disease symptoms that often lead to the death of the bird [111, 159]. Other viral genes are also important in determining the virulence of the virus, but the hemagglutinin cleavage site is by far the most important virulence trait in gallinaceous birds [81, 123].

Impact of host and virus strain on pathogenicity

The HPAIV phenotype by definition causes high mortality in 4- to 6-week-old specific pathogen-free chickens [188], but just because it is HPAI in chickens does not necessarily provide a predictor for disease in other species. Few studies have characterized the pathogenicity of a single isolate in a number of different species after experimental challenge. One of the broadest series of studies examined an H5N1 HPAI 1997 chicken isolate

from Hong Kong that was used as an experimental inoculum for a variety of avian species. The Hong Kong 97 strain caused high mortality in all of the gallinaceous species tested, including chickens, turkeys, quail, and pheasants, although differences in mean death time were observed among species [111]. Most other species tested had less severe or in some cases no clinical disease signs, although most were infected based on the ability to reisolate virus from challenged birds [112–114]. Predictions of virulence, outside of the gallinaceous species, could not be made for different orders of birds. For example, some geese when challenged had neurological signs and lesions that correlated with virus replication sites in the brain [112]. However, ducks tested from the same order of birds, Anseriformes, had limited infection in the respiratory tract but did not show any evidence of disease [112]. It seems clear that the virulence associated with hemagglutinin cleavability is not the only factor that determines virulence in other species. This has been clearly shown in ducks with the recent Asian H5N1 viruses. In a 2-week-old Peking duck model, the early H5N1 viruses from 1997 to 2001 could infect but did not cause morbidity or mortality. However, starting with some isolates in 2002, increased mortality was observed, with 100% mortality being seen with more recent viruses [104, 161]. The Asian H5N1 viruses all have an H5 gene from the same lineage and identical or nearly identical hemagglutinin cleavage site sequence with an insert of multiple basic amino acids, and all remain highly pathogenic for chickens. However, the internal genes for these viruses are variable, and it is believed that these internal gene differences account for the difference in virulence [78].

For mammalian species, including swine and humans, naturally infected with HPAIV, severe clinical disease is associated with severe atypical pneumonia, reflecting replication primarily in the respiratory tract, and systemic replication is not commonly observed. Other mammalian species, including ferrets, cats, and dogs, may have more systemic spread of the virus that contributes to high mortality for some strains of HPAIV [69]. The pathogenesis of HPAIV is difficult to characterize for all species, and as the virus changes, the clinical presentation of disease also often changes.

Hemagglutinin changes associated with high pathogenicity

The hemagglutinin cleavage site remains the best but not a perfect predictor of viral virulence in chickens and other gallinaceous birds. As previously mentioned, the presence of multiple basic amino acids upstream of the HA1 and HA2 cleavage site is correlated with virulence [122]. Only the H5 and H7 subtypes of AI are currently known to have an HPAI phenotype, for reasons that are not readily apparent. Sequence comparisons show the H5 and H7 subtypes to be distinctly different from each other. Although both H5 and H7 proteins maintain the general principle of the cleavage site being between arginine and glycine and multiple basic amino acids at the cleavage site resulting in an HPAIV phenotype, there are distinct differences between the subtypes. The typical cleavage site sequences of wild bird LPAIV of H5 and H7s viruses are different [121]. H5s viruses typically have a QRETR/G sequence with arginine at the -1 and -4 position. H7s typically have an NPKTR/G sequence with a lysine and arginine at the -1 and -3 positions. The change to virulence for H5s can occur by substitution of non-basic to basic amino acids or by an insertion of basic and non-basic amino acids at the cleavage site (Table 1.2). The chicken/Scotland/59 H5N1 virus has four basic amino acids at the cleavage site RKKR/G [27], presumably through site substitution that results in an HPAI phenotype. More commonly, additional basic amino acids are inserted at the cleavage site, with two, three, and four additional amino acids being observed. For example, the chicken/Hong Kong/97 H5N1 virus had a sequence of QRERRRKKR/G [153]. The mechanism of insertion of amino acids is not clear, but a duplication event appears likely for several of the H5 HPAIVs [109]. Other parts of the hemagglutinin protein can also play a role in the phenotype of the virus. The best example is the presence or absence of a glycosylation site at position 10–12 of the HA1 protein. In 1983, an LPAI H5N2 virus, chicken/Pennsylvania/1/1983, was isolated that had four basic amino acids, QRKKR/G, at the cleavage site. Six months later, an HPAIV emerged in Pennsylvania, chicken/Pennsylvania/1370/83, which had the same HA cleavage site, but this virus had lost a glycosylation site at position 10–12 in the HA1 protein. The glycosylation site is structurally extremely close to the HA cleavage

Table 1.2 Examples of genetic mechanisms for LP to HP change based on deduced amino acid sequence of HA proteolytic cleavage sites in H5 and H7 AIV.

Influenza virus	Subtype	Pathotype	Amino acid sequence	Mechanism[a]					References
				1	2	3	4	5	
Typical H5 LPAI	H5	LP	PQ. ….. . .RETR*GLF						[128]
A/turkey/England/1991	H5N1	HP	PQ . . . .RKRKTR*GLF	X	X				[128]
A/chicken/PA/1370/1983	H5N2	HP	PQ …….. KKKR*GLF	X				X	[128]
A/tern/South Africa/1961	H5N9	HP	PQRETRRQKR*GLF	X		X			[128]
A/chicken/Puebla/8623-607/1994	H5N2	HP	PQ. … RKRKTR*GLF	X	X				[37, 49]
A/chicken/Queretaro/14588-19/1995	H5N2	HP	PQRKRKRKTR*GLF	X	X				[37]
Typical H7 LPAI	H7	LP	PEIP ……. . . .KTR*GLF						[128]
A/chicken/Victoria/1985	H7N7	HP	PEIP . . . ….KKREKR*GLF			X			[128]
A/turkey/Italy/4580/1999	H7N1	HP	PEIPKG . . . .SRVRR*GLF			X			[19]
A/chicken/Chile/176822/2002	H7N3	HP	PEKPKTCSPLSRCRETR*GLF[b]				X		[154]
A/chicken/Canada/AVFV2/2004	H7N3	HP	PENPK … QAYRKRMTR*GLF[c]				X		[107]
A/chicken/Saskatchewan/HR-00011/2007	H7N3	HP	PENPKTTKPRPRR*/GLF[d]				X		[13]
A/chicken/Jalisco/12383/2012	H7N3	HP	PENPKDRKSRHRRTR-GLF[e]				X		[54]

[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.

[d]18 nucleotides from unidentified chicken gene coding 6-amino-acid insert.

[e]24 nucleotides from 28S chicken ribosomal RNA coding 8-amino-acid insert.

Modified from Swayne, D. E., D. L. Suarez, and L. D. Sims. 2103. Influenza. In: *Diseases of Poultry*, 13th edition, D. E. Swayne, J. R. Glisson, L. R. McDougald, V. Nair, L. K. Nolan, and D. L. Suarez, eds. Wiley-Blackwell: Ames, IA. 181–218.

site, and it is believed that the loss of the sugars allowed greater access to the cleavage site, making it accessible to the ubiquitous proteases that changed the phenotype of the virus [59]. This and other glycosylation sites have also been shown experimentally to be important in virulence [50].

The change from LPAIV to HPAIV for H7 viruses appears to have several important differences. First, all HPAI H7 viruses have insertions of 2 to 10 additional amino acids at the cleavage site. The mechanism for such insertions also appears to be different in many cases. Although a duplication event appears likely for some viruses, in several recent cases non-homologous recombination is the likely method of insertion. In the Chilean outbreak in 2002, the Canadian outbreak in 2004, and the Mexican H7N3 outbreak in 2012 an insertion of 30 nucleotides from the nucleoprotein gene, 24 nucleotides from the matrix gene, and 24 nucleotides from host chicken 28s ribosomal RNA, respectively, resulted in the increase in virulence [54, 107, 154]. Other cases of non-homologous recombination have been seen in experimental studies where nucleoprotein and host ribosomal RNA sequence was inserted at the cleavage site [60, 98]. In all five examples, the insertions had some basic amino acids, but they were a minority of the insert. In these examples the increased spacing in the cleavage site loop appears to be the more important factor for increasing virulence, as opposed to just the addition of basic amino acids. Almost all of the H7 HPAI outbreak viruses appear to have become HP by unique events at the cleavage site, which makes the prediction of minimum changes to define HPAI by sequence alone difficult for H7s.

Other variables that affect pathogenicity

The HPAIV is defined by an *in vivo* pathotyping test in chickens, applicable to any influenza virus, and/or by a sequence analysis of the HA cleavage site for H5 and H7 influenza. The best predictor of HPAIV is when a suspect virus has the same cleavage site as another known HPAIV.

In such situations the virus is reportable to the World Organization of Animal Health (OIE) as an HPAIV. However, an outbreak in the USA (Texas) in 2004 was a clear case where the phenotype and the genotype did not match up. In this case the Texas/04 isolate had the same HA cleavage site sequence as the A/chicken/Scotland/59 virus, and was reported to OIE as an HPAIV, but the virus was LP in the standard chicken pathotyping test [79]. Even though the two tests did not correlate, and high virulence was not seen in the field, the virus was still considered to be virulent, and this resulted in major trade sanctions on poultry exports for a limited period of time. Other examples of discordance between phenotype and genotype have previously been described [186], and a similar case was reported in Taiwan of H5 viruses with four basic amino acids where some were pathogenic after IVPI testing and some were not [74]. Currently no completely accurate molecular prediction scheme has been determined for HPAIV.

It is also clear from experimental studies that the age and route of inoculation as well as species can affect the virulence of AI virus in experimental infections. The age effect has been seen both in chickens and in ducks. For example, when 1-day-old SPF chickens were challenged intravenously with the LPAIV A/turkey/Oregon/1971, mortality was seen in seven of eight chicks. When the same virus was administered to 4-week-old chickens at the same dose and by the same route, mortality was seen in only one of eight chicks. In this example, the virus replicated to high titer in the kidney, which resulted in renal failure leading to death in most of the 1-day-old chicks. The same virus given by the intra-choanal cleft (intranasally) at the same dose caused mortality in only one of eight 1-day-old chicks [20]. This example shows that mortality can be greatly affected by the age of the bird and the route of inoculation. The intravenous inoculation route, which is not a natural route of exposure, probably seeded high levels of virus to the kidney, which led to the high mortality. The intravenous route of challenge, the standard for *in vivo* pathotyping in chickens, can result in sporadic deaths with some LPAIVs, typically because of replication in the kidney resulting in kidney failure [134, 135, 163]. Primary chicken kidney cells allow replication of LPAIVs, presumably because they produce trypsin-like enzymes that cleave the hemagglutinin protein, and this property allows LPAIVs to be plaqued without the addition of trypsin in primary kidney embryo cell lines [20].

In ducks it has also been shown that there is a marked difference in disease based on age, with younger ducks being more susceptible to severe infection. For example, several Asian H5N1 viruses cause high mortality in 2-week-old ducks, but the same viruses in 4-week-old ducks produce much lower or no mortality [104, 161]. Increased virulence in younger animals is commonly seen, although the reasons for the differences are not clearly defined. The immaturity of the immune response, both innate and adaptive, probably contributes to these differences. For example, the interferon response greatly increases in the embryo as it ages, and presumably the peak interferon response also occurs after hatching [87].

In some cases, virulence can be greater in older birds or in birds in egg production. A common example is swine-like influenza in turkeys. For turkey breeders in production, infection can cause severe drops in egg production, but for flocks not in production the birds often seroconvert with no clinical signs of disease [6, 34, 45, 155]. Increases in mortality have also been seen in layers with egg yolk peritonitis after LPAIV infection, which are not seen in immature birds [200].

Antigenic drift and shift

IAVs have two primary mechanisms to provide diversity in the viral population, namely a high mutation rate and the ability to reassort gene segments [86, 174]. Both methods provide an opportunity for the virus to rapidly change and adapt, which contributes to the ability of the viruses to establish infections in new host species. The ability to rapidly mutate and adapt is not unique among the RNA viruses, but some viruses can tolerate higher levels of sequence changes in at least some viral genes. IAVs, as has been previously described, can differ greatly in amino acid sequence, particularly in the surface glycoproteins, hemagglutinin and neuraminidase [95]. These differences in amino acid sequence result in differences in antigenicity, such that antibodies to H1 IAV will neutralize only H1 viruses, and not any other subtype of IAV. These antigenic differences

have major implications for vaccination, since vaccine protection is mediated primarily by specific antibodies being produced to the hemagglutinin protein, and to a lesser extent to the neuraminidase protein [77]. Therefore current vaccines are limited to providing only subtype protection, and to provide complete protection from IAV would require the addition of 16 different antigens representing each HA subtype.

Although neutralizing antibodies to one HA subtype of influenza should neutralize all viruses within the same subtype, differences in the specificity of the antibody greatly affect the level of protection observed. The impact of antigenic drift on vaccination with human influenza is a well-characterized problem that requires the vaccine seed strain to be evaluated every year to try to achieve the best possible match with the circulating strain [136]. Two different subtypes of IAV are endemic around the world in the human population, namely the H1N1 and H3N2. For both subtypes of virus, a single lineage of virus is present that can be traced back to the time when the virus was introduced to the human population [17, 18, 40]. Unlike what we see with animal influenza viruses, which will be described in more detail later, these two subtypes of virus have evolved with little difference in sequence based on geographic origins of the virus. This worldwide distribution is likely to be the result of widespread and rapid movement of humans between regions that efficiently transmits the virus and that allows only relatively minor variants of the virus to circulate at the same time. However, the viruses do change at a rapid and predictable rate, sometimes called a molecular clock [17]. The observed changes in the genome are not random, but are concentrated primarily in the surface glycoproteins [116]. Influenza viruses, like other RNA viruses, lack a proofreading mechanism in the replication of viral RNA, which results in errors in transcription leading to a high mutation rate [103]. The high mutation rate provides the opportunity for change, but many of the changes introduced by this error-prone transcription are deleterious to the virus, because it creates premature stop codons, changes in amino acids so the virus is less fit, or changes in a regulatory signal that affects virus replication [118]. Most of the deleterious mutations are lost during the selection process to achieve the fittest virus in a population. The mutation rate for all eight gene segments is probably the same, but because of positive selection, more changes in the HA and NA genes are conserved [116].

One of the primary selective factors on the HA protein is thought to be antibody pressure from the host, either from previous exposure to the virus or by vaccination [116]. For the human IAV H3 protein, five antigenic regions have been characterized where antibody to these regions can be neutralizing to the virus and therefore would be protective for the host during infection. These antigenic regions are on the globular head of the HA protein, with many close to the receptor binding site [182, 184, 185]. Antibodies to the antigenic sites can be neutralizing because they directly block access to the receptor binding site and prevent the virus from attaching to and initiating infection in the host. These antigenic regions, however, can tolerate a significant amount of amino acid diversity, and when changes to key amino acids occur, one of the neutralizing epitopes may be changed so that antibodies can no longer bind [182]. These changes in specificity of the antibody can result in a virus being better able to escape the ability of the host's antibodies to control infection, resulting in greater virus replication and transmission of these escape mutants. The accumulation of these amino acid changes at these antigenic sites is the antigenic drift that results in vaccines for IAV being less protective over time. For humans, the influenza vaccine seed strains, both IAV and influenza B virus, are evaluated yearly to determine whether the currently circulating field strains are still neutralized effectively by antibody produced to the vaccine strain. Comparison of virus sequence is used to identify when new viral variants are occurring and at what frequency [136]. From the sequence information, representative strains are used to produce antibodies to do more in-depth cross-hemagglutination inhibition (HI) studies. If the field strains in the cross-HI studies show a fourfold or greater difference in inhibition, this is evidence that the current vaccine seed strain may be ineffective. As the amount of HI data has increased, the use of computer programs to generate maps of antigenic differences, commonly referred to as antigenic cartography, has become common for both human and veterinary medicine [2, 137]. Vaccination for human influenza requires a close match of vaccine

to field strain, or protection from vaccination is adversely affected [48]. Antigenic differences of more than fourfold appear to be the range where the decrease in antibody specificity affects the protection seen from vaccines. The seed strains are typically changed every 3 to 4 years to compensate for this antigenic drift [136].

For poultry, antigenic drift also occurs, but the interpretation and importance of antigenic drift are much more complicated. The principles of changes at antigenic sites affecting the specificity of neutralizing antibody are the same for the immune response in poultry, but the trigger for when antigenic change necessitates a vaccine change is not defined. In part this is a difference in the pathobiology between influenza in humans and HPAI in chickens. With human influenza, viral infection is a mucosal infection of the respiratory tract, and with HPAI, the virus has both systemic and mucosal replication. Killed vaccines, which are commonly used in humans and poultry, provide high levels of serum IgG (or IgY, the avian counterpart to mammalian IgG) antibody, but little if any secretory IgA, which is the most effective antibody for the control of influenza in experimental mouse models [120]. The transudation of IgG (IgY) that crosses the mucosal surface can provide effective control of clinical disease, but it does not provide ideal protection [166]. In chickens with LPAIVs and for replication of HPAIVs on the mucosal surface, a similar immune response probably occurs. However, the severe clinical disease seen with HPAIV infection is primarily from the systemic replication of the virus, and subtype-specific antibody appears to efficiently block viremia and therefore the systemic replication of the virus [77]. The serum antibody protection appears to be affected less by antigenic drift in its ability to block viremia and prevent severe clinical disease, but it has been shown previously that the level of virus shedding is correlated with the relatedness of the vaccine to challenge strain [76, 162].

An additional concern with AIVs is the wide diversity of viruses that can infect poultry. Since most outbreaks of LPAI and HPAI result from independent introductions of viruses from the diverse wild bird reservoir, most epidemiologically unrelated outbreaks are antigenically different from each other even within the same subtype [38, 75]. This antigenic diversity, as described earlier, is broken down generally into North American and Eurasian lineages, and the selection of a vaccine seed strain should at a minimum consider matching the HA amino acid sequence as closely as possible to try to obtain the best protection and reduction in shedding [162]. However, many different factors are involved in vaccine seed strain selection.

One additional complication with AIVs and other animal influenza infections is that if an outbreak becomes widespread, geographic separation of viral populations can occur because of limits on the movement of animals and animal products that allows separate evolutionary paths to occur. The geographic separation has been observed with several outbreaks, including H5N2 LPAI in Mexico, H9N2 LPAI in the Middle East and Asia, and the H5N1 HPAI outbreak in Asia, Europe, and Africa [76, 187, 193]. The issue of different HA lineages again complicates vaccine selection, since antigenic drift can occur within a clade or lineage. The current A/goose/Guangdong/1/1996 lineage of H5N1 HPAIVs has separated into multiple lineages of virus described in a clade system based primarily on sequence differences, although this does translate into antigenic changes as measured by hemagglutination inhibition tests. Antigenic drift continues such that fifth-order clades are now defined. For example, the 2.3.2.1 viruses that emerged are now further defined based on sequence differences to 2.3.2.1a, 2.3.2.1b, and 2.3.2.1c [1]. Because of the antigenic differences between different lineages of viruses, China has been using surveillance information to target vaccination with updated reverse-genetics-based vaccines [80].

For long-lived animals, an additional concern with influenza infection is antigenic shift. Antigenic shifts are typically considered for human IAV, but have also been seen in animal IAV. Antigenic shift occurs when a large proportion of the host population has previous exposure, by either infection or vaccination, with a particular HA subtype, and then they become exposed to a different HA subtype [30]. Because the host population has little or no protective immunity to the new virus, it can rapidly spread in the new population, causing a widespread and sometimes severe outbreak of influenza called a pandemic. In the human population, four major pandemics occurred in the last century. The most severe was when an H1N1 virus emerged, probably replacing an H2 human influenza, in 1918, and

resulted in a major pandemic that killed over 40 million people [168]. The second pandemic of the century occurred in 1957, when the H1N1 virus was supplanted by an H2N2 virus. The third pandemic started in 1968, when an H3N2 virus supplanted the H2N2 virus [190]. The most recent pandemic was H1N1 influenza, which emerged in 2009. This virus, although the same subtype as the circulating seasonal H1N1 virus, was antigenically different enough to spread rapidly in the human population, and eventually supplanted the old H1N1 virus from circulation in humans [138]. The origins of new pandemic viruses generally are not clearly understood, although it appears that they can be caused by a completely new IAV being introduced into the human population or by a reassortment event between the circulating human strain and another animal IAV [190]. The 1918 H1N1 virus appeared to be a completely new virus, but the H2N2 and H3N2 viruses were reassortant viruses that changed multiple genes, including, most importantly, the HA gene [190]. The 2009 pH1N1 virus was closely related to SIV circulating in North America, but a reassortment event with an unknown virus contributed two other genes that allowed the virus to replicate and transmit well in humans [138].

The best example of antigenic shift in veterinary medicine is that of EIV. Historically, horses had been infected with an H7N7 subtype IAV that appeared to have circulated in horse populations for a long period of time. In 1963 a new subtype emerged, H3N8, which infected horses worldwide, and eventually completely replaced the historic H7N7 IAV, with the last isolate of that subtype being obtained in 1979 [24, 102]. For swine in the USA, H1N1 was primarily the only strain of influenza that circulated from 1918 to the late 1990s. However, starting in 1998, H3N2 viruses began to be isolated in the USA . These viruses were an unusual reassortant that had H1N1 SIV-like genes, human influenza virus-like genes, and AIV-like genes. The H1N1, H3N2, pH1N1, and even other reassortant viruses (H1N2 and H3N1) currently co-circulate in the USA [56, 57]. Because of the antigenic shift, vaccines for horses and swine needed to be updated to include the new viruses in order to achieve adequate vaccine protection. However, vaccine companies have not been very proactive about updating vaccines, in part because of regulatory concerns, and many equine vaccines include H7N7 as an antigen, although it has not circulated for over 35 years.

For poultry, antigenic shift has not been a major issue because of the short production lives of most commercially produced poultry. Because infection with AIVs had been uncommon, commercial poultry were not naturally exposed, and vaccination is still not widely practiced except against H5N1 HPAIV in China, Egypt, Indonesia, Vietnam, and Bangladesh. Therefore most poultry are completely susceptible to infection with any influenza subtype. Further details about avian influenza vaccines are provided in Chapter 15.

Conclusions

Influenza remains a major health issue for poultry, swine, and equine populations around the world. The biggest concern for poultry has been HPAIV infection, because of severe clinical disease and the negative impact on trade. However, LPAIV infections also remain a concern because they are able to cause disease and production losses, they occur more widely than HPAIVs, and for the H5s and H7s LPAIVs there is the ever present threat of mutation to HPAIV. AIVs are difficult to control because of the wildlife reservoir, the adaptability of the virus, and the lack of good control tools. The SIV issue continues to grow more complex as rampant reassortment of swine and human IAV makes control through vaccination difficult. EIV also continues to change antigenically, although only two major lineages currently circulate. However, current vaccination tools do not provide long-term protection, and in general remain poorly antigenically matched because vaccines are not updated appropriately. Efforts to increase our understanding of the virus and research to develop new methods for control should be a priority for the veterinary community.

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2 Diagnostics and surveillance methods

Erica Spackman, Giovanni Cattoli and David L. Suarez

Introduction

Detection and diagnosis of influenza A virus (IAV) infection in animals require a laboratory test since disease from IAV presents no pathognomonic signs. Diagnosis and surveillance of animal influenza focus on the detection of virus or type-specific antibodies. Whether one targets the virus or antibodies in testing depends on the goals of the testing. Further characterization of an isolate or antibody specimen may be undertaken in order to define the subtype or other biological features. The specific tests that are employed will vary depending on the species, the goals of testing, and the resources available.

Reflecting the importance of IAVs both for domestic animals and for public health, numerous diagnostic tests have been reported in the literature and are commercially available. In fact IAV is frequently used as the proof-of-concept agent for new diagnostic technology. In addition, because of the importance of IAV, some harmonization of diagnostic and detection methods has been established within certain species and domestic animal groups (e.g. poultry, horses). Standardization of testing methods for poultry is often undertaken at an international level (e.g. World Organization for Animal Health, also known as OIE) or at a regional or national level (e.g. federal government-issued guidelines, National Poultry Improvement Plan in the USA). In contrast, there is sometimes less guidance available for other species. Standard operating procedures and details of the established and most important validated diagnostic methods can be found in a number of references [10, 48, 61, 72] and on the OIE web site (www.oie.int).

Sample types

The type of sample and the processing methods are dependent upon numerous interrelated factors, such as the purpose of testing, the type of tests used, and the target species. It is not uncommon for a single sample to be tested by more than one assay, particularly when the results of a screening test, such as antigen capture immunoassays (ACIAs), must be confirmed by a second, more sensitive test, such as real-time reverse transcription polymerase chain reaction (rRT-PCR) or virus isolation (VI).

Oropharyngeal (or tracheal) swabs and cloacal swabs are the most widely used specimen types for avian species, although tissues are also collected in some cases. Tissues are not optimal for detection of low-pathogenicity (LP) avian influenza virus (AIV), but trachea and lung are recommended if tissue collection is undertaken. Numerous tissues may be collected for high-pathogenicity (HP) AIV, including lung, brain, heart, kidney, and spleen.

Oropharyngeal swabs, which include swabbing of the choanal cleft, are preferred to tracheal swabs for the following reasons: (1) material from the sinuses where the virus replicates is captured from the choanal cleft; (2) these swabs are less invasive and there is not a risk of causing damage to the trachea; (3) less skill is required, as the esophagus is easier to swab and can be confused with the trachea by untrained individuals. A study using rRT-PCR on specimens from experimentally infected animals has shown that oropharyngeal and tracheal swabs are equivalent for detection of influenza from avian species [69].

In most cases the optimal approach is to collect both oropharyngeal and cloacal swabs. Although

Animal Influenza, Second Edition. Edited by David E. Swayne.
 Published 2017 by John Wiley & Sons, Inc.

the tropism of AIV for the respiratory or enteric tract is often species specific, there are some strain-dependent exceptions, depending on how a lineage is adapted to a particular species. The general rule is that LPAIV in waterfowl (either domestic or wild) will have a higher tropism for intestinal replication, and therefore more virus will be shed by the cloacal route, resulting in better detection from cloacal swabs [2, 60, 73]. Conversely, in gallinaceous birds, including chickens and turkeys, LPAIV typically has respiratory tract tropism, so it is best to use oropharyngeal or tracheal swabs to collect infectious virus. Importantly, there are insufficient data from many other avian species (pigeons, gulls, shorebirds, etc.) to allow unequivocal recommendation of the use of one swab type or the other; therefore both should be collected. A recent example of an exception to the respiratory–gallinaceous and intestinal–waterfowl tropism rules of thumb is the 2013 lineage of H7N9 viruses from China that replicated well in the upper respiratory tract of both gallinaceous birds and waterfowl. Therefore, when undertaking surveillance for this lineage, oropharyngeal or tracheal swabs are the recommended sample for waterfowl as well as for chickens [49].

More generally, several studies have shown that maximal sensitivity in a population can be achieved by collecting and testing both oropharyngeal (or tracheal) swabs and cloacal swabs, although many investigators do not consider that the increased number of positive samples justifies the greatly increased cost of sampling [33]. However, an approach that has been adopted with wild bird samples involves placing both swab types in the same tube. This approach has been shown to increase the number of positive samples compared with cloacal swabs alone in two independent studies [30, 50].

Since the tissue tropism of IAV tends to be consistent for the respiratory tract in mammals, the optimal samples from mammalian species (swine, horses, and dogs) are nasal swabs. Oral fluids have also been shown to be effective for detecting IAV in swine herds [18, 28, 56]. As is the case for birds, lung tissue may also be used in post-mortem sampling.

Pooling of swab samples by placing numerous swabs in the same tube at the time of collection can help to reduce costs by consolidating samples,

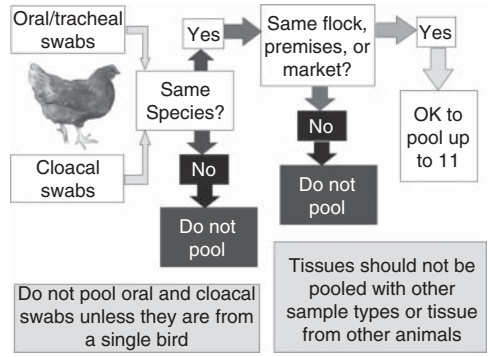

Figure 2.1 Swab pooling for specimens from avian species. Up to 11 oropharyngeal/tracheal swabs or cloacal swabs may be pooled per vial if they are collected from the same species and the birds are housed together as part of the same flock or at the same location or market.

but samples from different species and groups of animals should not be pooled (Figure 2.1). Tissues from different animals should not be pooled because if one animal has developed antibodies they can neutralize the virus in tissues from other animals if the two samples are processed together. In addition, tissues should not be pooled with swab material. Pooling of material later, in the diagnostic laboratory, can dilute positive samples and increase the risk of cross-contamination, and is generally not recommended. Up to 11 oral swabs from experimentally infected chickens have been successfully pooled for both rRT-PCR and VI [35, 64], and up to five swabs with fecal material from mallard ducks have been successfully pooled for rRT-PCR detection in a study using spiked swabs [26].

Sample collection, transport, and storage

The correct procedures for sample collection, transport, and storage are critical for obtaining accurate test results. Swab samples should be collected in a well-buffered, salt-balanced medium containing protein (e.g. brain–heart infusion broth or tryptose phosphate broth) [23, 64]. Influenza is not as stable in salt buffers without protein, such as phosphate-buffered saline, and the sensitivity of virus isolation will be reduced [23, 64]. Samples should never be transported dry, as this has been shown to reduce the sensitivity of rRT-PCR as well as that of VI [55, 64]. Antibiotics may be added to

transport media unless there is a need to test for bacterial agents.

During transport it is important to maintain the cold chain. Ideally, samples should be transported to the diagnostic laboratory within 24 hours of collection, and should be kept at refrigeration temperatures (approximately 4°C). Wet ice may be used to keep the samples cool. Freezing should be avoided, as freeze–thaw cycles will degrade RNA and viable virus. Swab material has been shown to be stable for up to 14 days at 4°C, while freezing for the same period reduced virus detection by rRT-PCR [25, 46].

The metadata associated with a sample are a key part of sample collection. The date of collection, type of sample, location (including global positioning system (GPS) coordinates when possible), clinical condition of the animal(s), age of the animal(s), species, and vaccination status (for domestic animals) should be recorded. This information should be kept with the sample. When collecting samples from wild birds for AIV surveillance, it is important to use the scientific name of the species, as common names can be regional and may not be recognized universally. Historically, countless samples have been labeled "duck", which is insufficient to improving our understanding of IAV biology, as there are numerous species of ducks, with highly variable habitats, migration routes, and genetics.

Since transport of diagnostic samples can be difficult due both to shipping regulations for potentially infectious material, and because the cold chain must be maintained, in situations where only molecular methods (e.g. rRT-PCR, sequencing) will be used, Flinders Technology Associates (FTA) cards (Whatman-GE Healthcare and Bio-sciences, Pittsburgh, PA) may be utilized. Liquid samples may be blotted on this specially manufactured paper card, and once the sample dries the virus will be inactivated and the RNA will be preserved. The card can then be transported with fewer shipping restrictions than samples which may contain live virus. Avian influenza virus RNA has been shown to remain intact for 5 months on FTA cards at ambient temperatures [1], although the sensitivity will be lower than if swab material is used directly [1, 31, 32]. An alternative preservation method for viral RNA that does not require the cold chain involves collecting cloacal swabs from wild birds in 100% ethanol for screening by rRT-PCR [57]. Although this was found to be successful for rRT-PCR, paired swabs needed to be collected in a traditional viral transport medium and maintained at low temperatures to attempt virus isolation [57], so this method is not suitable if virus isolates are needed. Another drawback is that 100% ethanol must be shipped as a flammable chemical. Other commercially available transport media will inactivate samples for ambient-temperature long-term storage, but in the absence of controlled scientific comparisons with avian samples, these products cannot currently be recommended.

Virus detection

Virus detection to identify an active infection can be achieved by attempting VI or by using ACIAs (Table 2.1). Alternatively, viral nucleic acids can be targeted by molecular assays (e.g. rRT-PCR) (Figure 2.2). Typically, rRT-PCR or ACIAs are used to screen samples, and then virus isolation is used to confirm the results.

Virus isolation

The reference standard for the diagnosis of IAV is VI, and although other methods may be used to make a presumptive diagnosis, VI is necessary to confirm the presence of virus in an index case and to undertake further characterization of the virus. The embryonated chicken egg (ECE) from a specific pathogen-free flock (or a flock that is negative for IAV or for IAV antibodies) is considered to be among the most sensitive host systems for the isolation of both avian and mammalian IAVs. Madin–Darby canine kidney (MDCK) cells are also widely used for the isolation of IAV from animal (avian or mammalian) specimens. Although IAV will replicate in other cell lines and in embryonating eggs from other avian species, ECE and MDCK are probably the most widely used systems. The choice of which of these is the optimal laboratory host system is dependent on the strain; some lineages will replicate only in ECE, some only in MDCK cells, and some will replicate well in either system.

One cannot always deduce which system is best based on the sample species of origin. For example, recent swine or swine-like viruses, including H3N2

Table 2.1 Characteristics of selected IAV diagnostic assays.

Assay	Target	Relative sensitivity	Relative specificity	Relative cost per sample	Time to result
Virus isolation	Viable virus	Very high	Moderate	High	1–2 weeks
Antigen detection immunoassays (commercial kits)	IAV protein	Low	High	Moderate	15 minutes
Real-time RT-PCR	IAV RNA	Very high	Very high	Moderate	3 hours
Agar gel immunodiffusion (AGID)	**1** Type A influenza virus nucleoprotein and matrix protein **2** Antibody to type A influenza nucleoprotein and matrix protein	Moderate	High	Moderate	48 hours
ELISA (commercial kits)	Antibody to type A influenza	Moderate	Moderate	Low	2–3 hours
Hemagglutination (HA) inhibition	**1** Identification of HA subtype **2** Antibody to a specific HA subtype	High	Moderate to high	Moderate to high	2 hours
Neuraminidase (NA) inhibition	**1** Identification of NA subtype **2** Antibody to a specific NA subtype	Moderate	Moderate to high	Moderate	3 hours

IAV = influenza A virus, RT-PCR = reverse transcriptase polymerase chain reaction.

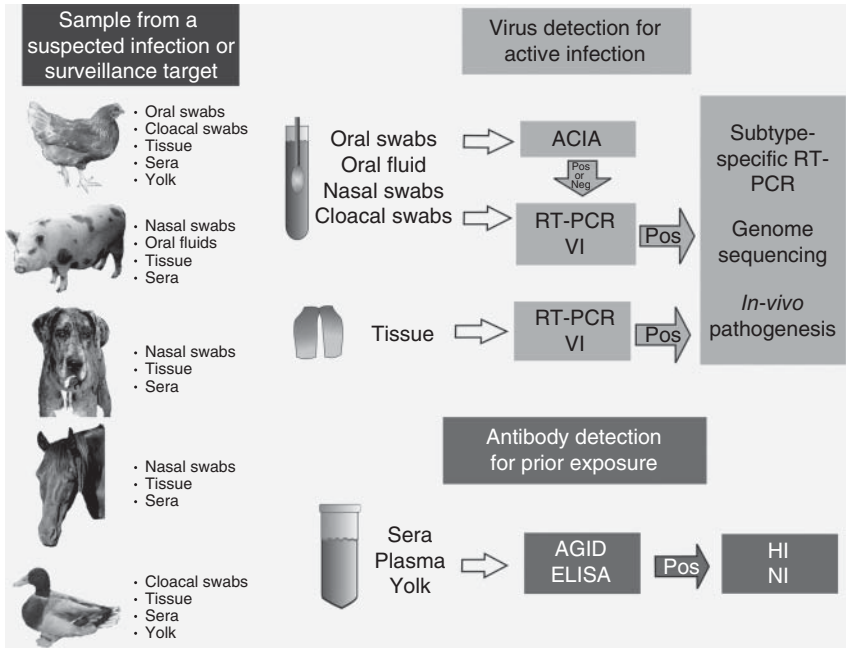

Figure 2.2 Outline of common approaches to influenza A virus (IAV) diagnostic testing. Active virus infection may be detected from swab material, oral fluids, or tissue by antigen capture immunoassay (ACIA), which needs to be confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) or virus isolation (VI), or may be directly tested by RT-PCR or VI. Positive samples and isolates are further characterized by subtype-specific RT-PCR and/or genome sequencing and, in some cases, *in-vivo* pathogenesis studies. Exposure to IAV may be evaluated by antibody detection by agar gel immunodiffusion (AGID) or enzyme-linked immunosorbent assay (ELISA). The subtype specificity of positive samples may then be determined by hemagglutination inhibition (HI) assay or neuraminidase inhibition (NI) assay.

isolates, and pandemic H1N1 lineage viruses may not grow efficiently or at all in ECE, so MDCK cells are preferred. Because turkeys can be infected with swine influenza viruses (SIVs), turkey samples are commonly processed for VI with both ECE and MDCK cells [63]. Numerous other cell lines will support the replication of IAV, but are not so widely utilized.

A limited number of comparisons between ECE and MDCK cells have been reported with samples from different host species. For samples from wild birds, the ECE system is apparently more sensitive, and titers were higher in ECE with samples that replicated in both systems [43, 44], but the data are less clear for samples from domestic poultry and domestic mammals, and MDCK cells are preferred for swine samples [81]. ECE and MDCK cells are considered to be similar in sensitivity for equine and canine samples [12, 20], although some canine isolates may grow preferentially in one system [20]. In some cases, both methods may be used. For example, samples from turkeys which are expected to contain swine influenza are often processed into both ECE and MDCK cells at some reference laboratories.

Other differences between the systems have been noted, such as cost. MDCK cells are less expensive [43], and it has been observed that the equine influenza viruses undergo more selection in MDCK cells than in ECE, which could be important in some situations [29].

Because of the high sensitivity of VI, this method may be used to detect IAV during any stage of an active infection. Depending on numerous host- and virus-related factors, virus may be detected within 24 hours of infection in an individual bird, and for several weeks post exposure in a flock, herd, or population of animals [70]. To achieve optimal sensitivity with either system, it may be necessary to serially passage a specimen (often referred to as a "blind passage") two or three times, but this substantially increases the time it takes to complete the test, and it also increases the risk of false-positive results due to cross-contamination of samples.

Although VI is very sensitive, it is not highly specific or selective, because other agents that may be present in a specimen will readily grow in ECE or cell cultures. For this reason, additional tests on fluids from ECE or MDCK cultures are required to confirm the presence of IAV. Fluids from eggs or cell cultures inoculated with the test material are usually first tested for hemagglutination (HA) by a standard hemagglutination assay [73], or for IAV with an ACIA. With avian samples, an HA-positive sample is often tested by the hemagglutination-inhibition (HI) assay or rRT-PCR to differentiate AIV from other hemagglutinating viruses, most commonly avian paramyxovirus type 1 (i.e. Newcastle disease virus) in poultry specimens.

The presence of IAV in avian or mammalian samples can be confirmed by type-specific tests such as ACIA, agar gel immunodiffusion (AGID) assay for IAV antigen, and rRT-PCR tests on undiluted egg or cell culture fluids. Alternatively, the subtype of the isolate can be identified by HI assay and neuraminidase-inhibition (NI) assays or by gene sequencing. Gene sequencing is the most accurate method of identifying the HA and NA subtypes of IAV, as cross-reactions and false negative results are associated with serological tests and RT-PCR.

Despite the high sensitivity of culture methods for detecting IAV, there are some practical considerations that should be taken into account. First, VI is relatively expensive, and in the case of ECE is not easily scaled up because procurement and incubation of eggs have to be scheduled well in advance. Second, when performing VI the infectious virus can be amplified to a high level, significantly increasing the potential for cross-contamination among samples and exposure of laboratory personnel to infectious virus. For this reason, VI is generally performed in laboratories with enhanced biosecurity (e.g. BSL-3, BSL-3Ag, or P3), especially if it is suspected that the specimen contains HPAI (or any high-consequence pathogen). Virus isolation also requires a high level of technical skill in order to perform the procedure and interpret the results, because culture host systems can support the growth of many different agents, which can complicate diagnosis, as noted above. Furthermore, virus isolation is dependent upon the correct handling of specimens. If the samples are not collected, transported, and stored under the correct conditions, the sample can be degraded and may contain inactivated virions that could lead to false-negative results. Finally, VI has the longest time-to-result of any IAV detection test. The VI procedure may detect virus within 48 hours, but negative samples may take 1–2 weeks to complete, depending upon

the number of passages used and how quickly the virus grows to a high enough titer to be detected by HA or other methods.

Finally, there are some situations where VI is preferred over other methods, such as when it is important to determine whether viable virus is present (e.g. when confirming that cleaning and disinfection have completely inactivated all virus), or when it is necessary to evaluate the antigenic characteristics of an isolate (e.g. to reveal the occurrence of antigenic drift). Virus isolation will also remain in the core IAV diagnostic arsenal because isolates will always be needed for biological characterization.

Antigen capture immunoassays (ACIAs)

Numerous commercial type A influenza ACIA kits in lateral flow device (LFD) and enzyme-linked immunosorbent assay (ELISA) formats are available, but licensing for veterinary use varies among countries. Before any kits were licensed for veterinary use in the USA and elsewhere, kits for human diagnostic testing (Directigen Flu A test, Becton-Dickenson, Franklin Lakes, NJ) had been used successfully in poultry and other species [11, 22, 80]. Within the past few years, several kits have been licensed for use in different countries worldwide.

The LFDs use a monoclonal antibody directed against the highly conserved IAV nucleoprotein to bind viral antigen on a filter strip or membrane. The results can be visualized by the appearance of a band or pattern on the test strip or membrane following a chromatographic immunochemical reaction. Due to the immense interest in the recent Asian H5N1 HPAI virus, commercial H5-specific tests have been developed, and other similar kits may become available, but reports from the field indicate that the sensitivity and specificity are not high. Development of subtype-specific antigen detection tests is challenging because the monoclonal antibodies used in these tests must be directed to the highly variable HA antigen, making the test less reliable than IAV-specific assays.

The primary limitation of antigen detection kits is their low sensitivity. Most kits have an analytical sensitivity of approximately 10^4 to 10^5 mean embryo infectious doses (EID_{50}) [11, 80]. Since birds that present with clinical disease or which die from AIV infection are likely to shed higher levels of virus, they should be targeted for testing. Although clinically healthy birds may in fact be infected and shedding sufficient virus for it to be detected, the chances of obtaining a false-negative result are sufficiently high for the routine testing of clinical healthy birds not to be recommended. Also, it should be recognized that sick and dead birds can shed inadequate virus titers to be detected by ACIA (Spackman, unpublished data).

An effective surveillance approach for AIV, originally used in the 2002 H7N2 LPAIV outbreak in the USA, was to periodically sample 10 birds from the daily mortality on chicken or turkey farms in the surveillance zone in order to identify infected flocks [22]. The targeting of daily mortality has become a standard approach for surveillance of several respiratory diseases of poultry in the USA. Positive results from ACIA tests correlate well with those of other tests, but negative results from ACIA are not reliable and need to be confirmed by further testing.

Advantages of the ACIAs are that they are very rapid, producing results within 15–20 minutes, and highly specific. In addition, commercial antigen detection tests are convenient, self-contained, and easy to use. Therefore they are ideal for use on the farm as a "pen-side" test. Antigen detection tests are also used in diagnostic laboratories as a rapid screening test for IAV in clinical specimens, and for identifying suspect IAV isolates in VI material where titers are likely to be adequate for ACIA detection. The cost per sample of running the commercial antigen detection tests varies according to the manufacturer, but is less expensive than VI and of similar cost to rRT-PCR. It should be noted that the range of sample types that can be used with ACIAs is limited; most of these tests only accommodate tracheal, nasal, or oropharyngeal swab specimens. The tests are species independent, but few data are available to support their reliable use in off-label species, and negative results must be interpreted with caution.

Molecular/nucleic acid-based tests

In recent years, the application of molecular methods for the detection of viral nucleic acid has become an important tool for the detection of IAV and identification of HA and neuraminidase (NA) subtypes. RT-PCR based tests are the

most widely used molecular method, particularly real-time RT-PCR. Alternative amplification methods are also available, including nucleic acid sequence-based amplification (NASBA), an isothermic method for amplifying nucleic acids [14–16, 42], loop-mediated isothermal amplification (LAMP) [6], and insulated isothermal PCR (iiPCR) [5]. Although NASBA and LAMP are similar in sensitivity to rRT-PCR, these methods have not been as widely adopted as rRT-PCR, and iiPCR is too new for predictions to be made as to whether it will attain widespread use. Commercial NASBA, LAMP, and iiPCR kits are available for IAV and selected subtypes (e.g. H5 HPAIV, A(H1N1)pdm09). A report on the LAMP assay is available from the OFFLU website (www.offlu.net); however, full validation data have not yet been published.

Numerous rRT-PCR and conventional RT-PCR tests have been reported for the detection of IAV in poultry, swine, dogs, and horses [27, 37, 39, 40, 53, 54, 66, 68]. RT-PCR tests to identify important HA subtypes, often H1, H1 A(H1N1)pdm09-specific, H3, H5 and H7 [13, 45, 65] and N1 or N2 [74] have been reported. The recent Asian H5N1 HPAIVs have probably been the most targeted, with numerous reports of HA and NA subtype-specific tests [24, 47, 51, 79], although few of the reported tests have been field validated. Test procedures that are maintained by government and regulatory entities or international networks and organizations (e.g. OIE, USDA) are often the most reliable, because they are continually monitored for performance with new IAV lineages (e.g. A(H1N1)pdm09, A(H7N9) 2013 LPAIV lineage from China), and are rapidly updated with validation as needed. USDA-licensed RT-PCR test kits are available for both avian and swine influenza viruses in the USA, with some availability in other countries. Commercial tests from local manufacturers are also available in China and Russia, but it is unclear how sensitive and specific these tests are. The growing availability of commercial tests provides a mechanism for the availability of standardized reagents, internal positive controls, and quality control between reagent lots. The disadvantages of all-in-one kits are that they are more expensive per test, and the primer and probe sequences are proprietary, so *in-silico* specificity analyses cannot be performed by end users.

Molecular methods offer numerous advantages for IAV detection. These include high sensitivity, which is similar to that of VI [3, 11, 52, 65], high specificity, scalability, the ability to accommodate any sample type with proper sample processing, and minimization of contact with infectious materials, as the virus is inactivated at an early stage of sample processing. Real-time RT-PCR, which is more widely used than conventional RT-PCR, offers additional advantages. First, it is among the most rapid molecular tests available, where results can be obtained in less than 3 hours. Second, it is more specific than conventional RT-PCR when used with a hybridization probe. Third, the potential for cross-contamination is reduced because samples are not manipulated after amplification.

The major disadvantage of both conventional and real-time RT-PCR is the high start-up cost for equipment, which has hindered some smaller laboratories from using this technology. Also, the reagents for rRT-PCR are expensive, and although RT-PCR is less expensive than virus isolation, the cost can still be prohibitive for some surveillance efforts. The reagents do require refrigeration, which can be a problem for testing in remote locations. The development of lyophilized reagents for rRT-PCR has been attempted, but compared with conventional RT-PCR the cost of the reagents is higher and the sensitivity is frequently lower [17, 75]. The cost of rRT-PCR is also affected by sample processing (RNA extraction), as this adds to the cost of materials and labor. Another disadvantage of rRT-PCR is that subtype identification has low accuracy due to the high variation of HA and NA sequences. Both false-positive results by cross-reaction with other subtypes and false-negative results have been observed, with an overall accuracy rate of 49.5% being reported [62].

The high sensitivity of RT-PCR does increase the risk of false-positive results by detecting low levels of cross-contaminants. Conversely, decreased sensitivity can occur with RT-PCR due to inhibition with some sample types if these are not processed properly. Both of these risks can be managed with proper controls, including no template RNA extraction controls to test for cross-contamination and internal positive controls [17, 19], and positive extraction controls to verify that inhibitors are not present.

Serological methods

Antibody detection is a common and relatively inexpensive method of surveillance for detecting exposure of animals to IAV. Numerous test formats are used for IAV antibody detection, including AGID assay, HI assay, and ELISA. Of the three assays, HI is the only absolutely quantitative format.

Serology can be performed with sera, plasma, egg yolk from avian species, and sera eluted from blood stored on filter papers (e.g. Nobuto strips) [21]. As with all diagnostic methods, the correct conditions for transport of samples are essential if accurate results are to be obtained. With the exception of blood stored on preservative filter papers, samples should be kept cool, and although antibodies are more robust to freeze–thaw cycles than live virus, freezing and thawing of samples should be minimized.

One of the primary applications of antibody testing for poultry is in the support of trade, to certify flocks or poultry products as free of exposure to AIV. For this reason, antibody tests are performed on millions of samples yearly from US poultry alone. Antibody testing in mammalian species can be used to evaluate exposure to IAV or response to vaccination.

ELISA

ELISAs for influenza A antibody detection are well established, and numerous ELISAs for different species (e.g. avian, swine, equine) are commercially available. Most of the IAV tests are targeted to nucleoprotein antibodies which are produced early after infection, and although these tests are reliable for identifying infected flocks, the ELISA results cannot be used to measure protective antibody levels, because nucleoprotein antibody is not neutralizing. Although both indirect (sandwich) and blocking formats have been used, the utility of the blocking format is broader, since the ELISA is not species specific, and it can therefore be used for surveillance in numerous avian and mammalian species (although performance data are only available for a limited number of species).

ELISA is a high-throughput format which is rapid and easy to use. Although commercial tests are more expensive than in-house-produced tests, the quality control and reagent production are undertaken by the manufacturer. On a per-sample basis, the materials for commercial ELISA cost about twice as much as those for AGID. The amount of specialized equipment required is minimal. An optical microtiter plate reader is needed to evaluate the results. However, many laboratories that run ELISAs routinely also have automated plate washers and even liquid handling stations for diluting samples. For AIV antibody detection, ELISA results need to be confirmed with AGID or preferably HI for H5 or H7, and currently only one AIV antibody ELISA is certified by the OIE.

Commercially available subtype-specific ELISAs are available for H1, H3 swine influenza H5 HA subtype, and N1 and N2 subtypes. However, their specificity is less dependable than that of assays which target type A influenza antibody, due to the variation in the HA protein. When there is a need for a subtype-specific ELISA, whether selecting a commercial test or a procedure that has been reported in the literature, it is essential to ensure that there are adequate verification and validation data for the target species and subtype.

Agar gel immunodiffusion (AGID)

The AGID assay has been used since the 1970s for IAV antibody detection [7]. The principle of AGID is to visualize the immunoprecipitation reaction of AIV antibody and antigen after diffusion in an agar matrix. Although AGID is most widely used in a diagnostic setting to detect antibody using a reference antigen, it can also be used to detect type A influenza antigen (e.g. to confirm the presence of IAV in ECE fluids or cell culture supernatants).

AGID is inexpensive, simple to run, and does not require unusual supplies or expensive equipment. However, preparation of the antigen and control sera with proper quality assurance is expensive and time consuming. For these reasons, many laboratories use antigen and control sera produced by reference laboratories, or that are commercially available. In addition, AGID requires moderate skill and training in interpretation of the test results. The results may be read within 24 hours, but it may take up to 48 hours for weakly positive reactions to become visible.

AGID has moderate sensitivity, and can detect antibody earlier post infection than other antibody detection tests because it reacts with IgM.

Antibody may be detected as early as 5 days post infection, and may be detected for many weeks or months post infection [73], although the response and duration of antibody are affected by both the host and the virus strain. The AGID test is suitable for testing serum, plasma, and egg yolk [8]. Importantly, however, AGID does not produce consistent results with serum from some avian and mammalian species [20, 59, 71].

Hemagglutination inhibition assay

The HI assay can be used as a confirmatory test for the presence of subtype-specific IAV in hemagglutinating egg fluids or cell culture supernatants, to further characterize IAV isolates by identifying the HA subtype, or to identify the subtype-specific antibodies to IAV in serum, plasma, or egg yolk [76].

Suspect isolates are identified by HI with a panel of subtype-specific antisera representing each HA subtype. Because false-positive reactions can be caused by steric inhibition when the reference reagent and test material have a homologous NA subtype (but have different HA subtypes) [58], more than one reference serum per HA subtype is often necessary to assure adequate specificity. The problem of steric inhibition can be overcome by the use of antisera prepared by DNA vaccines containing only the HA gene [38]. In addition, some cross-reaction can occur between HA subtypes, making the results more difficult to interpret. Therefore the specificity of the HI assay is highly dependent upon the quality of the reference reagents.

Specific to the procedure for isolating AIV, HI has historically been used to exclude the presence of avian paramyxovirus type 1 (APMV-1, also known as Newcastle disease virus) in the test material by testing for inhibition with APMV-1-specific antibody. A negative HI assay result with APMV-1 antiserum indicates that APMV-1 is not present and that the specimen is suspect for AIV.

Conversely, the HI assay may be used to identify the HA subtype of IAV antibodies in a specimen by using viruses of known subtype as the antigen in the assay. Again, a panel of all 16 HA subtypes is needed to evaluate all of the different possible subtypes, and the results must be interpreted carefully because of the possibility of cross-reactions, particularly with samples from wild birds where the animal may have been exposed to different subtypes over its lifetime.

Sensitivity is generally not a major concern when the HI assay is used to identify IAV isolate subtypes, because the test is used with amplified virus (as opposed to clinical specimens, where the concentration of virus may be low). However, sensitivity of the HI assay for antibody detection is more of a concern. Reduced sensitivity can occur when significant antigenic drift occurs within a subtype, resulting in low reactivity between the antigens used in the HI assay and antibodies found in test sera. Despite these concerns, the HI assay is still considered to be more sensitive than AGID [41], and it will detect IAV antibody for a longer period post exposure than AGID. Furthermore, the HI assay is not species specific.

From a practical standpoint, the HI assay is relatively expensive and labor intensive when used to identify isolates or when used as a screening test for detecting antibodies, because of the number of antigens or antisera required to test for all 16 HA subtypes. However, the advantages of this assay are that it is rapid (results are available within a couple of hours), simple to perform, and requires only moderate skill to interpret the test results. The HI assay can be useful in some specific applications. For example, for trade purposes some countries require HI testing for the H5 and H7 subtypes, and during an outbreak where the target HA subtype is known, an HI test can be used more efficiently because a specific antigen is targeted. A major advantage of the HI assay is that inactivated antigens can be used, eliminating the need for special biosecurity or biosafety measures in the laboratory.

Neuraminidase inhibition assay

The neuraminidase inhibition (NI) assay can be used to detect NA subtype-specific antibodies or to identify the NA subtype of an isolate. The principle of the NI assay is to inhibit the enzymatic activity of the neuraminidase with subtype-specific antibodies [4]. For characterization of new isolates, a panel of reference antibodies corresponding to all nine NA subtypes is needed to perform the NI assay. The test utilizes a colorimetric reaction which does not occur when the neuraminidase activity is blocked, indicating a match between the antibody and test virus subtype. As with the HI assay, sensitivity is

not a critical characteristic of the NI assay, as virus isolates are used instead of clinical samples. Also, like the HI assay, the specificity is moderate and depends on the quality of the reference sera or antigens used [78].

The current standard NI assay, the thiobarbituric acid (TBA) NI assay, is a more complicated procedure than the HI assay, and although it can be completed within a few hours, it is typically performed in reference laboratories because the substrate used in the test is expensive and the chemicals used are hazardous. The assay can be performed in a 96-well microtiter format or in tubes, but the microtiter assay requires special white-colored plates to make it easier to distinguish color differences. An alternative method for NI antibody detection, the enzyme-linked lectin assay (ELLA) [36], has been increasingly used recently [9, 34, 77]. ELLA is less expensive and uses safer reagents than the TBA assay.

Characterization of influenza isolates

Once an IAV has been isolated it may be genetically and biologically characterized if necessary. The amount of characterization necessary depends on the circumstances. For example, an isolate of an unusual subtype for a species, or from a species not normally associated with influenza infection, will have a higher priority. Isolates from routine diagnostics (e.g. isolation of an H3 from swine) is less likely to be extensively characterized.

Due to the low cost of sequencing, and rapidly improving technology, it has become common to produce the sequence of the HA and NA at a minimum, and often the full genome sequence is produced. Sequencing is the most accurate way to identify both the HA and NA subtype. In addition, partial sequencing of the HA cleavage site is starting to replace the *in-vivo* tests, such as the intravenous pathogenicity index (IVPI), to identify the presence of a multibasic amino acid cleavage site and to classify the AIV pathotype. A list of the multibasic cleavage sites of the HA molecule detected to date for low- and high-pathogenicity H5 and H7 avian influenza viruses is regularly updated and available at the OFFLU website (www.offlu.net/fileadmin/home/en/resource-centre/pdf/Influenza_A_Cleavage_Sites.pdf.). For index-case AIVs, particularly if they are H5 or H7, the *in-vivo* test (i.e. IVPI) should be applied to confirm the pathotype.

For all IAVs, the gene sequence can be used for a basic phylogenetic analysis that provides information about the most closely related isolates for which there are data, and can provide valuable epidemiological information. In addition, as more and more molecular markers for virulence and host range are identified in the literature, likely biological properties can potentially be identified. The Influenza Research Database (www.fludb.org) [67] and the GISAID-EpiFlu database (http://platform.gisaid.org), which contain sequences submitted to public databases, provide annotated lists of possible biological features based on published information for numerous host species. Other characterization may include pathogenesis studies in the host of origin or model species to evaluate potential host range or transmission characteristics. Receptor-binding studies are also becoming more common.

Education and training

The role of the farmer, owner, or animal handler in detecting IAV infections in domestic animals should not be discounted, as their recognition that there is a health problem is necessary for initiation of the diagnostic process. Therefore education of these personnel is of critical importance for early detection, because the signs of influenza can be subtle and non-specific (e.g. LPAI is sometimes first recognized in chickens and turkeys because there is a decrease in food and water consumption). In addition to a description of the clinical signs, education and training should include an explanation of why diagnosing influenza is important, and also describe how and when to increase biosecurity, and the appropriate biosafety measures that should be implemented.

Conclusion

One of the most critical aspects of implementing diagnostic and detection tests for any disease is fitness for purpose. The practical aspects of the test

are as important as its analytical performance. A test such as RRT-PCR may have superior sensitivity and specificity, but the rapid and portable nature of ACIA kits makes them ideal for on-farm testing, whereas RRT-PCR must be performed in a laboratory because of the sample processing required. It is also important to define the goals and outcomes of the testing. For example, active surveillance will have different diagnostic needs to surveillance during an outbreak. Other questions that need to be addressed include what action will be taken if a positive result is obtained, and the consequences of obtaining a false-negative or false-positive result. Finally, regulatory guidelines need to be considered when implementing IAV diagnostics, as these may dictate which tests can be used and how an outbreak or case is handled.

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3 The economics of animal influenza

Anni McLeod and Jan Hinrichs

Introduction

The epidemic of H5N1 highly pathogenic avian influenza (HPAI) that has spread across Asia, Europe, and Africa since 2003 was followed by the emergence and spread of several new influenza subtypes. Although the public has become increasingly well informed about health threats posed by animal influenza, there has been a continuing theme of panic and economic losses even where outbreaks have not occurred, or have been reported and rapidly stamped out, or have been caused by non-zoonotic influenza virus strains. The emergence of H1N1 pandemic virus in Mexico resulted in worldwide human vaccination campaigns in October 2009 [80]. H7N9 low-pathogenicity avian influenza (LPAI) has caused severe disease in humans and abrupt declines in poultry demand since its detection in China in February 2013 [61]. Although equine influenza outbreaks were not harmful to human health, the control program nevertheless caused business disruptions for the Australian horse industry, which led to severe financial losses and financial assistance payments by the Government of Australia in 2007 [69]. Losses caused by animal influenza have been large, and estimates of potential loss are enormous. Equally important, the zoonotic character and pandemic potential of new emerging animal influenza viruses has led to increased collaboration between human and animal health sectors, as well as putting pressure on stakeholders in the livestock sector to increase the safety of livestock production and linked value chains.

Much of the economic impact is driven by the fear that animal influenza viruses may over time lead to a global pandemic with sustained human-to-human transmission. A multitude of animal influenza viruses are known to circulate in different species, but not all of them are zoonotic. Several have low virulence, and only a subset of these are "notifiable" to veterinary health authorities which are then responsible for control interventions. Low-virulent animal influenza viruses decrease the productivity of infected livestock and may reduce the effectiveness of vaccinations against other common diseases of livestock. The newly emerged H7N9 LPAI virus is zoonotic. Table 3.1 gives an overview of the most common avian influenza virus subtypes in poultry. Reducing the prevalence of influenza in livestock reduces the opportunities for genetic reassortments which could potentially cause sustained human-to-human transmission. Control of zoonotic and non-zoonotic influenza viruses in animals therefore has benefits in terms of protecting human health. One recent example is the genesis of zoonotic H7N9 LPAI, which was facilitated by the widespread presence of low-pathogenic and non-zoonotic H9N2 LPAI in poultry production systems [60].

Pandemic prevention has attracted considerable funding from the international donor community, with the aim of reducing the number of infected animals and thus limiting human exposure. Between 2005 and 2009, US$3.9 billion had been committed by bilateral and multilateral donors for the control of pandemic influenza [83]. While strengthening of health services with these funds has probably led to benefits beyond pandemic influenza control, control interventions in livestock value chains have also caused negative economic impacts for value chains actors from movement controls and destruction of livestock. In order to use the animal influenza control funds effectively, it is important to understand the economic and

Animal Influenza, Second Edition. Edited by David E. Swayne.

Table 3.1 Influenza A virus subtypes that circulated in birds and were found sporadically in people [26, 54, 82].

Subtype	Disease in humans	Impact in animals relevant to economic analysis
H5N1	Fatal human cases reported	High mortality reported
H5N3	No human cases reported	High mortality reported
H5N6	Human cases reported	High mortality reported
H5N8	No human cases reported	High mortality reported
H7N9	Fatal human cases reported	No clinical signs reported
H9N2	Mild symptoms in humans reported	Varying mortality and morbidity reported
H10N8	Fatal human cases reported	?

social factors that affect the success and impact of measures used for control. The absence of apparent losses in livestock production represents a major challenge in engaging stakeholders in the livestock sector in human health-driven control programs for low-virulent influenza viruses. This chapter addresses the economic imperatives faced by decision makers who must deal with different animal influenza virus infections as livestock diseases, while remaining aware of the humanitarian and economic threat of a human pandemic.

Benefits and costs of controlling animal influenza

Three types of benefit justify animal influenza control. Animal influenza covers low-virulent swine and equine influenza viruses, LPAIV, and HPAIV.

Net benefits of avoiding a human pandemic

It is challenging to estimate the potential benefits of preventing a human pandemic. Estimates of the potential number of prevented human fatalities are highly sensitive to assumptions in predictive epidemiological models. Disruption estimates of other economic activities from social distancing and other prevention costs are similarly speculative in nature. The 2009 H1N1 epidemic was considered a mild pandemic, but still caused more than 18 000 laboratory-confirmed deaths during the pandemic phase [79], estimated by one study as equivalent to between 334 000 and 1 973 000 years of life lost (YLL) [76]. Although past pandemics can give rough guidance on potential lives lost, the "valuation" of lost lives presents another challenge. Non-monetary valuations such as YLL or disability adjusted live years (DALY) can be used to prioritize the use of resources among several diseases. Another monetary valuation approach is to use the statistical value of a life saved based on life insurance data. The application of this approach justifies the investment of US$1 billion in influenza risk mitigation if on average 654 people are saved per year [71]. Other economic estimates of potential impact are very large, and this has resulted in considerable international funding for animal and human pandemic control of mainly zoonotic animal influenza viruses, as discussed in the next section under potential impacts of human influenza.

Net benefits of minimizing human disease contracted directly from livestock

Human cases of and deaths from non-pandemic animal influenza viruses, although tragic, have so far been small in number and would not have justified huge international expenditure on disease control. There were 58 781 deaths recorded for malaria in 2013 [81], whereas H5N1 HPAI had caused 402 known deaths and H7N9 LPAI had caused 178 known deaths at the time of writing.

Net benefits from improved livestock productivity through avoiding disease

It is widely agreed that control of the disease at its source in livestock will be the most effective way to prevent the occurrence of a human pandemic of animal origin, and this chapter focuses mainly on the economic impacts of disease and control methods in the livestock sector. Control should be achieved as cost-effectively as possible, and with the minimum disruption to human lives and economies.

Many low-virulent animal influenza viruses, such as the zoonotic H7N9 LPAIV, the pandemic H1N1 influenza virus, and the non-zoonotic H9N2 LPAIV, often cause very mild or barely noticeable disease syndromes. Livestock keepers and traders are therefore often not aware of the invisible productivity losses. Control interventions for low-virulent animal influenza viruses require compliance by livestock keepers. The absence of visible losses does not create compliance incentives, and means that a wider range of livestock production and marketing issues need to be addressed.

A typical pattern of socio-economic effects beginning before an outbreak and progressing towards long-term control measures for notifiable animal influenza viruses is shown in Table 3.2. The length and intensity of each phase are influenced by the virulence of the animal influenza virus, the structure of the livestock sector, and the response capacity of the animal health system.

Market shock is the first economic effect, and may occur even without an outbreak, created by consumer fears. If an outbreak occurs, each element of the disease control process has associated costs and livelihood effects, beginning with reporting of disease, stamping out by culling and movement control, providing compensation for animals culled, and later perhaps the introduction of vaccination. The diverse character of livestock keeping and livestock keepers presents huge challenges in terms of designing control programs that maximize the benefits of containing disease while at the same time balancing the needs of small- and large-scale operators. As disease is brought under control, rehabilitation of the livestock sector begins. This is a straightforward process if an outbreak has been quickly stamped out, but more complicated if it is taking place under conditions of recurring outbreaks. Where there are complex livestock value chains and continuing disease, there is pressure for governments to introduce long-term measures that will restructure the sector in a more biosecure way. However, this carries the risk of excluding smallholders from livestock keeping, with associated loss of livelihoods. It also requires investment to revive animal health systems that have suffered from neglect.

Estimates of net benefits from avoiding disease in livestock need to balance the impact of disease against the impacts of control processes, and assess the differential impact by sector and along value chains. Ideally they will take into account all of the following:

1. Net impact of market shocks. Shocks occur when demand and prices are disrupted by consumer fears of disease or import bans of trading partners. The control process can also cause market disruption by restricting movement and sales or exaggerating consumer fears through ill-judged communication, and may have impacts far beyond the area of infection.
2. Net impact on livestock productivity. Productivity gains from controlling disease must be offset against the losses caused by the control process. These effects are greatest within areas where outbreaks occur, affecting producers and those immediately connected to them through value chains. There may be wider effects if depopulation is extensive.
3. Costs of dealing with diseased livestock. These include treatment (if any) and disposal of carcasses.
4. Direct costs of prevention and control processes. These include all of the human resource,

Table 3.2 Phases of disease and socio-economic issues for notifiable animal influenza outbreaks.

Socio-economic issues	Pre-outbreak	Outbreak(s)	Rehabilitation	Long-term prevention
Market shocks	■	■		
Culling/compensation		■		
Movement control effects		■		
Vaccination costs			■	
Restocking costs			■	
Restructuring investment			■	■
Long-term market access			■	■
Financing animal health			■	■

capital, and consumables needed to carry out surveillance, culling and disposal, movement control, and vaccination.

5 Costs of rehabilitation. Restoring the operation after an outbreak incurs a restocking cost above the normal costs for maintaining production cycles. In addition, it usually requires investment in more biosecure management by farmers, traders, and market managers, as part of the effort to prevent recurrence of disease.

6 Impacts of restructuring. Beyond the immediate impacts of dealing with disease, there may be changes in the structure of the livestock sector resulting from heightened animal health and food safety regulations, or restrictions in the places where production and processing may take place. These measures require investment and will result in improved productivity for some but reduced market access for others. They may also, although this has not yet been evaluated, result in a loss of animal genetic resource.

If the control strategy is well designed and implemented, the losses from control should be considerably less than those that would have occurred from an uncontrolled disease outbreak, but the impacts on different stakeholders may be uneven. Compliance with disease control regulations will depend on the benefit that each stakeholder group perceives from them. For example, providing compensation does not reduce the production loss from culled livestock, but shares the loss between producers and others in society, providing an incentive for producers to cooperate with culling teams.

A complete benefit–cost or cost-effectiveness analysis for global control of zoonotic animal influenza has not yet been attempted. Preliminary estimates have been made for H5N1 HPAI in some countries and regions at different stages of disease. Some of these give a detailed snapshot for a particular country and time, and others talk vaguely of potential costs running into billions, but none of them provide a complete picture.

An example of the scale of losses caused by H5N1 HPAI in the poultry sector was reported for the H5N1 HPAI epidemic in Nepal in 2013, and compared with the costs of short-term response measures as well as long-term investments in animal and human health service infrastructure [41]. Before the onset of the epidemic, the commercial poultry sector had experienced dynamic growth of the commercial poultry population by 50% within 3 years. An annual output of 25.4 million eggs and 1.9 million broilers was achieved, equivalent to US$388 million or 2.04% of Nepal's GDP. More than 1.7 million poultry were culled or died during the HPAI outbreaks in 2013, and control efforts prevented a much wider spread of the disease to more farms. The lost poultry had a domestic market value of about US$9 million.

About 40% of the high-value broiler breeding stock and about 15% of the layer breeding stock were lost. This resulted in supply shortages of replacement progeny for poultry meat and egg production. Nepal has a highly specialized commercial poultry production sector which depends on day-old parent stock imports. Lost parent stock resulted in reduced production for an extended time period after the containment of HPAI, due to the required growth period until birds are productive again and produce eggs for progeny stock. Production took 9 months to recover, and during this period value chain actors had to cope with revenue and income losses.

The value of poultry production declined during this time, resulting in a foregone output value of US$119 million, equivalent to 0.63% of Nepal's annual GDP. This represented a loss to the national economy. Poultry farmers suffered a loss in gross margin totalling US$38.8 million during the 9 months of reduced output. They may also have experienced lost value addition opportunities as the outbreak slowed down the recent dynamic growth in commercial poultry production. It is likely that the reduced domestic poultry production value was to some extent replaced by formal and informal imports from other countries, and by the replacement of poultry meat with other meat substitutes, as Nepal is a net importer of livestock.

Nepal's animal and human health service capacity and infrastructure had been supported with about US$23 million of donor funds earmarked for animal influenza control and prevention between 2006 and 2014. It is highly speculative to attempt to forecast the scale of poultry losses without these additional investments in the animal and human health services. However, the scale of poultry sector losses in 2013 in relation to US$23 million prior to control and prevention investments over a period

of 7 years does indicate the potential benefits in terms of prevented poultry losses if only one epidemic of similar scale is prevented.

One of the difficulties of making a comprehensive global estimate arises from the unreliable and non-specific data on mortality and morbidity losses of livestock from disease. Estimates to date suggest that approximately 232 million poultry had died or been culled in H5N1 HPAI outbreaks between the beginning of 2004 and October 2006 [27]. This figure is probably an underestimate, and does not include mortality from concurrent LPAI viruses. About 40% of all H5N1 HPAI disease events reported to the United Nations Food and Agriculture Organization (FAO) database lack data on mortality or culling quantities. Estimates based on proxy data such as household surveys or agricultural census figures are complicated by the fact that the disease behaves differently in each production system. The effects of LPAI on productivity are often either not noticed, or there is no obligation to report them. Market values of livestock differ substantially even within the same species. Production type and age details of died and culled livestock are not usually reported. Losses in the above-mentioned example from Nepal resulted from dead and culled poultry with market values ranging from less than US$1 for young broiler chicks to more than US$25 for productive broiler parent stocks.

The remainder of this chapter discusses in more detail the main economic effects that have been identified in this section. It covers the potential effects of animal influenza on humans that may be avoided by control of avian influenza in poultry, the contribution of livestock sector diversity to the impact of avian influenza, the effects of market shocks, the effects on food security and livelihoods, the costs and productivity losses associated with outbreak control, the restocking process, and the socio-economic effects of restructuring.

The potential impact of human influenza

It is likely that the next serious discontinuity in world development will originate from either a human influenza pandemic or a transformational world war [68]. Animal influenza has the potential to trigger the next human flu pandemic, and this is a major factor contributing to the concern about animal influenza. In social and humanitarian terms, human pandemics are devastating – witness the impact of the relatively minor global outbreaks of severe acute respiratory syndrome (SARS) in 2003, which killed less than 800 people [11], but seriously disrupted the economies of South-East Asian countries and Canada [14, 20], and the lives of their citizens. The human influenza pandemics in 1918–1919, 1957, and 1968 may have killed 100 million, 2 million, and 1 million people, respectively. In terms of YLL the 2009 H1N1 pandemic is estimated to be comparable to the 1968 pandemic [76].

Pandemic effects depend not only on the numbers of people killed, but also on the demographic distribution of illness and death. A high proportion of infection in economically productive age ranges, as in the case of HIV/AIDS, has the potential to cause long-term damage to economies. Should a human influenza pandemic occur, it is uncertain which age groups would be worst affected. With so many uncertainties, it is impossible to make precise estimates of the economic impact of any new pandemic. The World Bank [8] estimated that the potential economic losses for an influenza pandemic involving 71 million human fatalities would be around US$3 trillion. One of the long-term impacts of a pandemic could be to push large numbers of households below the poverty line [5], and the low level of investment in public health in the poorest countries [59] is a cause for concern.

The economic effects of a pandemic are likely to start with disruptions to businesses and economies, and will place unusually high demands on some services (through stockpiling essential items) and abruptly lower the demand for others (e.g. entertainment, restaurants, hotels). National and corporate plans for operation in times of pandemic aim to allow government and business to continue in the event that employees may be ill, caring for others, or unable to travel to work, and to ensure the availability of the most essential supplies. The 2009 H1N1 pandemic was estimated to have caused Mexico's tourism sector losses of US$2.8 billion within a 5-month period [62]. Considerable resources have been devoted to preparing for a pandemic. It is tenuous to attribute all of this preparation to zoonotic animal influenza virus.

If a human influenza pandemic occurs, it could originate from some other source. Equally, terrorist attacks might create conditions in which travel is impossible and work disrupted. Much of the expenditure on preparedness for disaster, however, would not have been made or planned without the present threat of a human pandemic originating from animal influenza.

Even without a human influenza pandemic, the economic costs of animal influenza have been large, and its control at source is essential. Various contributors to cost are discussed in subsequent sections of this chapter. A number of non-zoonotic animal influenza viruses, which normally would not merit much international attention, are the focus of greater vigilance and stricter control measures than might otherwise be the case, out of concern that they may mutate to zoonotic animal influenza viruses.

The globalized livestock sector

Poultry and pigs are perhaps the most globalized of all livestock. Poultry and pig production and trade have shown steady growth (Table 3.3), and projections suggest that demand will continue to rise. At the same time, both sectors are highly diverse, with production systems ranging from specialized high-intensity units using special-purpose breeds to low-intensity systems using hardy, indigenous breeds. The steady growth in pig and poultry production is a result of efficiency gains from breeding technology, with selection for specific characteristics and a specialization of the required production process for specific breed types and age groups. Selection for high-performance pure-line breeds, hybridization, and artificial insemination, as well as the distribution of production breeds via worldwide market networks, have been a driving force for developing highly productive animals [22].

Poultry production systems

The FAO and the World Organization for Animal Health (OIE) [18, 28] have defined four types of poultry production system, classified as sectors 1 to 4. Sector 1, industrial poultry with high biosecurity, is the system from which the majority of internationally traded poultry is derived. Sector 2 includes large-scale commercial producers with good biosecurity and the farmers under contract to big companies, who raise birds from day-old chicks (DOCs), using feed supplied by the contractors. Contract farming represents an opportunity for new market entrants, requiring technical skill but a lower level of investment than independent farming, because the contractor supplies many of the inputs. During the H5N1 HPAI outbreaks of 2004–2005, contract farmers in Thailand, Vietnam, and Indonesia were buffered from financial loss by their contractors [34, 66]. Sector 3 consists of small- to medium-scale commercial units, in which poultry are confined and fed, but biosecurity investment is low. This is a highly diverse sector. In developed countries, some of the high-value niche-market production, such as organic and free-range products, might be considered to fall within this group, as might specialist producers of rare breeds who keep them in free-range systems. In developing countries, sector 3 consists chiefly of small-scale commercial units with limited investment in facilities, rapid turnover, and a growing market. Their numbers are not high,

Table 3.3 Production and international trade of pig and poultry meat during the period 2002–2011

Year	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011
Pig production (million tons produced)	178	185	186	190	195	200	206	210	215	215
Pig trade (1 million head)	18 099	19 989	21 846	23 914	26 814	30 628	31 610	32 885	33 317	34 178
Poultry production (million tons produced)	147	151	156	162	166	176	185	190	199	206
Poultry trade (1000 live animals)	868	749	821	917	919	962	1054	1312	1396	1457

Modified from FAOSTAT.

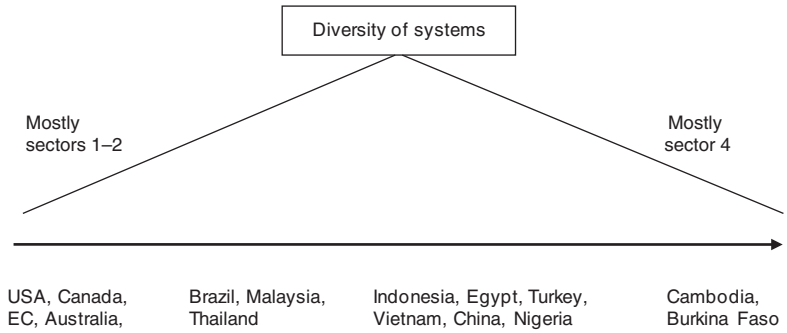

Figure 3.1 Poultry system continuum in 2006 with country examples. Modified from World Bank, FAO, IFPRI, and OIE. 2006. *Enhancing Control of Highly Pathogenic Avian Influenza in Developing Countries through Compensation: Issues and Good Practice*. World Bank: Washington, DC, USA. Available at www.fao.org/docs/eims/upload//217132/gui_hpai_compensation.pdf

typically accounting for around 10% of the poultry population in a country where the poultry sector is growing, but they represent a transition route out of poverty and a way of meeting a growing demand for poultry meat. Sector 3 includes large flocks of herded ducks that graze on the crop residues and snails of paddy rice systems in delta areas of Vietnam, China, Thailand, and Bangladesh [3, 13]. Until the rise of H5N1 HPAI, they represented a secure form of income that was tightly embedded in the farming systems of these specialized ecological regions. As "silent carriers" of H5N1 HPAIV, ducks became the focus of a debate about the future of certain poultry-rearing systems. In Thailand, government regulations were introduced that prohibited the raising of free-range ducks [70]. A very specialist group that may be included in sector 3 are the fighting cocks, banned in most countries, but still popular and highly valuable, and representing a unique part of the chicken gene pool. Sector 4 includes the backyard, scavenging system, in which birds may be housed at night or not at all, and small urban flocks kept in houses in the towns and cities of developing countries. Sector 4 flocks are small and their productivity levels are low, but they contribute to cash flow, and from minimal investment they can produce a return of up to 600% [55]. They contribute directly to household nutrition and to social capital, since they are exchanged as gifts and eaten on social occasions [45, 63], and their meat is often preferred to that of commercial broilers. They play a part in farm and household ecology by eating snails and insects [3, 30], and they are used in social and religious rituals. With regard to the latter function, chickens are considered irreplaceable in parts of South-East Asia. A number of endangered breeds are kept within this system. Millions of smallholders keep sector 4 flocks, but most are not recorded in formal registration systems. From the available data and estimates they appear to represent 10–99% of poultry and producers in different countries.

Sectors 1 and 2 predominate in industrialized nations, whereas developing nations, even those with strong commercial sectors, still have predominantly small flocks. Figure 3.1 shows the different situations that countries face. Those with the highest diversity of systems have the greatest challenge in controlling disease in a way that is both efficient and equitable.

Pig production systems

Although there is no documented formal system for characterizing pig production systems equivalent to that used by the FAO for poultry, it is possible to identity three distinct types of production.

Large-scale confined pig production, the equivalent of poultry sector 1, is highly specialized for specific production steps, such as farrowing, nursing, and finishing. High-value inputs are purchased, such as breeds from dedicated breeding pyramids using genetically improved lines in nucleus herds. Marketing of outputs is connected to sometimes internationally operating processing companies. All common biosecurity measures can be applied in this production system [23]. Timely slaughtering and transportation of pigs are crucial to maintain profitability, which makes this system extremely

vulnerable to demand shocks and movement bans for disease control.

Small-scale semi-intensive confined production, equivalent to poultry sector 3, has been practised by entrepreneurial smallholders in some countries in Asia and Latin America, and to a lesser extent in Africa, in response to the demand for pig meat in urban centers, and in some cases a preference for meat from certain traditional breeds [4, 44, 56, 58]. These systems are characterized by confinement of the pigs and the use of purchased breed and feed inputs. There is an increasing emphasis on marketing to higher-value urban markets via a more complex transport system. Compared with the scavenging system, this confined production system provides more opportunities for the application of biosecurity measures to prevent the spread of swine influenza virus.

Small-scale extensive pig production, equivalent to poultry sector 4, is found in rural and periurban areas of many African, Asian, and Latin American countries. Rural households in developing countries traditionally keep scavenging pigs in their backyards to make use of food waste and other agricultural by-products. This low-intensity system is often self-reproducing (i.e. farmers produce their own replacement animals). Although productivity is usually low, economic resilience and returns on low-cost inputs are generally high. A large variety of production is either for home consumption or marketed locally [6]. The majority of scavenging pigs are owned by subsistence farmers and kept to serve the function of savings rather than regular cash income. Backyard production is less vulnerable than commercial production to negative impacts of disease control measures, such as movement bans, because the optimal selling time for fattened pigs spans a larger time window compared with intensive production systems [39]. However, it is hard to apply biosecurity measures in these systems. A more specialized form of extensive pig-keeping is found in Europe, where pigs are raised for niche markets, including organic production and favored traditional breeds, and this system is more vulnerable to the impact of movement controls [65].

Market chains

Feed, vaccines, eggs, input breeds, live animals, carcasses, and feathers are traded through international market chains, so an outbreak of a poultry or pig disease in one country can have economic impacts in several other countries. The main international market chains are concentrated and integrated, whereas domestic market chains in developing countries involve many participants and a variety of contractual relationships. The length and international reach and complexity of poultry market chains make it important to consider the whole chain when identifying risks and assessing benefits and costs [24].

Live bird markets form an important part of the poultry sector, due to the preference for live poultry meat, and they sell a wealth of species and products, brought in by numerous traders and producers. Figure 3.2 shows the interconnectedness of the formal and informal sectors for broiler chickens in Thailand and for layers in Egypt. In both cases, day-old chicks from a single source lead into various products and market channels. In Thailand, three types of farms raise the meat birds – those owned by the company that produces the chicks, farms contracted to the breeding company, and independent farmers, often on a small scale. In Egypt, much of the production of eggs and spent hens lies within integrated systems (those owned by a single company), which are an important source of products for informal markets supplying domestic consumers.

Domestic market chains for small-scale confined pig production can also involve a complex web of interactions between small-scale producers, large-scale producers, and traders [49].

Market chains not only have a functional form, as shown here, but they also exist in geographical space. Where human and animal populations are dense, different market chains tend to be physically close. Live bird markets and small slaughter points are important interaction points for different chains. The same participants provide feed, veterinary services, and transport to more than one chain, and create physical contacts between them.

Market shocks

International markets

Outbreaks of animal influenza have occurred in the context of an already volatile international poultry and pork market, adding a new source of volatility.

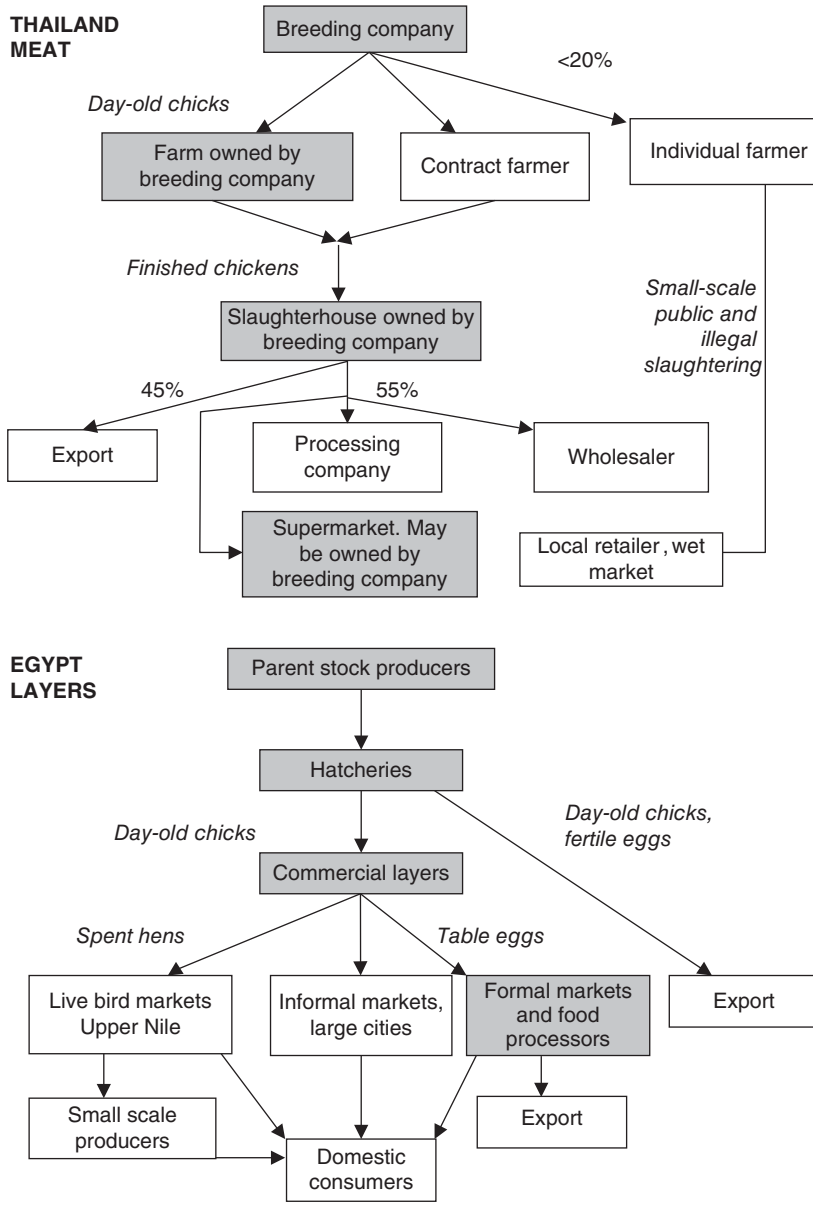

Figure 3.2 Poultry meat market chains in Thailand, 2003, and layer market chains in Egypt, 2006. Boxes in gray show integrated systems. Modified from Rushton, J. 2006. *Compensation for HPAI in Egypt*. Report produced for the FAO ECTAD Socio-Economics Working Group, November 2006. FAO: Rome, Italy.

Effects have been substantial in terms of shifts in prices, volumes, and location, driven by a fall in consumption of meat and eggs.

Restrictions on exports from Asian countries affected by H5N1 HPAI outbreaks in 2004 and the first half of 2005 contributed to a nearly 20% increase in international poultry prices over that period (Figure 3.3). Consumers switched to other protein sources, and export of live birds and chilled meat from major Asian producers, particularly Thailand and China, was banned. At the same time as international prices rose, domestic prices fell in the infected countries because of reduced domestic demand and the release of products intended for export onto their domestic markets [46]. Asian poultry populations decreased because of culling,

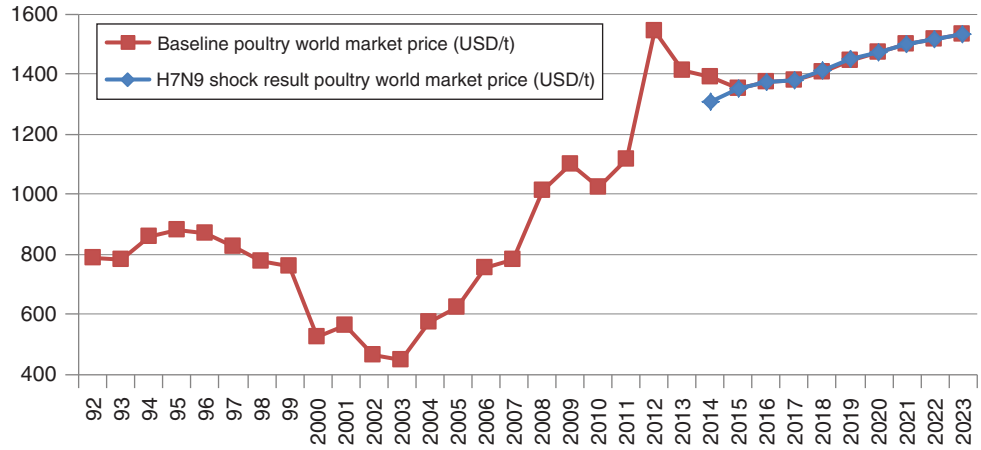

Figure 3.3 World poultry market prices and simulated impacts from 50% demand decline and export ban in Asia. Source: OECD-FAO. 2013. *OECD-FAO Agricultural Outlook 2013*. OECD Publishing. Available at 101718/agr_outlook-2013-en. Holger Matthey, Market and Trade Division. Used with permission from the FAO. *See Plate section for color representation of this figure.*

and between 2003 and 2004 there was an 8% fall in the volume of global poultry trade. Global trade bounced back in 2005, and rose again in 2006.

The news that a 2009 H1N1 pandemic was associated with pig farms in Mexico resulted in severe pork demand shocks and pork trade disruption. Chilled and frozen pork exports to Japan and the USA declined by 61% and 32%, respectively. About US$12 million in export revenue was lost within 6 months [62]. Similarly, LPAI outbreaks have also triggered market shocks. For example, in August 2006 an H7 LPAIV strain was detected at a poultry farm in the Netherlands, resulting in import bans from Taiwan and Hong Kong.

International market chains are not restricted to formal systems, but flow informally across the borders of neighboring countries in the Mekong Delta, Southern Asia, Africa, and the Middle East. This means that both disease and market shocks have the potential to cross borders. Nepal suffered a fall in local demand when India first had H5N1 HPAI, and a fall in price for live birds to 52% of the former level was reported informally from Mauritania after an outbreak in Nigeria.

Domestic market shocks

H5N1 HPAI has caused shocks to domestic markets in most countries that have suffered outbreaks, and in some that have not. The zoonotic H7N9 LPAI has caused severe market shocks in China. Once the first human case of H7N9 LPAI was reported at the end of March 2013, demand for live birds and poultry products declined, and as a result market prices dropped. Wholesale live bird markets in 27 prefectures in Eastern China were closed for between 33 and 63 days, which resulted in foregone poultry sales revenue of about US$69 million. Many market closures were not driven by plummeting demand, but were a risk reduction measure to decrease the number of human infections with H7N9 LPAI [61]. The China Animal Agriculture Association estimated losses of US$6.5 billion about 7 weeks after H7N9 LPAI was first reported, but it is unclear what factors were included in this estimate [86].

Typically, demand for poultry and pig products declines when an outbreak first occurs, with a resulting fall in price. This seems to be exaggerated if dramatic announcements of outbreaks are made by the media or governments, coupled with limited information on appropriate risk-avoiding behavior, although the extent to which communication promotes or may mitigate market shocks has not been fully analyzed. Sales together with official and unofficial culling lead to a fall in the productive animal population. It may take weeks before restocking is permitted, and even when it is allowed, establishing the sources of supply may be delayed. If consumer confidence is restored, market

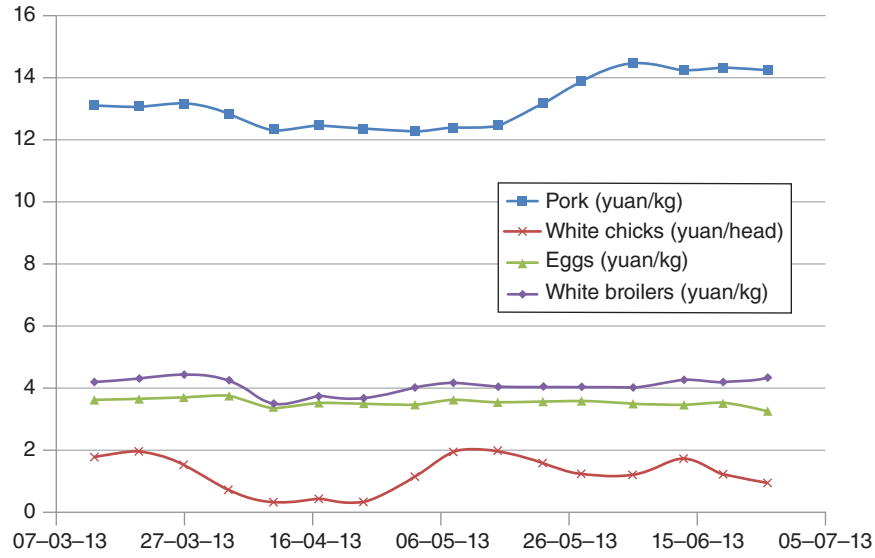

Figure 3.4 National pork and poultry prices in 2013 in China [12]. *See Plate section for color representation of this figure.*

Table 3.4 Yellow broiler wholesale market prices (in yuan/kg) in different provinces in China.

Province	13 March	20 March	27 March	3 April	10 April	17 April	24 April	9 May	15 May	22 May	29 May	5 June	14 June	19 June
Anhui	11.5	11.1	11.1	10.2	Market closure					Market closure				
Jiangsu	13.4	12.8	12	13.4						7.30	9.00	9.50	11.40	11.4
Jiangsu	12.8	12.8	12.8	12.8						Market closure				12.8
Jiangxi	11.2	11.6	11.6	11.2	10.60	Market closure						9.2		
Hubei	14.2	14.2	14	14								13.5	13.50	13.5
Guanxi	20.5	20.5	20.5	20.5	20.50	18.50	18.50	18.50	18.50		18.50	18.50	18.50	18.5
Xingiang	14.5	14.5	14.5	14.5	12.00									
Xingiang	9	9					9.00	9.00	9.00		9.00	9.00	9.00	
Xingiang	14.75	14.75	14.25		14.25	14.50		14.5			11.50			
Xingiang		15	15	15	15.00	15.00	15.00	13.50	13.50	13.50	13.50	13.50	13.50	13.5

Modified from China Institute of Animal Husbandry Engineering. www.chinabreed.com/market/poultry/.

prices rise again, sometimes to above pre-outbreak levels.

Examples of market shock are shown in Figure 3.4 and Table 3.4, for poultry and pork, meat and egg prices in China during the early phase of the H7N9 LPAI outbreaks in 2013. White broiler meat and egg prices were minimally affected on a national scale. The risk of H7N9 LPAI infection for humans was quickly associated with live bird markets, which are mainly supplied with premium-price cross-breed "yellow chicken." The "yellow-chicken" market collapsed as a result of both decline in demand and control measures, such as movement bans and market closures, to prevent human infections. In the remaining open wholesale markets the price of live chicken was 3–4 yuan/kg by mid April, one-third of the price before the onset of the H7N9 LPAI outbreak.

From a consumer perspective, analysis of this situation is complex. Consumer decisions to stop eating poultry or pig meat have a profound effect on markets. In turn, consumers may experience changes in diet and effects on the household food budget, as the prices of meat from one species and the prices and availability of substitute proteins shift. It is not clear to what extent different factors affect consumer perception of risk. A study in Vietnam suggested that there were differences between older and younger populations, and between rural and urban populations, in their perceptions of HPAI

risk [29]. In the European Union (EU), countries that suffered a similar level of H5N1 HPAI risk showed differences in consumer behavior (for example, the fall in demand in Italy was much greater than that in the UK). In Thailand, which has seen three waves of H5N1 outbreaks since 2003, progressive communication and quality control gradually minimized market shocks.

Substitution effects have been seen for alternative proteins. In Cambodia, when the prices of chicken meat and chicken and duck eggs fell during an H5N1 HPAI outbreak in 2004, the local prices of pork, beef, and fish all increased, and remained slightly higher than normal even after poultry prices had increased [77]. In Vietnam, the price of pork rose from 15 000 VND/kg (approximately US$1) to 24 000 VND/kg in November and December 2005, at the lowest point in the poultry market [1]. Diseases other than influenzas also affect the prices of substitute proteins. For example, foot and mouth disease (FMD) and bovine spongiform encephalopathy both contributed to low world prices for beef between 2001 and 2004 [50].

Direct impacts on food security (reduced energy intake, protein, or micronutrients) do not seem to be a major effect of market shocks caused by animal diseases. Indirect effects through loss of livelihoods are more of a concern than direct effects, as they reduce the ability to purchase alternative proteins, and may continue for several weeks during an outbreak and into the rehabilitation period. A market shock may result in these impacts being felt over a wide area. Poultry and eggs can be sold at short notice for a rapid source of cash in small quantities, to buy food and other daily household needs, and poor households are as likely to sell poultry products as they are to eat them, particularly in urban and periurban areas [35]. The issues of food security and livelihoods will be examined in more detail in the next section.

It has been suggested that different types of market might be expected to adjust in different ways to market shocks [73]. In a closed economy, the relative strength of fall in demand and fall in supply determines the final impact, since there is no opportunity to compensate for a fall in the poultry population by importing. A net importing country can use imports to buffer shifts in domestic supply and demand. For an exporting country, a ban on exports may be damaging to the whole sector and not only those firms involved in exports. A country where no outbreaks have occurred may still suffer demand shifts. The impacts of H7N9 LPAI outbreaks in China on world poultry market prices were limited. Figure 3.3 shows the volatility of world poultry prices, with declines during the onset of H5N1 HPAI outbreaks in 2003. Compared with the Organization for Economic Cooperation and Development (OECD)-FAO projections for 2013, a scenario of 50% decline in demand and a complete export stop for all South-East Asian countries was assumed. In this scenario the world poultry market would drop by 5.8% [52]. The effect of collapsing domestic poultry sectors and subsequently reduced imports into South-East Asian countries (China, Thailand, Vietnam, Lao PDR, Cambodia, and Myanmar) would more than offset the effect of export bans for poultry products from exporting countries in South-East Asia.

Mexico, a major pork exporter, lost chilled and fresh pork cut exports to Japan and the USA of about US$2 million per month during the second half of 2009. At the same time, Mexico stopped importing swine products at a scale of US$36.1 million from the USA and Canada [62].

In the cases where market shocks have been studied in detail, the impacts, although dramatic, seem to be quite brief. More serious effects on markets may result from longer-term disease prevention measures, and this issue is discussed later in the chapter.

Food and livelihoods insecurity

Livelihoods insecurity and in some cases food insecurity may be caused by a market shock, by disease, or by disease prevention and control measures, in particular those associated with depopulation and market restrictions. Losses of productive animals and markets and reduced prices all have effects on producers, while consumers may suffer from lack of food products or raised prices.

An animal influenza outbreak would have to be extremely prolonged and extensive, and occurring in a closed-market economy, to cause a national food security problem. It is extremely unlikely that it would directly cause national food insecurity (i.e. by reducing the food available to eat) unless the country was already on the brink of a food crisis,

or was a small island state that found it difficult to restock.

However, household food security may be affected in the immediate area of an outbreak or in a wider area affected by market closure. At the height of the H5N1 HPAI outbreaks, based on conservative estimates, 78 million people in Africa and 280 million people in Asia were in food-insecure households that kept poultry [35]. In such households, loss of poultry through HPAI may create a food security problem, mostly as an indirect effect of loss of livelihood [21]. At that time, poultry products constituted less than 1% of daily calorific intake in Africa and about 3% in Asia, accounting for 5–15% of protein consumption and 20–50% of meat consumption. They appeared to be most important to the diets of the poor in the poorer countries of Central Asia and the Middle East. The source of livestock products consumed by poor households varies by area and income group. At the time of the H5N1 HPAI outbreaks, in the more remote areas in Vietnam, the proportion of poultry consumed and used within the household was 91%, whereas it was only 9% in areas with good access to markets [72]. Regardless of location, people in very poor households are more likely to sell livestock products than to eat them.

Because of this variability in consumption habits, losses of poultry through disease or culling, or increases in the price of poultry products due to market shocks, have impacts that vary by location. Smallholders in Turkey affected by H5N1 HPAI outbreaks mentioned that they had to buy eggs instead of producing them to eat [30], but they regarded this as an inconvenience rather than a crisis. In studies of the impact of H5N1 HPAI conducted in five South-East Asian countries in 2005 [9, 16, 18, 33], direct loss of food did not emerge as a concern for any of the farmers questioned, but many of them reported income losses. Smallholder poultry development schemes have resulted in increased consumption, improved nutrition, and increased income [17]. Income from poultry is often managed by women, and income controlled by women often goes directly into child nutrition or education.

The studies of the impact of H5N1 HPAI in South-East Asia showed that these effects differed by production system. The total value of dead and culled birds was greatest for producers in specialized large-scale production systems using improved breeds. Producers with smaller flocks but equally intensive systems experienced a smaller absolute loss, but it could be a large proportion of their total asset, and they were likely to be in debt if they lost a flock. Scavenging poultry keepers lost the smallest number and value of birds, but they were the most likely to be excluded from official compensation schemes, because birds died before culling teams arrived or were not properly registered. Data for Vietnam suggest a pattern of effects for a country that has been badly affected by H5N1 HPAI and that subsequently imposed strict control measures. In total, 58 out of 64 provinces were affected in 2004–2005 [64]. A survey of smallholders showed that the average losses per farm affected by H5N1 were between US$70 and US$108, and the loss per bird was around US$2.70, including slaughter, disposal, and some down time [15]. Based on a poverty line of US$1 per person per day and a family size of five, this is equivalent to 2 to 3 weeks' income for a very poor family. Four months after an outbreak in 2005, a survey found that 27% of backyard farmers and 19% of small commercial poultry keepers had not restocked and were considering leaving the poultry sector [34]. Sector 3 farmers who continued to keep birds fed them the minimum rations necessary to sustain them [1], and this resulted in a considerable loss of feed sales. Poultry sales were banned in many markets of the Red River and Mekong Deltas (the areas of the country where poultry production is most dense) from December 2005. Some traders remained in the markets to deter others from occupying their space, while others traded in other commodities with mixed success. In Ho Chi Minh City, the largest city in the south, as a result of biosecurity measures imposed on markets, there was a concentration from 134 wholesale markets selling fresh eggs to 75, and from 1300 small shops and 250 markets selling chicken to six poultry "selling points" and one shop selling frozen poultry meat [1].

Thailand is estimated to have lost 29% of its total chicken population in the first wave of the outbreak, including around 18 million native chickens, and more than 20% of the duck population [16]. It was particularly difficult for the duck farmers to return to production, as biosecurity regulations were introduced requiring them to register and

invest in improved housing. Cambodia [77] and Laos [75] experienced outbreaks at about the same time, but with much more localized effects. In Cambodia, the main impacts seem to have been caused by the market shocks described previously. However, the affected households experienced a severe loss of assets. Between July 2003 and July 2004, the mean number of birds fell by 44% and the number of households owning 0–10 birds rose from 5% to 25%. In two provinces of Turkey, farmers ranked loss of poultry eggs and meat for their direct consumption as the most serious impact of culling, and loss of income from poultry within the top three impacts [30]. Loss of companion animals and the stress of the culling operation were also mentioned by more than 50% of the farmers who were interviewed.

Livelihood losses have also been experienced by others in the market chain, including traders, DOC suppliers, and those who work in the livestock industry. Unless deaths from H5N1 HPAI or culling are sufficiently widespread to cause serious depopulation, losses for those other than poultry producers tend to be an effect of market shocks or movement controls rather than direct effects of poultry deaths.

Impacts of outbreak stamping-out measures

The discussion in this section focuses mainly on animal influenza in poultry, particularly H5N1 HPAI and H7N9 LPAI, because most published material on the direct costs of controlling animal influenza relates to those two viruses. However the principles are applicable to any animal influenza of poultry or pigs.

Stamping-out measures for HPAI and H5/H7 LPAI outbreaks have included movement control, culling with compensation, disinfection, and disposal of carcasses. When these measures have been successful in containing disease, no further measures have been needed, but when disease has become widespread, vaccination has been used. Table 3.5 shows the timeline for an H5N1 HPAI outbreak in a Turkish community in 2006. It shows the progression from the first announcement of disease in the country, through reporting of a suspected outbreak, laboratory confirmation, culling, and compensation. The potential impact of each element of stamping out will be discussed in the sections that follow.

Reporting and confirmation

Timing is critical in outbreak control, as every delay increases the likelihood that disease will spread and all of the associated control costs will increase. Although an outbreak of HPAI in an isolated rural flock may die out without veterinary intervention, an LPAI or an HPAI outbreak linked with a dynamic marketing system will spread rapidly. The situation shown in Table 3.5 is commendable, with the veterinary services being notified on the same day that the first suspect case was noticed, and the test result becoming available 5 days later. This is symptomatic of a country that had recently experienced a human death, and where good laboratory facilities existed.

Table 3.5 Timeline for an H5N1 HPAI outbreak in Turkey.

Outbreak event	Date	Behavior
National television broadcast	January 2006	Three children died of HPAI in Eastern Turkey, causing instant panic countrywide
Birds start dying in own flock or village	February 2006	Some panic sales
Veterinarian is called	Same day	Farmer reports to Mukhtar, who calls veterinarian
Veterinarian collects samples	Same day	
Laboratory test result	5 days later	Announcement via Mosque loudspeakers
Culling team arrives	1 day later	Some not willing to hand over birds; people told what compensation they would receive
Compensation is paid	*1 month later*	Compensation paid to those who registered their name and tax number

Modified from Geerlings, E. 2006. *Rapid Assessment of HPAI Socio-Economic Impacts in Turkey*. Report produced for the FAO ECTAD Socio-Economic Working Group, November 2006. FAO: Rome. Italy.

The absence of clinical signs of H7N9 LPAI infections in chickens has made the clinical detection and reporting of H7N9 LPAI in poultry farms almost impossible. Most cases are detected in live bird markets. Time lags between sampling of birds and laboratory confirmation have often resulted in culling of newly arrived birds that were not yet present in the market but that might still have acquired infection from virus in the market environment.

Ingredients of a good reporting system can be summarized as follows.

Farmer awareness of disease-reporting pathways

Efforts have been made in various countries to improve reporting, using a combination of funding of veterinary services for surveillance, engagement of animal health workers, communicating messages to farmers via television, radio, posters, and leaflets, and community involvement. It is quite widely agreed that communication needs to be 10% or more of the total budget for control of a notifiable animal disease, and involves messaging, negotiation at all levels, and advocacy, backed up by a sound disease control strategy that is seen to deliver results [78]. The greatest investment to date has been in broadcast or printed messages, usually slanted towards protection of people. The cost of mass communication depends on the concentration of potentially affected livestock keepers and the methods by which messages must be broadcast. For instance, mass media campaigns for H5N1 HPAI prevention and control in Vietnam have cost US$1 million a year [38], but where communication can be achieved through industry groups, private veterinarians, and the Internet, the cost should be lower. Table 3.5 shows the importance of religious channels and other community institutions where a communication infrastructure already exists.

Compulsory disease reporting, legislative back-up, enforcement capacity, and incentives to report

A report on the Australian system of financing disease control [2] highlighted the need for both "carrot" (compensation) and "stick" (penalties for non-reporting, including withholding of compensation) approaches to improve reporting. However, finding an effective balance between carrot and stick is not easy. It often requires legislation change and improved enforcement capacity, which may not be costly, but may be extremely time consuming. Compensation, which will be discussed in more detail later, requires good management procedures and can also be expensive. There may also be a need to invest in infrastructure to improve the ability to identify suspect cases. In Hong Kong, improvement in biosecurity measures in live bird markets, including reporting of suspect cases, has required long negotiation with market stakeholders, provision of better stalls and hygiene facilities, and severe penalties for those who do not comply with regulations.

An effective animal health information system

An animal health information system may be considered to be effective if disease information that is added to it is timely and accurate, and decisions made on the basis of the information improve disease control. For a country engaged in international trade, an effective information system will improve trade prospects by inspiring confidence in trading partners. Not only the hardware and computer software but also the human aspects need to be considered. In Vietnam, the animal health information system has been upgraded as part of the effort to control H5N1, at an estimated initial cost of US$340 000 to upgrade laboratory and province-level systems for two-thirds of the provinces [42]. The human element requires competent field-based personnel and good links between farmers, private animal health staff, and government veterinary staff. The information needs of each stakeholder in the system, and their sometimes complex relationships with each other, need to be understood and catered for [19]. Regardless of legislation, reporting of initial suspicion is more likely to occur between individuals who know and trust each other and who have at least a basic understanding of the problem. In industrial production systems and for most farmers in developed countries, the most trusted contact is likely to be the farmer's regular private veterinarian. For less intensive production systems in developing countries, the link between farmers and the state veterinary service is through paraveterinarians and community leaders [18, 25].

In Vietnam, efforts have been made over several years to strengthen the community animal health worker (CAHW) networks. Pilot initiatives have

used regular meetings and training to promote links between CAHWs, farmers, and district veterinary staff, and these were expanded during 2006 in response to H5N1 HPAI. The cost of setting up a network in one district and training staff was estimated to be around US$3000, with an additional US$1200 a year to run it (data from project financing estimates of the World Bank Avian Influenza Emergency Recovery Project). A review of classical swine fever control [48] estimated that the cost of contracting CAHWs to do surveillance work supervised by district veterinary staff would be in the range of US$3000–4600 per district per year, excluding any additional costs for training or setting up a communications network. In Indonesia, a system was set up for participatory surveillance and reporting at district level. The set-up costs for this system were US$23 million between 2005 and 2009 [57].

Competent laboratory staff in properly equipped laboratories

Laboratory testing for precise identification of animal influenza virus strains has been beyond the capability of many affected countries, leaving them with the options of either sending samples to approved laboratories for testing or, if the need for testing was likely to be prolonged, upgrading their own laboratory facilities and training staff in how to perform new tests. The cost of building or upgrading a laboratory ranges from US$500 000 to US$50 million, but cannot all be attributed to animal influenza control, as the laboratory can also be used to test for other livestock and poultry diseases. The costs of virus isolation or real-time PCR tests are approximately US$10–20 per sample [67]. In Thailand, where detailed house-to-house surveys were conducted as part of an active surveillance program, the laboratory diagnostic testing associated with each survey was reported to cost approximately US$1 million [42]. In Hong Kong, the costs of surveillance and monitoring for HPAI in 2006 were approximately US$0.12 per bird sold [67].

Movement control

The implementation of movement bans for LPAI between supplying farms and closed markets is more challenging than classic movement bans around farms with outbreaks. H7N9 LPAI has mainly been detected in live bird markets in China. Culling and disinfection in these markets, together with closures, have also been the standard measure to halt the spread of the virus. Market closures have reduced the infection risk for humans who usually visit these markets. Typical movement ban diameters of 3 or 5 km would often not include the majority of farms supplying the affected market. Specialized poultry and other livestock value chains are dynamic, complex, and trade over long distances. Traders and farmers have a strong incentive to ensure timely movement and processing of the animals. In the case of broiler chicken, adding a few days to the fattening period can cause space and welfare problems on farms, as well as rapidly reducing profit margins.

In the Turkish H5N1 HPAI outbreak described in Table 3.5, there was panic selling of birds before control measures were put in place. This is a common occurrence, and birds may continue to be moved after a government regulatory authorized movement ban has been imposed. OIE recommends that movement control measures should be put in place at the earliest suspicion of an outbreak [85]. Table 3.5 does not mention movement controls, but this may have been because the farmers interviewed were smallholders consuming poultry products at home or selling only to local markets. Control measures may include the requirement for movement permits, closure of live animal markets, and even quarantining of animals on farms in infected areas. Costs associated with movement control include the costs of imposing it and the costs of lost sales and of feeding animals beyond their normal production cycle. In a country with predominantly large-scale industrial production systems it is easier to monitor them, and the penalties for non-compliance can be made very severe. The main costs involved are those of communication, inspection of vehicles, and issuing of movement permits. In countries with many smaller-scale farms it is almost impossible to impose control, as poultry and pigs are moved by foot, bicycle, taxi, and van, carried in plastic bags and small crates. The veterinary service often does not have the power to stop and search, so this must be done by the police.

Costs of lost sales follow a similar pattern to those from a market shock, but when markets are closed or animal movements are restricted there

may be additional costs of maintaining market space against competitors, or of feeding animals beyond their normal production cycle. Market stall rentals for wholesale live bird markets amounted to US$14.6 million during closures for H7N9 LPAI in China [61]. In Hong Kong in 2001, all live poultry markets were closed for 1 month for H5N1 HPAI. As Hong Kong had no dedicated slaughter plants for chickens, the outlets for poultry were lost. The government culled market-weight birds on farms, and made a payment to farmers for the birds [67] In Vietnam, for H5N1 HPAI, Ho Chi Minh City and Hanoi closed the live bird markets within the city and restricted entry of birds from other provinces. Birds could only be sold from approved slaughterhouses. Before the H5N1 HPAI outbreaks, households with low-intensity scavenging poultry flocks typically sold birds and eggs at the local market, or to neighbors or assemblers at the farm gate. Birds sold to assemblers were taken to wholesalers or consumers [1]. After the H5N1 HPAI outbreaks the birds could no longer be taken beyond their communes and local markets. Small-scale poultry farmers had a wider range of marketing channels, some of which are now closed [1]. Previously, they could have supplied supermarkets, but this is no longer possible, due to new regulations. Some farmers changed to pig production, while others reduced their agricultural activities. Sector 1 producers have been the most adaptable to movement restrictions, with expansion of packaged meat to supermarkets, and even opening up their own selling points for poultry products [1].

Culling and compensation

Culling of infected birds on farms normally extends to those that are infected and in close contact, but "close contact" may cover as much as a 3-km ring around the first premises to be infected. Culling of poultry in markets and tracing back to supplying contact farms has in the case of H7N9 LPAI in China been neither possible nor fully implemented. Table 3.5 shows a typical scenario where culling for H5N1 HPAI is undertaken on the farm by a government team after a short advance notice period. In this case, although farmers were given information that there would be compensation, some of them were still reluctant to present birds for culling. Women and children in particular were very distressed about the procedure.

Economic impacts of culling can be summarized as follows.

Costs of carrying out the culling, disposal, and disinfection

Culling and disposal are normally carried out by the government, and the cost should always be borne by the government, as should the cost of disinfection. Since disinfection takes place after the farm has been empty of poultry for a period of time, it may be carried out by the farmer, who should then be recompensed for their time and materials. Costs depend on the production system and the process used, with some economies of scale and experience. With a zoonotic animal influenza there is a need for protective clothing to prevent infection of humans. Culling costs in different types of markets in three high-risk South-East Asian countries were budgeted for by the FAO for H7N9 LPAI emergency preparedness purposes. It was found that a culling team could cull up to 3000 birds per day at a cost of US$1300 per team week.

Loss of production

Approximately 232 million birds are estimated to have died or been culled because of H5N1 HPAI. An average market value for an adult chicken just before an outbreak might be around US$5, giving a very rough total estimate of US$1.16 billion. However, this does not take into account the full production value lost before restocking, or the much higher value of parent stock, geese, turkeys, and fighting cocks. The aim of culling is that although more poultry may die in the short term as a result of culling, less will die in the longer term, as culling will reduce spread and produce fewer infections. If compensation is provided for culled poultry, the loss is shared between the government and producers; otherwise it is entirely borne by producers. Losses to others in the market chain are not compensated under official schemes for control of any animal disease in any country. Psychological distress for farmers and cullers is often a side effect. Farmers are never paid in full, and often not at all, for the loss of income while they wait to restock. Where the time to restocking is long because of government regulations, as in China, or because of the seasonality of outbreaks,

as in Turkey, the effect of "down time" may be quite severe. An unusual positive effect was experienced in 1998 after poultry farms in Hong Kong had been depopulated and cleaned, with farmers reporting that the first batches of poultry grew much faster than those before the outbreak [67].

Compensation is a transfer payment between the government and farmers. The value of dead birds does not change if compensation is provided for them; the loss is simply borne by a different stakeholder. However, a well-designed compensation scheme should improve compliance with culling requirements, and may encourage disease reporting, ultimately reducing the spread of disease and overall loss. No estimates have yet been made of the impact of compensation on disease reporting or spread in the countries affected by H5N1 HPAI. Many of them had little experience of compensation schemes for animal disease. Moreover, those that have experienced widespread or persistent problems (e.g. Vietnam, China, Indonesia, Egypt, Nigeria) are mainly countries that fall into the central area of Figure 24.1, with high diversity in their poultry systems, whereas the countries with most experience of compensation schemes are those with a predominance of large-scale industrial farms. Cost estimates to date report the amounts paid to farmers, but not the administrative overheads of the schemes. In Thailand, as of March 2004, farmers had been compensated for about 61 million heads of poultry [18], resulting in the payment of US$46.5 million. In Nepal, compensation rates were increased subsequent to the outbreaks in 2003, and are in the range of US$0.52–5.15 depending on the poultry category. The set compensation rates partly covered the market value of culled poultry, ranging from 9% for adult backyard poultry to 67% for adult white broilers [40].

The compensation scheme needs of sectors 1 and 2 differ from those of sectors 3 and 4 [85], and this has implications for costs and cost sharing. For example, in sectors 1 and 2 the emphasis is on precise valuation of birds, which means that the costs of a valuation process need to be considered. Contract farmers may need to be compensated, although they may not be the legal owners of birds, because they have incurred production costs. In sectors 3 and 4, although the value paid for birds should not be too low, speed of payment is more important, and it may be acceptable to use a blanket valuation on broad categories of birds, so long as compensation is paid quickly. There is a need to disperse money widely, sometimes in cash, and this carries administrative overheads. Sectors 1 and 2 are most interested in rapid disease control to restore markets, and may be willing to endure severe culling to achieve this. Sector 3 is concerned about markets, but may not see the need for severe culling, and debt may become widespread unless compensation is paid quickly. There can also be difficulties with restocking, which will be discussed later. For sector 4, one of the biggest challenges is to include farmers in official culling schemes so that they can be compensated, since compensation has rarely been paid for dead birds. Systems of registration and disbursement need to be planned with consideration for country and local conditions – there are no one-size-fits-all solutions.

In terms of funding, countries where sectors 1 and 2 are strong tend to be those with cost sharing, using public and private sector payments into a joint animal health fund from which payments can be made for compensation as well as other animal health expenses. These funds are associated with strict regulations on biosecurity. In theory, producer groups in sector 3 might also contribute to such funds, but there are no examples of good practice that can be drawn upon. For sector 4, compensation will always need to be funded by the government, and for reasons of efficiency ideally from central or earmarked funds.

Culling in a reduced area with ring vaccination in a wider area is a possibility, provided that the vaccination is administered safely and effectively. In 2006 it was estimated that in the areas of China with moderate poultry density, using ring vaccination in a 5-km zone with limited culling to stamp out an outbreak of H5N1 HPAI, instead of culling all poultry within a 3-km zone, had the potential to prevent the destruction of poultry valued at approximately US$84 000 for meat birds of moderate value, from an investment of about US$14 000 [67]. Ring vaccination costs may be shared between the government and farmers, or borne entirely by the government.

Preventive vaccination

Countries that have experienced widespread and repeated outbreaks of HPAI have developed

long-term control strategies that minimize the need for culling. Large-scale vaccination programmes for LPAI and HPAI viruses have been implemented in Mexico (H5N2), Italy (H7N1), and Pakistan (H7N3) [74]. Vietnam, Indonesia, China, and Hong Kong have introduced wide-scale vaccination against H5N1 with varying levels of coverage and success. At least 125 billion doses of H5N1 vaccine have been used between 2004 and 2012 in China, Indonesia, and Vietnam [10]. Large-scale vaccination campaigns need substantial resources, and therefore require good planning management of technical, operational, and financial issues in order to be sustainable over long time periods. Several frameworks and planning tools [10, 41, 47] are available in the literature to structure the planning and decision making of veterinary authorities with regard to including vaccination in a control strategy for H5N1 HPAI. The decision of a veterinary authority to vaccinate may be affected by a number of factors. The following questions (adapted from [47]) provide a simple checklist for both LPAI and HPAI viruses.

Is vaccination technically viable?

Existing vaccines against H5N1 HPAI give good protection in chickens and ducks, although there is a continual need to be vigilant for emerging new strains, and to improve the formulation of vaccines. H7N9 LPAI vaccines for poultry are not yet available.

Is vaccination of animals a cost-effective measure for reducing human cases?

For zoonotic animal influenza viruses, such as H5N1 and H7N9, the decision to use animal vaccination will be largely driven by human health imperatives. Short-term human health benefits have to some extent been demonstrated by large-scale H5N1 HPAI vaccination campaigns in China, Vietnam, Indonesia, and Hong Kong. Long-term cost-effectiveness will depend on the required time period and effectiveness of phasing out vaccination programs.

Does vaccination benefit the livelihoods of vulnerable people?

There is likely to be a positive effect of vaccination against HPAI viruses if it minimizes both disease spread and depopulation, particularly in situations where adequate compensation is not available. For maximum impact it needs to be accompanied by clear signals that restocking is welcomed, reopening of markets, and reassuring consumers that vaccinated birds are safe to eat. There will also be a positive effect on livelihoods if vaccination minimizes the disruption of other sectors linked to livestock, such as leisure and tourism, but this effect has not been estimated for HPAI. In Vietnam, the numbers of cases of human deaths have fallen since the introduction of mass vaccination together with other control measures, and this has presumably had a positive effect on tourism. However, Thailand and Malaysia, two countries that were concerned about effects on tourism, chose not to vaccinate.

Vaccination that reduces culling and poultry deaths may also be a valid strategy for preserving biodiversity if endangered species kept in sector 4 flocks, or valuable genetic stock in grandparent flocks, can be vaccinated and excluded from culling.

Vaccination does not remove the effects of market shocks or movement control and, in some cases, farmers may be better off having their flocks culled and receiving rapid compensation, rather than having them vaccinated and being unable to sell them.

Will vaccination have an adverse effect on international trade?

For a country with predominantly sector 1 producers, the preferred option is to try to stamp out an outbreak without vaccination, but as the Netherlands and Canada have discovered, the costs involved can be very high. Thailand was among the top five exporters of poultry meat before the onset of H5N1 HPAI, which resulted in bans on the import of Thai chicken products. Thailand has successfully eliminated H5N1 HPAI, but the EU did not lift the ban on fresh chicken imports from Thailand until 8 years after the first reports of H5N1 HPAI. France and the Netherlands vaccinated parts of their poultry stock as a preventive measure [41].

Is there an assured source of funding for continued vaccination?

An advantage of preventive vaccination over emergency activities is that many of the associated costs can be planned in advance. However, a vaccination program, once begun, may need to continue for

several years. China, Vietnam, and Indonesia have used H5N1 vaccine for a decade. Investment may be needed in staff training for field operations, improvements to laboratory facilities, and establishment or upgrading of cold chains. Recurrent costs must cover mass campaigns or the costs of making vaccine continuously available to sector 1 and 2 farmers. They also need to cover the cost of serosurveys to monitor effectiveness. Reported costs of vaccination tend to emphasize the campaigns and ignore the costs of monitoring and quality control. Ex-ante assessments of H5N1 HPAI vaccination in Indonesia and Vietnam indicated that the costs of labor, equipment, distribution, and post-vaccination monitoring would be 15–25% of the total costs of vaccinating commercial layer and broiler chicken farms. H5N1 HPAI vaccination in Indonesian backyard flocks required 72% of the overall campaign costs for labor (43%), distribution, and storage [41]. Population modeling of vaccination scenarios for backyard poultry has estimated that a maximum immunization rate of 52% of all backyard birds could be achieved with a two-shot vaccination campaign [41]. Immunization coverage would fall to 19% within 17 weeks, because of the high turnover in these flocks.

Can vaccination be delivered in a cost-effective manner that takes into account the production systems in which it is applied?

In confined, intensive, medium- to large-scale production systems, the most cost-effective method of vaccination is for the farmer to make arrangements and pay for the vaccine to be administered by the farm's usual veterinarian and farm workers. Even medium-scale farmers will take the initiative to do this if vaccine of reasonable quality is available [66]. In smaller and extensive flocks and herds, vaccine will need to be funded by the government and probably delivered through official campaigns, employing animal health workers when they are available. If birds and animals can be housed before the vaccination team arrives, the time taken and cost will be considerably reduced [41]. Vaccination against H5N1 HPAI in larger hatcheries before the chicks are widely distributed into smaller commercial flocks would significantly reduce distribution and labor costs while ensuring a higher level of coverage. Initial promising results have been obtained with operational research in Egypt. However, it is likely that booster shots will need to be delivered for long-lived layer poultry, which will still require the maintaining of a costly distribution system covering wide areas [53].

Post-outbreak rehabilitation

Even during contingency planning for outbreaks it is valuable to think beyond outbreak control to the rehabilitation process, because it may affect the decisions made during the stamping out of the outbreak. For example, providing compensation *in kind* rather than as cash, or linking it to restocking, would signal an intention to promote restocking, and would also mean that payment of compensation is delayed. Economic considerations during the rehabilitation phase include the financing and management of restocking, and the potential to invest in biosecurity improvements before restocking takes place. Smallholders find it more difficult than large commercial farmers to restock and to adopt adequate biosecurity measures [21].

Restocking

Two considerations in restocking are finance and the source of the animals or birds. In confined large- and medium-scale systems, finance is either internally generated or provided by credit, and replacement stocks are sourced from commercial producers. After a severe outbreak, if breeder farms have been culled it takes time to re-establish a production cycle. Normally cycles are staggered so that there is a regular flow of chicks or piglets. When breeder farms are destocked, production needs to be restarted in a staggered fashion, and this means that it takes time to build up to full flow. In Canada, contingency planning for stamping out H5N1 HPAI had to take into account that there would be delays in providing day-old chicks to some farmers, and this might justify higher compensation rates for farmers experiencing a longer down time [7].

For extensive flocks and herds, birds and animals for restocking are usually sourced locally. Farmers may split their flocks and keep part of the flock with a relative to avoid risk, or restock from within their own village.

Small-scale intensive farmers are likely to rely on credit to maintain the production cycle and will

experience financial problems particularly if birds or animals die close to the end of the cycle, when much has been invested in feed. In Vietnam in 2004–2005, the government encouraged banks to extend credit periods so that farmers could restart poultry production. In Indonesia, many small- and medium-scale farmers had problems in paying or rescheduling credit [36]. Some became contract farmers in order to obtain the inputs to restart production. In Lombok Island of Indonesia, contracted broiler growers were forced to destock for a month during the 2003–2004 outbreaks when contracts were suspended, but were paid for destocked birds at the contract price set by the contractors prior to the outbreak [66].

In Turkey, after the H5N1 HPAI outbreak in 2006, many people whose birds had been culled did incomplete restocking for a variety of reasons – for example, due to concern about contracting H5N1 HPAI, or fear that the government would cull their birds again, or because they did not know that restocking was permitted, or because they normally partly destocked during the winter and preferred to restock after the wild bird migratory season [30].

Increasing biosecurity in farms and markets

When the poultry sector is being rehabilitated after a serious outbreak, farmers and governments are likely to think about upgrading their biosecurity. Ideally they would do so in advance as a preventive measure, but this seldom happens except in large-scale confined systems where continuous improvements are necessary to keep pace with international food safety requirements. Biosecurity and hygiene improvements may be needed in feed supplies, on farms, in transport systems, at live bird markets, at slaughterhouses, and at meat retailers.

On farms, different approaches are needed for poultry that is going into long market chains rather than local sale. Poultry sectors 1 and 2 and large-scale pig producers regard investments in biosecurity as part of normal business practice, and view the potential loss of markets as a much greater concern than the cost of upgrading biosecurity. Government-run farms do not face the same commercial imperatives. In Vietnam it was necessary to make major biosecurity improvements to 12 government breeding farms, with costs per farm of US$10 300–75 500 for construction, US$12 750–41 875 for equipment, and around US$1500 for training [67]. In Lombok in Indonesia, a contract farmer who needs to upgrade biosecurity to meet contractor requirements might spend US$3000, in 2002 figures, for a 2500-head broiler house meeting the requirements of the contracting firm [66]. To be a contract farmer to one integrator in Vietnam, a poultry keeper must agree to construct a coop on the farm, build a good road so that trucks can access the farm all year round, provide the birds with clean water and light, and raise chickens, ducks, and pigs apart from the family residence [1].

Improving biosecurity in duck systems can require a complete change of management system, if previously free-ranging ducks herded in rice fields are required to be enclosed or housed indoors. Not only does this entail investment and increased recurrent costs, but also it could result in loss of crop yields, or the need to introduce pesticides and fertilizer. Farmers might consider continued vaccination preferable to making such drastic changes. Sector 3 chicken systems are likely to need improved night houses with netting (under US$200 in Vietnam for a small unit), more regular cleaning, disposable or washable footwear, and exclusion of visitors. In developed countries, niche markets such as organic and free-range systems may benefit from specialist approaches based on knowledge of seasonal risk from wild birds, since the main concern in these systems is not the cost of extra biosecurity measures, but the potential loss of premium markets if birds can no longer be kept outside. In sector 3, improved biosecurity is most likely to be achieved if it is linked to progressive registration and inspection of farms. In Hong Kong there has been progressive tightening of biosecurity requirements for farms, including wild bird proofing, construction of disinfectant baths to clean equipment, and transportation and other measures. In an offer to farmers wanting to leave the industry, a sum of about US$19 300 for a farm of 10 000–20 000 caged meat birds was provided to compensate for the improvements made by farmers [67].

Extensive herds and flocks may need different approaches, with community as well as individual household measures, since investment in confinement is not likely to be economically viable.

In all cases there is a need to fine-tune the advice and training given to farmers and their advisors with training. One survey [66] found that adoption of biosecurity measures by poultry farmers could be affected by industry structure, whether farmers were contracted to agribusiness firms or operating independently, type of products, and the stage of development of the poultry industry. For instance, a contracted grower might adopt biosecurity measures when required to do so by a contractor, whereas an independent farmer might choose to diversify away from poultry rather than make investments in biosecurity.

Governments face a dilemma about whether they should ban live poultry markets or insist on better hygiene. These markets provide a livelihood for many people, and freshly killed meat from previously inspected birds is preferred by many consumers. Detection of H7N9 LPAI virus in live bird markets in China has put pressure on all stakeholders to implement improved biosecurity in order to prevent virus amplification and spread. Traders and vendors have an incentive to avoid excessive losses from market closures and demand shocks, and to regain consumer confidence. Few examples of costs are available in the published literature, and local solutions to biosecurity upgrading are not always transferable. Solutions for live bird markets are often not restricted to investments in infrastructure and increased recurrent costs for cleaning and disinfection. Achieving operational biosecurity requires the sensitization and participation of traders and vendors. The establishment of a vehicle cleaning station in Guangdong cost about US$80 000. Planning and construction of a new live bird wholesale market on the outskirts of Hanoi cost more than US$2 million in international donor funds. The market was constructed in 2009–2010, with a capacity of more than 20 000 birds per day [38].

Longer-term measures for prevention and control of animal influenza

Prevention measures for animal influenza that are robust in the long term combine an improved response to outbreaks with a reduction in the risk that they will occur. Benefits should include reduced market shocks and increased market stability, increased consumer confidence, risk sharing, and hence a greater buy-in to animal health. However, sustained investment is needed, and some of the changes that are likely to be introduced have the potential to exclude people from making a livelihood from the poultry sector. The most likely measures include biosecurity upgrading (which has already been discussed), restructuring of market chains, and investments in the animal health system to provide support to poultry production.

Restructuring

When a market chain is restructured, changes may occur in several features of the chain. These changes are summarized below.

Changed location

Parts of the chain may be re-sited away from areas with high densities of humans and other livestock. Examples of this include moving wet markets and slaughterhouses outside of cities, and banning poultry keeping in cities. There was a shift of poultry production away from Bangkok between 1992 and 2000, with densities decreasing across a 50-km radius away from the city, encouraged by tax incentives [31]. One consequence of moving slaughtering facilities away from cities is likely to be a larger number of supermarket outlets in cities. The impact of re-siting can be negative (due to facilities being less accessible to poor consumers and small traders) or positive (due to creation of employment in rural areas, and the moving of smells and water contamination away from human residences).

Reduced complexity

One consequence of increased attention to biosecurity may be separation of more and less formal chains – that is, a reduction in complexity compared with the situation illustrated in Figure 3.2. As some chains become more regulated, with higher food safety standards, their contact with other chains tends to decrease. In Vietnam, the formal and informal chains have seen some separation particularly in terms of the sources of birds for high-value retailers. Gains from increased trade have largely accrued to sectors 1 and 2 [1]. Increasingly it is being recognized and defined in

international guidelines that the status of disease freedom required for international trade may apply not only to a country but also to a zone or a compartment, with the latter being effectively a biosecure integrated market chain.

Increased concentration and changed composition

The commercial poultry sector is already concentrated in terms of ownership and numbers of premises, and there is a tendency towards further concentration in countries whose poultry sectors are modernizing. H5N1 HPAI elimination policies have induced structural change in Thailand and Vietnam. The free-grazing mobile duck system was prohibited, and duck owners had to convert to a housed system in Thailand. Contract farming of broiler chickens has to some extent been phased out, and larger integrator companies have moved to vertical integration in order to increase control and biosecurity at all stages of production, encouraged by government regulations introduced for H5N1 HPAI prevention [37]. Survey results indicated that 29% of the farmers who had produced broiler chickens in 2003 had given up broiler production in 2007. A similar survey among farmers who had kept layers in 2003 revealed that 44% of them had switched to other activities [51]. In Vietnam, the government provided incentives for the modernization of poultry production which resulted in an 8% point increase in the semi-industrial chicken share in the total national production within 1 year in 2006 [43].

There is a trend towards mechanization in developed and emerging economies. In developing countries, the low price of labor currently favors the use of people rather than machines, but the need to implement ever more rigorous quality management systems for food safety may tip the balance. If this happens, the potential for restructured poultry chains to offer employment to displaced smallholder farmers will diminish rapidly. Cessation of backyard poultry production in Vietnam alone could lead to income foregone of the order of US$550 million a year, equivalent to 2.5 million "full-time" jobs at the minimum rural wage rate [43]. In Thailand, 3% of the poultry population were ducks [32], but biosecurity measures and breeding restrictions are making it increasingly difficult for the extensive systems to survive.

More formal relationships

Relationships in poultry market systems include integration, where one firm owns several parts of the chain, written contractual arrangements, verbal (but still firm) contracts, and more casual arrangements. The need for higher biosecurity along a market chain tends to push relationships towards formal contracts with lower transactions costs. In vegetable market chains it has also tended to push the risk towards small-scale producers at the end of the chain. It is not impossible for small-scale producers to be included in formal market chains, but they may need to upgrade from sector 3 to sector 2 in terms of their biosecurity, and to be operating from a secure financial basis in order to cope with delayed payments. They will also face fierce price competition with larger producers unless they can offer a differentiated product such as a traditional or organic bird. One possibility for smallholders would be to increase the strength of their producer associations to negotiate with buyers. There has been some success with this approach in Latin America with horticulture products, but there are no obvious examples to follow in poultry production.

Some of the countries tempted by restructuring will be those where domestic demand for meat is growing. There is still a strong demand for fresh or chilled rather than frozen meat, and this offers hope for domestic production. The tendency is to assume that this demand will best be met by fewer, larger, efficient units that may also form the basis for breaking into export markets. However, in a country where demand is growing, sectors 3 and 4 supply consumers who cannot afford to access supermarkets, and sector 3 has the flexibility to expand and contract quickly to meet shifting demand. An abrupt change in the composition of the sector is likely to harm consumers as much as small producers, and may create a demand gap that must be filled by imports from neighbors with uncertain animal influenza status.

Investment in animal health systems

Management of influenzas in dynamically growing pig and poultry sectors will take support from animal health systems in surveillance, rapid response to suspected cases, and preventive measures and border controls. This calls for both

investment and some rethinking of the systems. H5N1 HPAI has highlighted the particular difficulty of managing infectious diseases in decentralized financing and decision-making systems. The containment and eradication of transboundary diseases require standardization and guidelines that cut across international, national, and administrative boundaries [18]. It would be absurd to suggest wholesale re-organization, since there are strong arguments for maintaining decentralization for functions other than epidemic disease control, but it is possible to learn from the examples of good practice in managing the funding of animal health control and major decision making on legislation and enforcement. Even in a decentralized system, funds for animal health can be pooled at a central point and their use authorized by a trusted group of decision makers. As the commercial sector grows, it becomes increasingly possible to develop a fund with contributions from central government, decentralized government, and private industry that is managed according to strict guidelines agreed by representatives of all of the contributors.

It is more problematic to provide funding to support small-scale and less biosecure herds and flocks whose owners are not in a position to contribute to a national fund. It may be in the interest of the commercial sector to subsidise them. It is also in the public interest to support the formation of local producer groups through which training and services can be delivered and centered on animal health paraprofessionals. There has already been considerable progress in learning what supports and what hampers the ability of paraprofessionals to support communities [25], and this needs to continue even if it is expensive. It will be a particular challenge to direct energy towards service for poultry, since small poultry flocks receive the least animal health inputs of any livestock. Despite strong efforts to legitimize the position of paraprofessionals, their relationship with the government is still irregular. One way to build their capability is to give them regular work, with formal contracts, in surveillance, quality control, and vaccination. Finally, with the increasing tendency toward emerging zoonotic diseases, it may be possible to forge closer links between animal and human health paraprofessionals, particularly for surveillance.

Conclusions

This chapter has briefly highlighted the main issues in the economics of animal influenza control, particularly of H5N1 HPAI, H7N9 LPAI, and 2009 H1N1 pandemic influenza. Avian influenza did not initiate the changes that are taking place in the global poultry sector, since trends towards concentration and reorganization were already under way, but it has brought them to the attention of people worldwide and accelerated the changes.

This review has described the chronology of social and economic issues that must be addressed at different stages of disease management. It has drawn attention to the different concerns of confined and extensive, small-scale and large-scale production systems, with differing levels of biosecurity and commercialization, that all play a part in the economic and social fabric of the countries in which they exist. When these systems are examined, it becomes clear that the control strategy for an individual country, while it follows certain general principles, must be tailored to the mix of systems in the country and their stage of development, as well as to local financial and human resources.

There is a strong case not only for continued international financing of animal influenza control, but also for fine-tuning of recommendations to make them more cost-effective within specific situations. In order to support this process, work needs to continue in learning more about the benefits and costs of control processes. Areas of particular interest for continued investigation are summarized below.

Economics of surveillance systems

Emerging diseases, particularly those of a zoonotic nature, are a continuing threat, and their economic impact increases sharply with delays in first response. A clearer understanding is needed of the incentives for reporting, including more effective use of compensation in developing country smallholder production systems, and deeper engagement at community level in decision making about disease control. Regional cooperation in surveillance must also be improved, given the extent of official and unofficial cross-border trade in poultry. Increased coordination and collaboration between

human and animal health surveillance systems following a "One Health" approach has been estimated to result in potential savings of 20–40% for 139 low- and middle-income countries [84].

The future of smallholder poultry and pig production

Small-scale intensive production of poultry and pigs has been promoted as a pathway out of poverty, but it is not clear under what conditions it can continue to play this role. If biosecurity requirements continue to be raised, will there be the potential for small-scale production with high biosecurity levels? Extensive systems are likely to remain untouched for much longer, since they play a different economic role. It is not yet clear whether improved biosecurity would be possible for these systems, what would be required to implement it, and the cost and management implications.

Investment needs for health systems

In view of the likelihood of continued changes to the structure of the poultry sector, and to a lesser extent that of the pig sector, and continued threats from emerging diseases, there is a need to examine the way in which animal and human health systems are designed, coordinated, and funded. Those in developing countries were poorly prepared and underfunded to respond to H5N1 HPAI.

The World Bank estimated annual funding needs of US$1.9 billion to bring the global zoonotic disease prevention and control system up to OIE and WHO standards. These funding needs are significantly lower than the assessed historical costs of emerging and re-emerging zoonotic diseases of about US$6.9 billion per year. High rates of return can therefore be expected from investment in health systems in order to prevent pandemics [84]. However, the mobilization of this level of funding remains a challenge.

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4 Trade and food safety aspects for animal influenza viruses

David E. Swayne

Global production and trade of horses, pigs, poultry, and their products

Production and trade

Poultry and pigs are the most frequently raised farm animals and, on a global basis, they are the main source of animal protein in the human diet through meat, and also eggs in the case of poultry (Table 4.1) [29–31, 98]. The majority of animal protein is produced and consumed domestically, but 9.4% of swine meats, 13.4% of poultry meats, and 2.6% of eggs are distributed through global trade. Pig meat is the single most commonly consumed source of animal protein in the human diet (109 100 thousand metric tons [TMT]), but if poultry meat (92 800 TMT) and eggs (66 400 TMT) are combined (159 200 TMT), chicken is the major single contributor (Table 4.1) [31]. Trade of live animals is less than that of meat products. However, the seemingly large quantity of trade in live chickens is primarily as day-old chicks, with 1.4 billion exported yearly. The major producers and exporters of pigs, poultry, and their products are Brazil, the USA, the European Union (EU), and China (Table 4.1). By comparison, trade in horses is very small, and primarily involves live animals, with a minor percentage related to trade in meat.

The principal poultry species raised is the chicken, but significant numbers of turkey, duck, goose, Japanese quail, guinea fowl, and various ratite species are also raised, depending on culture, customs, national production system, and markets. In developed countries, most production and consumption is through specialized, integrated commercial farms and cold chain distribution. In addition, there is a smaller contribution from poultry raised through rural (village), organic, and live poultry market systems that supply some consumers with specialty products such as live or fresh-killed birds. In contrast, in many developing countries the integrated commercial poultry production sectors with cold chain distribution are smaller. The majority of poultry are raised in village or semi-commercial sectors, and live poultry markets supply the local population with poultry meat and eggs.

Standards for safe trade

Influenza A viruses (IAVs) infect and cause disease in agricultural animals, principally in horses, pigs, and a variety of poultry. As a result, IAVs have an impact on global trade in live animals and their products, including meat, meat products, eggs, feathers, hides, offal, fat, and miscellaneous other products.

Domestic production and distribution systems are critical to meeting the culinary demands of consumers as well as supplying the global market with other products, such as live animals, hatching eggs, pet food, offal, fat, and fiber (i.e. feathers). These systems include not only the economically viable production of agricultural animals, but also contribute to the control of animal diseases and the prevention of disease spread. This is achieved by implementing sanitary standards and effective disease control programs. Under the Agreement on the Application of Sanitary and Phytosanitary Measures (the "SPS Agreement") of the World Trade Organization (WTO), the World

Animal Influenza, Second Edition. Edited by David E. Swayne.
 Published 2017 by John Wiley & Sons, Inc.

Table 4.1 Global production and export of agricultural animals and their derived products for species that have endemic or high susceptibility to influenza A viruses (FAO statistics).

Animal	Live (million head)[e]	Production (slaughtered)[e]				Exports			
		Number (million head)	Meat (1000 metric tons [TMT])	Other	Top five producers[c] (in descending order)	Live[a] (1000 head)	Meat[a] (1000 metric tons [TMT])	Other[a]	Top exporters[c] (in descending order)
Horses	58.5	4.8	750			330	144		
Pigs	969	1140	109 100[d]		China, European Union, USA, Brazil, Russia, Canada, Japan, Mexico, South Korea, Ukraine	36 500	10 300		USA, European Union, Canada, Brazil, China, Chile, Mexico, Belarus, Australia, Vietnam
Poultry, meat Eggs	23 400	64 100	105 600	71 900 TMT eggs (1.4t eggs; 6.9b hens)	USA, China, Brazil, European Union, Mexico, Russia, India, Iran, Indonesia, Malaysia[d]	1 524 000[f]	13 600[c]	1847 TMT in shell eggs	
Chicken Meat	21 200	59 800	92 800	66 400 TMT eggs (1.3t eggs; 6.7b hens)		1 446 000[f]	12 470	2000 TMT canned meat; 1809 TMT in shell eggs	Brazil, USA, European Union, Thailand, China, Turkey, Argentina, Canada, Ukraine, Belarus
Ducks	1300	2900	4300			10	159		
Geese		699	2800				33[b]		
Pigeon and others	32					0.6			
Turkey	476	640	5600			78 000	987		

[a] Data are for 2011 [30] except where otherwise stated.
[b] Combined data for geese and guinea fowl.
[c] Data are for 2013 [98].
[d] Data are for 2013 [29] except where otherwise stated.
[e] Data are for 2012 [31] except where otherwise stated.
[f] Most exported as day-old chicks.

Organization for Animal Health (OIE) is recognized as the international standard-setting organization for animal health and zoonoses by the WTO. It establishes science-based sanitary standards for the prevention of disease spread through international trade in terrestrial animals and their products [61]. This has been achieved through the development and review of standards by specialized commissions, followed by the adoption of these by the OIE delegates, who are the chief veterinary authorities of the 180 member countries. These measures are necessary to ensure safety of animal health and public health as a result of international trade, as well as to prevent unfair trade [106]. Trade measures are established by each national authority, as regulations, on the basis of the international standards that they have all adopted. However, the WTO has a dispute settlement mechanism for cases where trading partners cannot agree on trade conditions on the basis of the OIE standards [106]. The OIE Terrestrial Animal Health Code provides the sanitary standards on animal health and food safety for international trade.

High-pathogenicity avian influenza (HPAI), H5/H7 low-pathogenicity avian influenza (LPAI), and equine influenza are OIE-listed diseases for which reporting of infections and disease is required [59, 61]. They have a specific chapter on animal health status and trade recommendations in the *Terrestrial Animal Health Code* [61], and recommendations on diagnostic tests and vaccines in the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* [59]. However, swine influenza is not an OIE-listed disease, and therefore does not need to be reported to the OIE, unless outbreaks of this disease meet the definition of an "emerging disease" [60]. However, the OIE provides recommendations on diagnosis and vaccination for swine influenza in the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* [59, 61]. Avian influenza, previously termed "notifiable avian influenza" (NAI), in poultry is reportable to the OIE, and it includes all HPAI and the H5/H7 LPAI, but does not include other LPAIs (i.e. H1-4, H6, and H8-16 subtypes).

IAV as a non-tariff trade barrier

IAV infections and disease have resulted in interruption of trade in horses, pigs, poultry, and their products, for both legitimate (based on international standards) and non-legitimate reasons. As an example of legitimate trade restrictions, countries can impose trade barriers to importation of live poultry or untreated products from HPAI-affected countries, zones, or compartments (CZC) into HPAI-free countries, or horses from EIV-affected countries into EIV-free countries, as long as the importing country can demonstrate non-discriminatory adherence to the OIE international standard. The importing country must have adequate surveillance to demonstrate freedom, application of the OIE recommendations, or the application of a scientific risk assessment to demonstrate the right to impose a trade barrier to protect animal and/or human health within the country. An example of a non-legitimate trade barrier is a recent WTO case regarding the prohibition of importation of poultry and poultry products by India from the USA on grounds of H5/H7 LPAI reporting. The WTO ruled in favor of the USA as India's restrictions (i) were not based on OIE Terrestrial Animal Health Code, (ii) were not justified on the grounds of a scientific risk assessment, (iii) were a case of arbitrary and unjust discrimination between WTO members, (iv) were more trade restrictive than *in-country* measures, and (v) did not recognize areas that were disease-free or had a low disease prevalence [107].

Unfair trade barriers can have a huge economic impact. For example, immediate restrictions applied by several countries on importation of pigs and pork products from Canada, Mexico, and the USA during the initial outbreaks of H1N1pdm09 IAV in humans were not based on scientific evidence, but were the result of an erroneous assumption that the virus was being transmitted by pigs and their products, exacerbated by use of the moniker "H1N1 swine influenza" by the scientific community and the media [37]. The H1N1pdm09 virus was first identified in human respiratory infections in Mexico, and then spread to other countries through human travel, with subsequent cases resulting from human-to-human spread. IAV was not present in swine meat, and despite clear information from the Centers for Disease Control and Prevention (CDC), the World Health Organization (WHO), and the OIE that the H1N1pdm09 virus was not spread by pork and that humans could not become infected with the virus if pork products were properly handled and cooked,

trade barriers imposed against North American pig products still occurred [60]. Initial losses included an immediate global drop in Lean Hog Futures of 15% between 27 April and 30 April 2009 [89], and, by the end of 2009, Mexico had a $27 million pork trade deficit, and the USA experienced a 11% drop in value of pork exports compared with 2008 [53, 66].

Risks for spread of influenza A viruses to animals through trade

Scientific evidence should be used to evaluate the risk of introduction of IAV through trade in animals and animal products using an import risk analysis tool [57]. Such a process provides the importing country with an objective and defensible method of assessing the infection and disease risks associated with the importation of animals and animal products, but must be based upon transparency and scientific data in order to be defendable. The outcome of such an analysis can be prohibition of importation, the allowing of importation of some or all products with mitigation, or the allowing of importation under the current system of the exporting countries. The components of a risk analysis include hazard identification, risk assessment, risk management, and risk communication [57]. The scientific data that are needed to determine the IAV infection and disease risk from importation of animals and animal products should include an understanding of the pathogenesis of infection and the natural route of exposure, the species of the susceptible host, and the prevalence of the IAV infections in the country and product(s) under consideration.

Pathogenesis of different influenza A virus infections has an impact on trade risk

Equine influenza virus (EIV), swine influenza virus (SIV), H5/H7 LPAI virus (H5/H7 LPAIV), and HPAI virus (HPAIV) do not cause persistent infections. The infection period is typically limited to 7–10 days, but can be as long as 21 days, with the latter duration being used by OIE as an incubation period for IAV, allowing for a specific quarantine and testing period to verify the IAV-negative status of individual animals or groups of animals [59, 61]. In addition, IAVs are generally host adapted, with sustained easy transmission of EIV being limited to animals within the family Equidae, and that of SIV being limited to animals within the family Suidae. However, in the case of AIV, sustained easy transmission can occur between birds of different orders, such as Galliformes (terrestrial poultry) and Anseriformes (waterfowl), but may require an adaptation phase [78].

EIV, SIV, and H5/H7 LPAIV in horses, pigs, and poultry, respectively, cause an acute respiratory infection and disease, with clinical signs reflective of the respiratory tropism of virus replication and disease, and the absence of IAV in meat and most viscera [54, 55, 80]. In addition, H5/H7 LPAIV can infect the gastrointestinal tract, but does not spread systemically. Clearly, the movement of IAV-infected animals creates the highest risk of spread, because the virus is replicating in and being shed from the respiratory tract (EIV, SIV, and H5/H7 LPAIV) and gastrointestinal tract (H5/H7 LPAIV) into the environment. In addition, any item within that environment, such as equipment and supplies, clothes, and shoes, can be contaminated with the virus and become an efficient means of transporting the virus between premises. Therefore the highest risk of transmission for EIV, SIV, and H5/H7 LPAIV is during the acute infection process, and would involve live animals and offal containing respiratory tissues, but the risk of transmission through raw meat products is negligible [109].

By contrast, HPAIV infections of terrestrial poultry (e.g. chickens, turkeys, quail, pheasants, partridges, and guinea fowl) cause a disseminated, systemic infection with a high mortality rate. The HPAIV is present in the respiratory and alimentary tracts, visceral organs, brain, skin, skeletal muscle (meat), bone, and blood, and virus is shed in nasal excretions, saliva, and feces [2, 3, 64, 80]. Since 1996, the Guangdong lineage of HPAIV (H5N1, H5N6, H5N8, etc.) has been shown to cause systemic infection in non-gallinaceous poultry, such as ducks, geese, and emus [6, 40, 65, 96]. Previously, ducks and geese had been shown to be resistant to infection by other HPAIVs [2, 83]. The systemic nature of HPAIV infections in poultry means that all products, including offal, feathers, and meat of poultry, are at risk of containing HPAIV. This risk could be mitigated by heat treatment such as rendering (of offal and animal feed meat meal), cleaning and heat treatment (of feathers), cooking (of meat), or pasteurization (of eggs).

Exposure risks

EIV, SIV, and H5/H7 LPAIV

Transmission of respiratory trophic IAV between and infection of susceptible hosts is initiated by inhalation of the virus or viral contact with the mucous membranes, especially those of the upper respiratory tract, and this is the natural route of animal-to-animal transmission. However, the anatomical connection between the oral cavity and the nasal cavity via the pharynx in mammals, and the choana in birds, may contribute to respiratory exposure to IAV during eating, drinking, or respiration [39].

HPAIV

Natural and experimental infections with the Guangdong lineage of H5N1 have been reported in various carnivores, including the house cat (*Felis catus*), tiger (*Panthera tigris*), leopard (*Panthera pardus*), and stone marten (*Martes foina*), and in domestic dogs (*Canis lupus familiaris*) [38, 44, 67, 75, 108] and scavenging birds, including the brown-headed gull (*Larus brunnicephalus*), great black-headed gull (*Larus ichthyaetus*), black-headed gull (*Larus ridibundus*), large billed crow (*Corvus macrorhynchos*), and mountain hawk eagle (*Nisaetus nipalensis*) [27, 50, 72, 87]. Many of these infections have been associated with close contact with or consumption of infected poultry or wild birds. Pigs, which are omnivores, have been infected naturally and experimentally with the Dutch H7N7 and the Guangdong lineage of H5N1 HPAIV [18]. During the Dutch outbreak in 2003, infections in pigs occurred on farms with infected poultry, and in some cases the pigs had been fed broken eggs obtained from the infected chickens [51]. This suggests infection either through close contact with infected poultry or through the consumption of raw infected materials. Experimentally, feeding of H5N1 HPAIV-infected chicken meat to pigs resulted in asymptomatic infection, initiated through the tonsil and extending into the upper respiratory tract, or experimental infections have been produced in the upper respiratory tract by direct intranasal inoculation of virus [49]. However, natural infections of swine with H5N1 HPAIV have been rare [14], and in most studies experimental inoculation has inconsistently resulted in infections of swine, most being asymptomatic [18, 36, 74]. In chickens, which are also omnivores, H5N1 HPAIV produced infections and high mortality when present in liquid administered by intranasal or crop gavage inoculation, or in water or infected meat consumed orally, or in infected meat given by crop gavage [45].

In the case of carnivorous or omnivorous animals, infections could theoretically have resulted from gastrointestinal and/or oral mucous membrane exposure, or virus-containing droplets generated during the tearing of carcasses while feeding could have infected the conjunctiva and/or upper respiratory mucosa. However, the dose of virus needed to cause infection varies greatly depending on direct respiratory or oral exposure. For example, chickens exposed to H5N1 HPAIV via gastric inoculation or oral consumption in either meat or water required a mean chicken embryo infectious dose (EID_{50}) which was 1000–10 000 times higher than that needed to cause infection by intranasal inoculation. This indicates that H5N1 HPAIV infection in birds is primarily a respiratory transmitted virus that favors a respiratory route of exposure, via respiratory droplets and airborne fomites such as dust, but infection following oral exposure is possible if birds consume high doses of HPAIV, such as occurs during cannibalization of infected carcasses [45]. Similarly, in a previous study using ferrets and mice, gastrointestinal exposure was less successful in producing H5N1 HPAIV infection than was intranasal inoculation when using the same dose, or required a higher gastrointestinal dose to produce infection than did respiratory exposure [48].

Risk of animal influenza A virus to humans through trade

Animal IAV infections have occurred infrequently in humans, as evidenced by a lack of documented EIV cases, less than 1000 documented cases of AIV over the past 50 years, and sporadic SIV cases in humans, usually all with limited subsequent human-to-human transmission [10, 21, 33, 43, 63]. In contrast, hundreds of millions of human cases of influenza A virus infection are caused by H1N1 and H3N2 human seasonal IAVs worldwide each year [84, 88]. The majority of the human AIV infections have resulted from two HPAIV lineages (i.e. the 1996–2016 Guangdong H5N1 lineage and the 2003 Dutch H7N7 lineage), and an H7N9 LPAIV Eurasian

lineage [4, 25, 104]. Even rarer human infections have been caused by other LPAIVs and HPAIVs, suggesting that infectivity of AIV for humans is at least in part strain dependent. Human SIV infections have resulted from H1N1, H1N2, and H3N2 viruses, with the most recent cases being from H3N2 variant viruses through exposure at agricultural fairs in the USA [28]. These facts indicate that human risk of IAV infections is much greater from human seasonal IAV than from animal IAVs.

Sufficient epidemiological data are available from the Eurasian-African H5N1 and Dutch H7N7 HPAI epizootics and the H7N9 LPAI epizootic with accompanying human infections to allow some important conclusions to be drawn concerning human exposure risks and portals of virus entry that have resulted in human infections, with most transmission of animal IAV resulting from direct animal–human contact and from respiratory tract exposure. The Dutch H7N7 HPAI epizootic resulted predominantly in clinical cases of conjunctivitis, with flu-like illness reported infrequently, suggesting that the portal of entry was most frequently through the conjunctival mucosa [42]. For the clinical cases, individuals on depopulation crews and poultry veterinarians had higher infection rates (41.2% and 26.3%, respectively) than poultry farmers and their families (14.7%) and other individuals (7.7%). This suggests that the risk of human H7N7 infection during the outbreak was associated with the highest quantity of IAV exposure via infected poultry. During the ongoing H5N1 HPAIV and H7N9 LPAIV panzootic, human infections have resulted from very close direct or indirect exposure to infected poultry (live or dead) in the household, village, or live poultry market [47, 62, 73], with presumed entry of the virus through respiratory and/or oropharyngeal tissues [26]. However, one case was linked to presumed virus exposure via consumption of uncooked duck blood and organs, and another case was linked to the defeathering of H5N1 HPAIV-infected dead swans [102, 103]. Although the case involving consumption of uncooked duck blood could suggest exposure via the gastrointestinal tract after ingestion of the virus in the raw food, evidence that HPAIV replicates in the human intestinal tract is lacking, and entry could have occurred via contact with the oropharyngeal mucosa and the upper respiratory tract [26]. However, exposure to H5N1 HPAIV-infected poultry does not always produce human infections. A Cambodian study described a lack of H5N1 HPAIV infections among villagers who were in frequent close contact with H5N1 HPAI-infected poultry, suggesting that the transmission potential from poultry to individual humans is very low [100]. Furthermore, despite the presence of HPAIV in meat of infected poultry [80], human infections have not been linked to poultry meat consumption, most likely because humans consume poultry meat as cooked product, and such cooking kills the virus [79, 90, 91]. In addition, the exposure dose needed to produce human HPAIV infections via consumption of raw product, based on the ferret model, is much higher than the dose required to produce infection via the respiratory tract [8]. These data suggest that most human infections required exposure to large quantities of AIV via direct or indirect respiratory exposure to live infected poultry, and that infection was dependent upon the AIV strain. Although some AIV strains are more likely than others to infect humans, the risk of human infection from any AIV strain is very low. In addition, various undefined host factors, such as young age and the presence of secondary disease conditions, may increase susceptibility to AIV infections [12, 19, 94]. Similarly, the lack of IAV in meat of SIV-infected swine, EIV-infected horses, and H5/H7 LPAIV-infected poultry means that infection via consumption of meat is negligible.

The zoonotic aspects of IAV infections are discussed in Chapter 5.

Risk of the spread of animal influenza A virus through trade

Human activity is the most frequent means of spreading animal IAV. This includes movement of infected animals and potentially some products, or of IAV-contaminated equipment and supplies, between different premises, compartments, regions, and countries. Within individual countries, the state/provincial and national veterinary authorities regulate movement, in relation to animal health and products derived from such animals, in order to minimize the spread of animal and zoonotic disease agents such as IAV. The OIE *Terrestrial Animal Health Code* provides sanitary

recommendations for safe international trade, and emphasizes safety and risk assessment with regard to importation of animals and animal products [61]. The goal is to prevent unacceptable risks to animal and human health while avoiding unjustified or politically motivated trade barriers. The development of transparent, objective import regulations based on international standards is essential for the prevention of introduction of IAV and for the protection of animal health, and potentially human health, while allowing fair and safe trade to continue.

Different levels of risk for spread

The level of risk of spreading IAV through trade is dependent upon several factors, including the following:

1. the ability to demonstrate freedom from IAV in the CZC through adequate surveillance and diagnostics
2. the type of IAV present, such as respiratory tropic (i.e. EIV, SIV, and H5/H7 LPAIV) versus systemic viruses (i.e. HPAIV)
3. importation of live animals or the specific type of products traded
4. the use of any type of mitigation, such as vaccination in live animals or IAV inactivation by heat treatment.

Rigorous guidelines for conducting an importation risk analysis can be found in Chapter 2.1 of the *Terrestrial Animal Health Code* [57].

Since the mid-2000s, the OIE has recognized different risk levels for IAV when importing live animals, or products derived from such animals [56]. For example, if the exporting CZC is free from IAV, based on appropriate test methods and adequate surveillance sampling, and the CZC has been consistently transparent in reporting animal health issues to the OIE member countries, importation of animals and animal products should not be prohibited based on IAV status.

In general, the highest risk of spreading an IAV (EIV, SIV, H5/H7 LPAIV, and HPAIV) through trade is from movement of live animals that are acutely infected and shedding the virus, and importation should only occur from IAV-free CZC, or if not free from IAV, importation should only be allowed after adequate mitigation steps have been taken to reduce the risk of IAV importation, such as quarantine and testing to show that the specific group of imported animals are free from IAV infection. However, the importing country cannot restrict trade if the specific category of IAV is already present within that country. An example of IAV importation through trade of live animals occurred in Australia during 2007, when imported subclinically infected vaccinated horses were held in quarantine, but a failure to follow quarantine protocols permitted EIV to breach the quarantine compound, resulting in spread of virus via respiratory fomites into the Australian horse population [11, 101]. The total cost of this 2007 outbreak of EIV in Australia was AUS$ 381 million. At the time of writing, only Australia, Iceland, and New Zealand are free of EIV.

The lack of EIV, SIV, and H5/H7 LPAIV in meat of infected horses, pigs, and poultry, respectively, means that importation of such from a non-IAV-free country is of low risk.

Different levels of risk for avian influenza virus through trade

With regard to AIV, if the exporting CZC is affected, the importation risk from HPAIV in poultry is greater than that from H5/H7 LPAIV, due to the systemic nature of HPAIV infections in poultry (i.e. the virus is present throughout the bodies of infected birds), and the greater economic impact of HPAI compared with H5/H7 LPAI.

With regard to the specific commodities, the importation risk varies according to the specific product, and is listed from highest to lowest as follows:

1. live poultry (other than day-old poultry)
2. live birds other than poultry
3. day-old live poultry
4. hatching eggs
5. eggs for human consumption
6. egg products
7. products derived from poultry, such as semen, raw meat, and other untreated products
8. poultry products that have been treated to inactivate AIV.

If the product is from an AIV-affected CZC, treatment to inactivate AIV can be utilized to eliminate the risk, provided that the exporter has taken appropriate steps to prevent recontamination of the final product, as recommended by the OIE. Furthermore, importation of HPAIV-infected raw

meat or other products may not result in infections unless the product is fed to susceptible hosts (e.g. feeding of raw scraps to backyard poultry, or placing garbage containing raw scraps in an area accessible to susceptible wild birds). In addition, the quantity of virus needed to infect poultry by feeding raw meat is 10 000–100 000 times greater than the quantity needed to cause infection via respiratory tract exposure, and must be factored into the risk analysis [45]. Previous risk analyses have determined that the probability of introducing H5/H7 LPAI through chicken meat imports ranges from insignificant to negligible [68, 109].

Cross-border transfer of AIV has occurred as a result of both legal and illegal trade of live poultry, other live birds, and avian-derived products. Prior to the 1970s, the lack of consistent restrictions on bird imports resulted in accidental introduction of exotic avian diseases such as Newcastle disease and avian influenza. The linkage of the 1972 Newcastle disease outbreak in poultry in Southern California to unrestricted movement of exotic birds from Central America into the USA led to the development and augmentation of quarantine and testing requirements for imported pet birds and poultry in the USA (in 1974), the UK (in 1976), and other countries [1, 97]. Following the implementation of justified import restrictions and the quarantine and testing of imported birds, AIVs have been isolated on infrequent occasions from smuggled or illegally imported birds, or legally imported birds inside quarantine stations [85]. In contrast, AIV isolations from poultry products have been reported even less frequently. In the past decade, AIVs have been isolated from poultry carcasses and meat, including H5N1 HPAIV from frozen duck meat legally imported from China into South Korea (in 2001) [96] and Japan (in 2003) [52], H10N7 LPAIV was isolated from lungs and tracheas in chicken and duck carcasses illegally imported from China into Italy (in 2006) [5]. In 2007 an outbreak of H5N1 HPAI occurred on a single turkey farm in Suffolk, in the UK [24], and although the source of the introduction was not clearly evident, epidemiological evidence indicated that the introduction had most probably occurred through importation of infected fresh turkey meat from Hungary that originated from a subclinically infected flock. The authors of the study proposed that transmission had resulted from a series of low-probability events, with gulls consuming infected scraps discarded at the processing plant, and then roosting on a neighboring turkey house which had poor biosecurity, leading to an HPAI breakout in the turkey house.

A variety of H5N1 HPAIVs isolated from birds and humans between 2003 and 2004 have been demonstrated in meat obtained from both naturally and experimentally infected chickens, Japanese quail, ducks, and geese [41, 77, 96]. In one experimental study, A/chicken/South Korea/ES/2003 (Korea/03) H5N1 HPAIV was present in breast and thigh meat in high titers of intranasally inoculated chickens, and the HPAIV was transmitted to naive broilers by feeding breast meat from infected chickens (Table 4.2), but A/chicken/Pennsylvania/1370/1983 (PA/83) H5N2 HPAIV was not transmitted by feeding similar infected chicken meat [80]. This discrepancy in transmission was the result of differences in the challenge dose. The probability of imported raw product initiating HPAI outbreaks via consumption by a susceptible host is low. However, the incident in the UK, along with experimental feeding studies in chickens, emphasizes the need for sanitary standards that prevent accidental importation of HPAIV-infected products that could possibly lead to HPAI outbreaks. Therefore the importation of raw

Table 4.2 Data for specific-pathogen-free chickens vaccinated subcutaneously at 1 day of age with either a recombinant fowl poxvirus containing an H5 AI gene insert (rFP-AI-H5) or inactivated H5N9 AI oil-emulsified vaccine (A/turkey/Wisconsin/68), and challenged intranasally 3 weeks later with H5N1 HPAIV (A/chicken/South Korea/ES/03). Meat samples were taken on day 2 after inoculation from euthanatized (vaccine) or dead (sham) chickens.

Group	Virus isolation from meat (log_{10} EID_{50}/g)		Virus dose/bird when fed infected meat (log_{10} EID_{50})
	Breast	Thigh	
rFP-AI-H5	–[a]	–	ND
Inactivated vaccine	–	–	ND
Sham	7.3	ND[a]	7.8[b]

[a] – = no virus isolated, ND = not done [80].

[b] Feeding the meat from the sham chickens to naive 3- to 4-week-old chickens produced lethal infection in 9 out of 10 chickens.

poultry meat from an HPAIV-affected CZC into an HPAIV-free CZC is an unacceptable risk, and trade barriers may be warranted.

Mitigation of trade risks

The best method of ensuring safe and fair trade for IAV-free countries is importation from other IAV-free countries [56]. Freedom from IAV can only be demonstrated for countries that conduct serological and virological surveillance utilizing sensitive testing and targeted or random statistically based sampling methods [56], and that have historically demonstrated transparency in reporting animal diseases to the OIE. Freedom from IAV can be demonstrated for a country or parts of a country, such as a zone (i.e. a region with definable geographic features) or a compartment (i.e. a functional unit separated from other units by biosecure management practices) [69]. However, under the SPS Agreement, the importing country cannot impose upon the exporting country sanitary measures that are not based on international standards. If a country chooses to apply more stringent measures above and beyond international standards, these should be supported by a scientifically valid risk assessment. In addition, if an importing country is affected by a disease, it can only require measures that are equivalent to those applied nationally. Various types of mitigation strategies can reduce the importation risks for IAV, and are discussed below.

Reducing the live animal trade risk

Mitigation of the live animal risks for an exporting country could be achieved through the application of safety measures, such as zoning or compartmentalization, or for the importing country by requiring vaccination, testing, and/or quarantine before movement into the country.

Vaccination

Vaccination either reduces or prevents replication of EIV, SIV, H5/H7 LPAIV, and HPAIV in the respiratory and gastrointestinal tracts of susceptible animal hosts [13, 81, 95, 99]. In addition, vaccination of poultry prevents systemic infection with HPAIV [86]. On a practical level, high levels of HPAIV were isolated from breast meat of non-vaccinated chickens, ducks, and turkeys after intranasal challenge with various H5N1 HPAIVs, but no virus was recovered from meat or viscera of birds that had been vaccinated 3 weeks prior to challenge with the same viruses [6, 80, 93]. These studies demonstrated that proper vaccination can reduce the risk of HPAIV in poultry meat and viscera.

Inactivation methods for influenza A viruses in animal products

A variety of treatment methods can be applied to products from IAV-infected CZC to inactivate the IAV and make the products safe. Heat is also commonly used to inactivate a variety of pathogenic and non-pathogenic viruses, bacteria, fungi, and protozoa in food products. Such heat applications are typically achieved by cooking, pasteurization, or rendering processes. The physico-chemical properties are similar across all IAVs, and include thermal inactivation properties.

Meat and related products

As HPAIVs produce systemic infection in poultry with virus in meat [80], initial thermal inactivation studies focused on the development of a reproducible microassay method for measuring virus inactivation in meat samples from chickens that had been infected intranasally with HPAIV [76]. Using a minimal cooking temperature of 70°C, infectious Korea/03 H5N1 HPAIV was detected in infected meat 1 second after treatment, but not after 5 seconds [76]. A detailed thermal inactivation study of Korea/03 H5N1 HPAIV established inactivation line equations, D_t values (time required to reduce the infectious titer by one $\log_{10}$ at a specific temperature), the Z value (the increase in temperature required to reduce the D_t value by one $\log_{10}$), minimum cooking times at various temperatures, and comparisons with minimum USDA Food Safety Inspection Service time–temperature guidelines for a 10^7 reduction in *Salmonella* (Table 4.3) [91]. This study estimated a maximum titer of $10^{8.7}$ mean chicken embryo infectious doses (EID_{50}) of H5N1 HPAIV per gram of chicken meat (breast or thigh). As shown in Table 4.3, the inactivation line equation for HPAIV in chicken breast and thigh meat predicts

Table 4.3 Time predicted for an 8.7-$\log_{10}$ reduction of Korea/03 HPAIV titer in chicken meat at a given internal temperature, and number of $\log_{10}$ reductions of Korea/03 HPAIV titer achieved in chicken meat cooked according to minimum current USDA FSIS time–temperature guidelines for a 7-$\log_{10}$ reduction in *Salmonella* [91].

Temperature		95% PI upper limit for D_t value (s)[a]	Time predicted for an 8.7-$\log_{10}$ EID_{50} reduction in Korea/03	Minimum FSIS time–temperature guideline[b]	Predicted number of $\log_{10}$ EID_{50} reductions in Korea/03 achieved[c]
°C	°F				
57.8	136	215.8	31.3 min	63.3 min	17.6
58.9	138	125.0	18.1 min	39.7 min	19.1
60.0	140	72.4	10.4 min	25.2 min	20.9
61.1	142	41.9	6.1 min	16.1 min	23.1
70.0	158	0.50	4.4 s	21.9 s	43.8
73.9	165	0.073	0.64 s	<10 s[d]	13.7/s

[a] D_t values with upper limit of the 95% prediction interval were calculated from combined breast and thigh meat model line equation (y = $\log_{10}$ D_t value), $y = [(-0.2157)(\text{temperature °C})] + 14.6773 + (2 \times \text{RMSE})$, where RMSE (root mean square error) = 0.0621.

[b] From the time–temperature table for chicken meat with 1% fat.

[c] Assuming that the required internal temperature is maintained for the length of time specified in the FSIS time–temperature table.

[d] The required lethality is achieved instantly at this internal temperature.

inactivation of HPAI in a 100-g sample after a holding time of 4.4 seconds at 70°C (158°F), or a holding time of 0.64 seconds at the standard USDA cooking temperature of 73.9°C (165°F). In terms of the USDA/FSIS guidelines for *Salmonella* reduction, the established time–temperature combinations exceed those predicted to inactivate H5N1 HPAIV in chicken meat. Multiple studies have confirmed that cooking meat to a core temperature of 73.9°C (165°F) will inactivate IAV within the meat or on the surface [79, 90, 91].

Egg products

With H5/H7 LPAIV, virus is shed from the cloaca (the common exit chamber for the digestive and reproductive tracts), and can thus be found on the surface of eggs laid by acutely infected hens [79]. However, the isolation of H5/H7 LPAIV, as well as non-H5/H7 LPAIV, from the internal contents of eggs has not been reported to date. As a result, eggs produced in an H5/H7 LPAI-affected CZC could be imported into an AIV-free country if the eggshell surface has been sanitized to eliminate any H5/H7 LPAIV, and the eggs are transported in new packing materials [56].

In contrast, it would be risky to import sanitized eggs from an HPAI-affected CZC into an IAV-free country, because the virus is not only present on the surface of eggshells, but could also be present within the internal contents of eggs laid by HPAIV-infected hens before they die [79]. However, heat treatment of liquid or dried egg products can inactivate HPAIV and make the product safe for trade. Pasteurization processes, which utilize lower temperatures than those used for cooking, are typically used to inactivate micro-organisms in egg products. This allows retention of egg functional properties such as albumen and yolk color, and viscosity traits. In experimental studies, the maximum reported titer of HPAIV in the internal contents of chicken eggs laid by infected hens was $10^{4.5}$, $10^{4.9}$, and $10^{6.1}$ EID_{50}/mL [7, 79, 82]. A recent study of thermal inactivation of HPAIV in liquid and dried egg products predicted that seven of nine standard commercial pasteurization time–temperature combinations would effectively inactivate 10^5 EID_{50}/mL of HPAIV, and that six of the nine processes would provide an extra safety margin of 10^2 EID_{50}/mL or greater (Table 4.4). Although H5/H7 LPAIV has not been demonstrated in the internal contents of eggs laid by acutely infected hens, pasteurization processes were assessed for their ability to inactivate any theoretical H5/H7 LPAIV (Table 4.5). The thermal inactivation data from the egg product study predict that the standard commercial pasteurization processes would inactivate more than $10^{2.3}$ EID_{50} of H5/H7 LPAIV per mL of egg product, and seven of the nine processes would inactivate the theoretically impossible $10^{17.6}$ or greater EID_{50} of H5/H7

Table 4.4 Estimated pasteurization times for eggs contaminated with HPAIV, and estimated number of $\log_{10}$ reductions in HPAI achieved by industry pasteurization standards.

Product	Standard USDA pasteurization process for *Salmonella*				Reference
	Temperature (°C)	Time (min)	HPAIV		
			D_t value (min)	Process lethality[a]	
Whole egg	60.0	3.5	0.45	7.8D	[79]
Homogenized whole egg	60.0	3.5	0.45, 0.56	7.8D, 6.3D	[17, 79]
Fortified egg yolk	61.1	6.2	0.23	27D	[17]
	62.2	3.5	0.14	25D	
Plain egg yolk	60.0	6.2	0.06[b]	103D[b]	[17]
	61.1	3.5	0.03[b]	117D[b]	
10% salted egg yolk	62.2	6.2	0.06[b]	103D[b]	[17]
	63.3	3.5	0.04[b]	81.5D[b]	
10% sugared egg yolk	62.2	6.2	0.05[b]	124D[b]	[17]
	63.3	3.5	0.02[b]	175D[b]	
Dried egg white (6.5–8% moisture)	54.4	$10^{4–4.15}$	400.2	19.1–27.3D	[92]
Liquid egg white	55.6	6.2	2.1	3.0D	[79]
	56.7	3.5	0.55	6.4D	
Egg substitute (with fat)	56.7[c]	4.6	5.6	0.8D	[16]
	57.7[d]	36.3	2.3[b]	2.7D[b]	
	59.0[e]	4.0	0.75	5.3D	

[a]Log reduction for virus at specified pasteurization temperatures and times.
[b]Estimate was made based on Z-value equation.
[c]Imitation egg without fat pasteurization standard.
[d]Liquid egg white pasteurization standard.
[e]Proposed pasteurization process.

LPAIV per mL of egg product. The OIE's *Terrestrial Animal Health Code* provides an appendix with a summary of the recommended time and temperature combinations for making various poultry products safe [58].

Other products

Goose feathers (down) are a common exported commodity from Asia, and are used as insulation or filler for pillows, quilts, sleeping bags, coats, and other apparel. Because AIV and other IAVs are very susceptible to heat, detergents, and a variety of chemicals [9], feathers or other similar products can be treated with steam or detergents to kill any IAV that might be present. However, such treatments must follow standardized processes that provide uniform treatment to the product and kill the virus without altering the physical qualities of the feathers.

Offal from livestock and poultry are used in meat meal for pet foods. Mitigation of the import risk for offal containing respiratory (EIV, SIV, and H5/H7 LPAIV), gastrointestinal (H5/H7 LPAIV), and all viscera, skin, and meat (HPAIV) tissues used in meat meal for pet foods could be achieved by heat treatment to kill the IAV, depending on the host source of the product. Typically, meat scrapes, offal, skin, and fat are ground, mixed, and heat treated for 30 minutes at 118°C to inactivate multiple pathogens, in a process termed rendering, to produce meat meal to be used as a protein source in animal feeds [46]. This process greatly exceeds the minimum requirements for thermal inactivation of IAV, based on modeling with H5/H7 LPAIV, and such a treatment has even been shown to destroy the viral RNA [46].

Food safety risks?

Natural and experimental HPAIV cases have demonstrated the systemic nature of infections in

Table 4.5 Estimated pasteurization times for eggs artificially contaminated with H5/H7 LPAIV[a] and estimated number of $\log_{10}$ reductions in H5/H7 LPAIV achieved by industry pasteurization standards.

Product	Standard USDA pasteurization process for *Salmonella*				Reference
	Temperature (°C)	Time (min)	HPAIV		
			D_t value (min)	Process lethality[a]	
Whole egg	60.0	3.5	0.19	18.4D	[79]
Homogenized whole egg	60.0	3.5	0.19	18.4D	[79]
Fortified egg yolk	61.1	6.2	0.13	47.7D	[15]
	62.2	3.5	<0.13	>47.7D	
Plain egg yolk	60.0	6.2	0.71	8.7D	[15]
	61.1	3.5	0.67	5.2D	
10% salted egg yolk	62.2	6.2	0.50	12.4D	[15]
	63.3	3.5	0.38	9.2D	
10% sugared egg yolk	62.2	6.2	0.23	27D	[15]
	63.3	3.5	0.13	27D	
Dried egg white	54.4	$10^{4–4.15}$	720	13.9–19.6D	[79]
Liquid egg white	55.6	6.2	2.7	2.3D	[79]
	56.7	3.5	0.55	6.4D	
Egg substitute (with fat)	56.7[b]	4.6	1.0	4.6D	[16]
	57.7[c]	6.3	0.8	7.9D[b]	
	59.0[d]	4.0	0.5	8D	

[a]Log reduction for virus at specified pasteurization temperatures and times.
[b]Imitation egg without fat pasteurization standard.
[c]Liquid egg white pasteurization standard.
[d]Proposed pasteurization process.

poultry, including the presence of virus in meat and eggs [2, 3, 6, 40, 64, 65, 80, 96], but IAV is not present in the meat of animals infected with EIV, SIV, and H5/H7 LPAIV [54, 55, 80]. There have been 694 H5N1 cases, 90 H7N7 cases, and one H7N3 HPAIV case of human infections, with 401, 1, and no fatalities, respectively [20, 22, 105]. Most human infections with HPAIV have resulted from close contact with live or dead HPAIV-infected birds [63]. Although consumption of an infected food (raw duck blood pudding) has been associated with one human case of H5N1 HPAIV infection [103], there were insufficient epidemiological data to confirm that consumption of the infected product was the transmission route [26]. Several factors limit the potential impact of HPAIV on food safety. First, unlike free-living bacteria such as *Salmonella typhimurium* and *Escherichia coli*, which can continue to grow in food products post-harvest or post-slaughter, HPAIV can only grow in living animal cells. HPAIV replication stops after the host animal dies, limiting the possible viral load in the food product. Second, the receptors needed for attachment and replication of HPAIV are present predominantly in the lungs, and have not been described in the human digestive tract. This suggests that HPAIV is more likely to infect humans via the respiratory system. Finally, proper cooking will inactivate any HPAIV that might be present [26, 91]. Taken together, these data suggest that HPAIV is not currently a significant food safety issue for humans. However, because most cases of HPAIV infection in humans have been linked to direct contact with infected birds, known HPAIV-infected flocks should not be processed for food in the home setting, live poultry markets, or slaughter plants. Human exposure could occur when catching, handling, transporting, or slaughtering diseased birds. Human infections could presumably occur through generation and inhalation of small droplets, dust, or aerosols containing the virus, or by touching the nasal, conjunctival, or oral mucous membranes with contaminated hands.

H5/H7 LPAIV, non-H5/H7 LPAIV, and HPAIV infections differ in both birds and humans. In birds, H5/H7 LPAIV and non-H5/H7 LPAIV produce limited respiratory and gastrointestinal infections, and virus is not detected in poultry meat or the internal contents of eggs [79, 80]. In humans, there have been 476 documented H7N9 infections and 18 other LPAIV infections in the past 30 years, with the majority presenting as respiratory infections, and some presenting as conjunctivitis only [22, 32]. The fatality rate has been low except with the H7N9 LPAIV, which has caused 175 deaths [104]. Because the poultry infections last only 7–10 days, H5/H7 and non-H5N7 LPAIV-infected flocks have been safely marketed after recovery from infection. Controlled marketing of recovered flocks allows farmers to recoup financial losses while reassuring farmers, workers, and consumers about their safety [34, 35]. Clearance of AIV infection can be confirmed in the flock by testing for AIV in the normal daily flock mortality by real-time reverse transcriptase polymerase chain reaction or pen-side antigen capture tests on oropharyngeal or tracheal swabs before marketing and processing the birds [23, 70, 71]. In addition, the processed carcasses should not contain respiratory or digestive tissues.

Conclusions

Poultry and pigs are the most frequently raised farm animals, and on a global basis they are the major source of animal protein in the human diet through consumption of meat and eggs. Domestic production and distribution systems, as well as imported products, are critical for meeting the culinary demands of consumers as well as supplying markets with other products, such as live animals, meat meal, offal, fat, hatching eggs, and feathers. The OIE *Terrestrial Animal Health Code* provides sanitary standards for international trade, and emphasizes science-based risk assessment for safe importation of animals and animal products. The goal is to prevent unacceptable risks to animal and human health while avoiding unjustified or politically motivated trade barriers.

Human activities are the most frequent means of spreading IAV. This has occurred through movement of infected animals and to lesser extent of their products, or IAV-contaminated equipment and supplies, between different premises, compartments, regions, and countries. The level of risk of spreading animal IAV through trade is dependent upon several factors, including the following:

1. the presence or absence of IAV in a CZC as demonstrated through adequate surveillance of and diagnostics for susceptible host animals, as well as transparent reporting
2. the type of IAV present, such as respiratory virus (EIV, SIV, H5/H7 LPAIV) or systemic virus (HPAIV)
3. the commodity traded, such as live animals, raw or cooked offal (including lungs and other respiratory tissues), meat, and other products
4. the use of any type of mitigation, such as vaccination in live animals, or treatments to inactivate IAV.

Because HPAIVs cause systemic infection in poultry, the risk of transfer through trade is greater than for H5/H7 LPAIV, EIV, and SIV. Cross-border transfer of IAV has occurred through both legal and illegal trade of live animals and animal-derived products, and emphasizes the need for sanitary standards to prevent accidental importation of IAV into IAV-free CZC. EIV, SIV, and H5/H7 LPAIV importation can be mitigated during live animal trade by quarantine and testing of susceptible animal species, and use of vaccines to reduce susceptibility to IAV. Furthermore, the risk of importation of these viruses through meat is negligible, as these viruses are not present in non-respiratory tissues (i.e. meat). By contrast, HPAIV produces systemic infection in poultry, with virus detected in all tissues, including meat. Various mitigation strategies can be used to reduce risk, such as vaccination of animals in an HPAI-affected CZC, or the use of inactivation processes such as cooking or pasteurization of products obtained from an affected CZC.

From a human health perspective, IAV infections are primarily acquired from aerosol and fomite exposure to human-origin IAV, and a small number of cases from SIV; a relatively smaller number of AIV cases are mainly HPAIV of the Guangdong HA lineage (H5N1 and H5N6, 1996–2016) and the H7N7 Dutch lineage (2003), and H7N9 LPAIV in China, and there are no confirmed cases of EIV. Most SIV cases have resulted from human exposure at swine exhibitions (e.g. county and

state fairs in the USA) with concurrent SIV infections in show pigs. Most HPAIV infections have occurred following direct or indirect exposure to infected poultry at live poultry markets or village production systems. Taken together, the current data indicate that animal IAV is primarily an animal health issue rather than a human health or food safety issue.

Acknowledgments

The author thanks A. Thiermann for critical review of the manuscript and suggestions for improvement.

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5 Public health implications of animal influenza viruses

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Introduction

The emergence and spread in humans of animal-origin (novel) influenza A viruses (IAVs) can lead to a global influenza pandemic, with accompanying widespread global morbidity and mortality [52]. It is likely that past influenza pandemics occurred after direct transmission to humans of an animal-origin IAV, or after genetic reassortment between human and animal-origin IAVs in a mammalian host. Because of the unpredictability and impact of past pandemics, the member states of the World Health Organization (WHO) agreed to report novel influenza virus infections in humans as a requirement of the 2005 International Health Regulations [238] which were implemented in 2007. Prior to this, reporting of animal-origin or novel influenza A cases in humans was discretionary and often incomplete.

In order for a novel IAV that infects humans to cause a pandemic, it must cause disease and, more importantly, acquire the capacity for sustained human-to-human transmission. Although such events are rare, largely due to host range restrictions for IAVs resident in different species, influenza pandemics in humans have struck unexpectedly on four occasions since 1900 (Table 5.1). Genetic analyses of these four pandemic IAVs identified genes of animal origin in each of them. For example, sequencing 8 genes of the 1918 H1N1 pandemic virus revealed that this virus probably emerged by adaptation of a wholly avian influenza virus (AIV) to mammals [166, 167, 192, 193]. In contrast, both the H2N2 virus that caused the "Asian influenza" pandemic in 1957–1958 and the H3N2 virus that caused the "Hong Kong influenza" pandemic in 1968–1969 originated through genetic reassortment between previously circulating human influenza A and low-pathogenicity avian influenza virus (LPAIV) gene segments (Table 5.1). The H2N2 virus contained hemagglutinin (HA), neuraminidase (NA), and polymerase basic protein 1 (PB1) genes from an avian virus, whereas the H3N2 virus contained HA and PB1 genes from an avian virus [109, 132, 175].

The devastating 1918 H1N1 pandemic resulted in an estimated 50–100 million deaths worldwide during three observed "waves" of disease [138]. The 1957 H2N2 and 1968 H3N2 pandemics were milder, but nevertheless resulted in an estimated 70 000 and 34 000 deaths, respectively, in the USA [148]. The 2009 H1N1 pandemic caught the world by surprise not only because it began in Mexico, and pandemic preparedness efforts in that decade had focused primarily on H5N1 highly pathogenic avian influenza viruses (HPAIVs) that had become endemic in birds in at least six countries, but also because all 8 genes of the 2009 H1N1 pandemic virus were of swine origin [79]. Although the 2009 H1N1 pandemic was relatively mild, it was estimated to have resulted in approximately 200 000 deaths globally, with the main impact being in younger individuals [56]. Following each of these four pandemics, the viruses that caused them became established in the human population and continued to evolve through antigenic drift

Animal Influenza, Second Edition. Edited by David E. Swayne.
 Published 2017 by John Wiley & Sons, Inc.

Table 5.1 Subtypes of animal influenza A viruses causing zoonotic infections and subtypes causing influenza pandemics since 1900.

Zoonotic disease: sporadic infections with novel influenza A viruses recently causing mild to fatal human illness	**Previous pandemic emergence though adaptation and/or reassortment**
Low pathogenic avian influenza viruses	*Adaptation of an avian-origin influenza virus*
H6N1, H7N2, H7N3, H7N7, H7N9, H9N2, H10N7, H10N8	1918 H1N1 pandemic virus
	Generation of a human–animal reassortant virus
	1957 H2N2 pandemic virus
	1968 H3N2 pandemic virus
Highly pathogenic avian influenza viruses	*No pandemics have yet arisen from HPAI viruses*
H5N1, H5N6, H7N3, H7N7	
Variant influenza viruses	*Transmission to humans of a swine influenza virus*
H1N1v, H1N2v, H3N2v	2009 H1N1 pandemic virus

over time, causing many human epidemics and a substantial cumulative public health impact. For example, it has been estimated that circulation of seasonal influenza viruses leads to an annual toll of more than 200 000 hospitalizations and 3000–49 000 deaths in the USA alone, and 300 000–500 000 deaths worldwide [27, 194, 231].

Because of the wide diversity among IAVs (i.e. 18 HA subtypes and 11 NA subtypes) that are resident in non-human hosts, it is impossible to predict which subtype will cause the next influenza pandemic and when the next pandemic will occur. Nevertheless, we know that the likelihood of another influenza pandemic is high because H5NX HPAI viruses and LPAI of the H5N2, H6N1, H7N2, H7N3, H7N9, H9N2, H10N7, and H10N8 subtypes circulate widely in poultry and have demonstrated an ability to jump the host species barrier to infect humans. In addition, a variety of H1N1, H1N2, and H3N2 reassortant IAVs that are endemic in swine populations have also infected humans, and can pose a significant pandemic threat, as evidenced by the 2009 H1N1 pandemic.

Although AIV infections had rarely been detected in humans prior to the H5N1 HPAI outbreak in Hong Kong, earlier human challenge studies using 10 different AIVs indicated that humans could be infected by at least three of the virus subtypes used, namely H4N8, H6N1, and H10N7 [11]. During the 41 years between 1959 and 2000, only 72 human infections with animal-origin IAVs were reported [75, 100, 228, 235]. Increases in global human influenza surveillance coupled with greater use of sensitive molecular methods for detection of animal-origin IAV infections led to over 1400 human cases being reported between 2000 and 2014 [100]. Other factors that probably contributed to the recent marked increase in detection of human cases include rises in global population densities of people, pigs, and poultry, along with increases in global travel and commerce [100, 101]. Most of these recent human infections by animal-origin IAVs were caused by three different subtypes of influenza, namely H5N1 HPAIVs, H7N9 LPAIVs, and H3N2 variant (swine-origin) IAVs [100].

H5N1 HPAIVs are panzootic in poultry, have so far caused more than 700 reported human infections, and arguably pose the most ominous threat to global public health due to a 60% case fatality rate among infected humans. Potentially devastating consequences could ensue if these viruses were to gain the ability to transmit efficiently among humans. However, other avian and swine IAVs are also of significant concern. During the past 2 years over 470 human infections caused by H7N9 LPAIVs have been reported to the WHO. It is notable that H7N9 human infections are accumulating at a

much faster rate than H5N1 HPAI cases. This is probably because H7N9 viruses possess mutations that confer partial mammalian adaptation and a greater ability to infect humans than H5N1 viruses. Although the precise number and nature of mutations required for efficient human-to-human transmission of contemporary H5N1, H7N9, and other AIVs are unknown, it is well understood that additional adaptive mutations and/or genetic reassortment with human IAVs might render these viruses more transmissible in humans. While these H5N1 and H7N9 AIVs pose significant pandemic threats, recent human infections coupled with human serologic studies indicate that Eurasian H1N1 swine IAVs also pose a pandemic threat to humans, due to the absence of antibodies to these viruses in the human population [95]. Here we discuss zoonotic IAV infections in humans, and their public health implications.

Diagnosis of animal-origin influenza A virus infections

Influenza virus infections are difficult to identify reliably by clinical examination and routine laboratory findings alone. Therefore specific diagnostic tests must be used to determine whether a patient is infected, and to guide patient management. Such tests include molecular detection methods, virus isolation and identification, direct detection of influenza virus in clinical (respiratory) specimens, rapid point of care tests, and serological tests.

Molecular methods are now widely applied to diagnose influenza virus infections, and have become the "gold standard" for virus detection, due to the increased sensitivity of these tests compared with virus culture and other older methods. Reverse transcription of viral RNA followed by amplification with polymerase chain reaction (RT-PCR) has been in wide use for a number of years [108, 234]. More recently, PCR methods using fluorescent probes for detection and/or quantification of amplified DNA in real time have been widely adopted [70, 135]. The use of real-time RT-PCR (rRT-PCR) shortens the time to results to approximately 4 hours, increases the sensitivity and specificity of diagnosis, allows quantification of the gene target, and decreases the risk of PCR cross-contamination through the use of a closed system [108]. By using primers and probes that target conserved genes, such as the influenza matrix (M) gene, along with those targeted at a specific set of IAV HA and NA subtypes, it is possible to determine the type and subtype of an IAV infection within hours of the arrival of clinical specimens in the laboratory. Due to the rapid evolution of the HA and NA genes of IAVs, it is necessary to constantly evaluate the available sequence data to determine whether it is necessary to update primers and/or probes over time.

Despite the high sensitivity and specificity of the rRT-PCR assay, and the ease and rapidity of point-of-care assays, it is crucial to obtain viral isolates to test for changes in antigenicity and antiviral susceptibility, and to obtain whole-genome sequences for zoonotic IAV infections. This information is essential for public health purposes, and the virus isolates are used for production of pre-pandemic and pandemic influenza vaccines. It is important to note that viral isolation for clinical specimens obtained from human individuals with HPAIV infections must be performed in laboratory facilities with Biosafety Level 3 enhancements, and that WHO guidelines for the safe handling of specimens from suspected H5N1 cases have been issued [241]. Specific rRT-PCR testing performed under Biosafety Level 2 conditions is the preferred method for diagnosis of human infection with animal-origin IAVs [33]. In the USA, all state public health laboratories, several local public health laboratories, and the Centers for Disease Control and Prevention are able to perform influenza rRT-PCR testing for a variety of animal-origin IAV infections.

Prior to the widespread use of PCR assays for virus detection, isolation of IAVs in cell cultures or eggs followed by hemagglutination-inhibition (HI) testing to identify the type and subtype of virus was the "gold standard" for influenza diagnosis in humans. Viral isolates were then typed, subtyped, and further characterized antigenically and genetically. Virus isolation results generally are not available for a week or longer, although some laboratories use a rapid culture method that allows virus to be obtained within 18–24 hours [262]. Fluoroimmunoassays, radioimmunoassays, and enzyme immunoassays can also be used to obtain a result within a few hours, but these assays are often less sensitive than virus isolation, require

specialized laboratory equipment and reagents, and are used much less frequently [108].

A number of commercially available "point-of-care" tests are available that use an immunoassay to detect influenza viral proteins in specimens. Most of the currently available point-of-care tests detect both IAV and influenza B virus (IBV), and distinguish between them. However, some may detect only IAVs or IBVs, or do not distinguish between them. In general, these diagnostic tests are most useful for determining within 15 minutes whether influenza is the cause of outbreaks in institutional settings or circulating in populations of patients. These tests have been reported to have a wide range of sensitivities (50–70%) and specificities (90–95%) for detecting seasonal influenza infections [36], and are less sensitive for detecting novel, animal-origin IAV infections in humans [8, 9, 72]. Recently, new rapid point-of-care diagnostic tests have become available with higher sensitivities (>60–70%) for detecting seasonal IAVs in respiratory specimens. These include tests that utilize an analyzer device to detect seasonal influenza viral antigens, and a rapid molecular assay with higher sensitivity than antigen detection tests. Other molecular assays are available that detect influenza viral RNA in respiratory specimens within 1–2 hours, but these tests must be performed in clinical laboratories that are able to run diagnostic assays with moderate complexity.

Human infections by animal-origin IAVs can also be detected by measuring increases in influenza-specific antibody between acute and convalescent serum samples from patients. Due to the need for paired sera, serodiagnosis of infection is necessarily retrospective and therefore is not useful for patient management. Techniques for measuring antibody against influenza in sera include HI, virus neutralization, and enzyme immunoassays. In general, these tests are considered sensitive, and may provide the only means of documenting influenza infection in situations where respiratory specimens are not available. The microneutralization (MN) test is generally more sensitive and specific than the HI test for detecting antibodies, and has become the "gold standard" for detection of antibodies to AIVs in human sera. This assay is sensitive and specific, can yield results in 2 days or less, and can detect virus subtype-specific antibody at titers that are not detected by the HI assay [174].

Human infections with LPAIVS

Sporadic human infections with LPAIVs of different subtypes (i.e. H6N1, H7N2, H7N3, H7N7, H7N9, H9N2, H10N7, and H10N8) have been reported to cause human illness (Table 5.2). The majority of these LPAI virus infections have been linked either to direct poultry contact or to indirect exposure, such as visiting a live poultry market. While some human infections have been linked to exposure during LPAI poultry outbreaks, other cases have occurred after poultry exposure but without identified outbreaks in birds, and for some human LPAI cases the source of exposure remains unknown.

LPAIV subtypes have been reported to cause illnesses of variable severity in infected humans, ranging from mild upper respiratory tract disease to moderate or severe lower respiratory tract complications; some infections result in full recovery, while others result in death (Table 5.2). Prior to the emergence of H7N9 LPAIVs in China in 2013, the most common clinical finding with LPAIV infection was conjunctivitis, which was observed in adults with H7 and H10 subtype virus infections. Influenza-like illness (ILI) with fever and upper respiratory tract signs and symptoms has also been reported in patients with H7N2, H7N3, and H9N2 LPAIV infections, while H6N1 LPAIV was reported to have caused mild pneumonia in one patient [218]. In addition, moderately severe to fatal lower respiratory tract disease has been reported with H9N2, H7N2, and H10N8 LPAIV infections in human individuals with immunosuppression or other comorbidities (Table 5.2). Human infections with LPAIVs, HPAIVs, and other animal-origin IAVs are described by subtype below, beginning with the ongoing outbreak of H7N9 LPAIV infections in China. Information about LPAIVs and HPAIVs in animals can be found in Section II of this book.

Human infections with H7N9 LPAIVs

In February and March 2013, the first three cases of human infection with H7N9 LPAIV occurred in Eastern China [77]. These three H7N9 cases were adults who experienced severe pneumonia and died of respiratory failure, raising concern that an H7N9 pandemic might emerge. Large outbreaks of human H7N9 LPAIV infections occurred subsequently in China during the spring of 2013 and

Table 5.2 Infections with LPAIVs reported to cause human illness (from data up to December 2014[a]).

Subtype	Patient characteristics	Clinical illness	Illness severity	Countries	Year(s)
H6N1	Young adult	Influenza-like illness, mild lower respiratory tract disease	Moderate	Taiwan	2013
H7N2	Adults	Influenza-like illness, lower respiratory tract disease, conjunctivitis	Mild to moderate	USA, UK	2002, 2003, 2007
H7N3	Adults	Conjunctivitis	Mild	UK, Canada	2004, 2006
H7N7	Adult	Conjunctivitis	Mild (one case)	UK	1996
H7N9	All ages	Influenza-like illness, lower respiratory tract disease, multi-organ failure	Mild to severe, majority with severe to critical illness with mortality of around 40%	China; exported cases identified in Hong Kong SAR, Taiwan, Malaysia, and Canada[a]	2013, 2014
H9N2	Young children and adults	Influenza-like illness, lower respiratory tract disease	Mild to moderate	China, Hong Kong SAR, Bangladesh	1998, 1999, 2003, 2007, 2008, 2009, 2011, 2013
H10N7	Adults	Conjunctivitis and influenza-like illness	Mild	Australia	2010
H10N8	Middle-aged and elderly adults	Severe pneumonia, respiratory failure, multi-organ failure	Critical illness, fatal outcome in two of three cases	China	2013, 2014

[a]Two cases in Canada occurred in January 2015.

the autumn and winter of 2013–2014, and are continuing. Initially, human H7N9 LPAI cases were identified in provinces in Eastern China, with later cases also identified in Southern China. Human H7N9 cases were subsequently reported from a wider geographical area in China, including the far north-west. Human cases of H7N9 LPAIV infection linked to exposure and infections in mainland China have also been identified in Hong Kong SAR, Taiwan, Malaysia, and Canada.

Most cases of H7N9 LPAIV infection have experienced severe pneumonia requiring hospitalization, and the majority of patients were admitted to an intensive-care unit with respiratory failure resulting in high mortality [76, 126]. H7N9 is the first LPAIV that has consistently caused a high frequency of severe and fatal human illnesses. By the end of December 2014, over 470 H7N9 human cases had been reported, with a case fatality rate of approximately 38%. Although this large number of cases occurred during a relatively short time period, one study suggested that the number of reported cases is a substantial underestimate of the number of human infections with H7N9 LPAIV [249]. Human H7N9 LPAI cases have peaked during conditions of colder temperatures and lower humidity in the autumn and winter months in both years, suggesting seasonality similar to that of human seasonal influenza.

The age spectrum of reported cases is wide, with a median age of approximately 60 years, and with about twice as many male cases as female cases [100]. This is in contrast to a median age for H5N1 HPAI virus infections of 18 years, and a gender distribution that is more nearly equal [156]. The reasons for these differences between H5N1 and H7N9 cases are not understood, but are thought to be due to the fact that older men are more likely to frequent live poultry markets. A case–control study reported that the presence of chronic medical conditions was a risk factor for H7N9 LPAIV infection [3]. Risk factors for death of H7N9 patients include older age and chronic lung disease [103]. Pandemic influenza concerns have also been raised by the identification of sporadic clusters of human cases of H7N9, including some clusters in which limited, non-sustained human-to-human H7N9 virus transmission may have occurred [126, 165].

H7N9 viruses have been isolated from some live poultry markets that were visited prior to the onset of illness by the human cases, and epidemiological and virological studies have suggested that many urban cases of H7N9 LPAIV infection were linked to exposure at live poultry markets [10, 42, 92, 126]. A case–control study reported that exposure in live poultry markets, even without direct poultry contact, was asignificant risk factor for H7N9 LPAIV infection [133]. Another study reported that direct poultry contact and environmental exposure to poultry were risk factors for H7N9 LPAIV infection [3]. A serological study of poultry workers in areas where human cases were reported revealed that approximately 6% had antibodies to H7N9 viruses, whereas no antibodies were detected in the general population [247]. Furthermore, one study estimated that closure of live poultry markets reduced the mean daily number of human cases of H7N9 LPAIV infection by 97–99% in four major cities in Eastern China during 2013 [252].

Most patients with H7N9 LPAIV infection who were treated with oseltamivir received late treatment, generally during hospitalization after a median of 7 days after illness onset [76]. Some of these patients developed oseltamivir-resistant H7N9 LPAIV infections associated with an R292K substitution in the viral neuraminidase; this substitution also results in highly reduced inhibition by peramivir. In addition, some of these patients had received corticosteroid treatment, had prolonged viral shedding, and experienced critical illness and fatal outcomes [96].

Importantly, some human H7N9 virus isolates have been demonstrated to have tropism for both avian-like (α2,3-linked sialic acid) and human-like (α2,6-linked sialic acid) receptors in the respiratory tract, and can infect epithelial cells of the upper and lower respiratory tract and replicate efficiently in *ex-vivo* bronchus and lung cultures [39, 204, 215, 256]. In addition, studies of experimental infection in ferrets with H7N9 LPAI viruses suggest that respiratory droplet transmission of these viruses occurs more readily than for H5N1 HPAIVs, but less well than for seasonal IAVs [14, 245, 259]. This dual tropism for both avian- and human-like receptors indicates that H7N9 LPAIVs will probably continue to circulate in domestic birds and in live poultry markets, and cause human infections. This feature, along with other molecular markers for mammalian adaptation that are present in H7N9 LPAIV genomes, raise ongoing concerns about the

potential of H7N9 LPAIVs to further adapt and cause a pandemic.

Human infections with other H7NX LPAIVs

Prior to the dramatic H7N9 LPAI outbreak in China during 2013, there had been few reports of human infections by H7NX viruses, and most of these were clinically mild cases. For example, in 1996 a 43-year-old woman developed unilateral conjunctivitis 1 day after a piece of straw had contacted her eye while she was cleaning a duck house containing 26 apparently healthy ducks. An H7N7 LPAIV was isolated from a conjunctival swab specimen taken from the woman [121]. In the USA, during a multi-state H7N2 outbreak among turkeys and chickens in commercial poultry farms in 2002, a culler involved in the disposal of poultry developed ILI with fever and upper respiratory tract symptoms, and was confirmed serologically to have been infected with an H7N2 LPAIV [38]. In the UK in 2006, a worker at a farm with an H7N3 poultry outbreak was diagnosed with conjunctivitis, and an H7N3 LPAIV was isolated from conjunctival, nasopharyngeal, and throat swabs [119, 146]. Subsequently, four human infections with H7N2 LPAIV were detected by RT-PCR by testing of conjunctival and respiratory specimens from adults who had contact with poultry linked to H7N2 outbreaks in the UK during 2007 [69]. Three of these cases were hospitalized with lower respiratory tract disease, and one had conjunctivitis [69]. However, in the USA the source of H7N2 LPAIV infection could not be determined for an immunocompromised adult male who was hospitalized with febrile upper and lower respiratory tract illness and in whom H7N2 LPAIV was isolated from a respiratory specimen [153]. There are no controlled clinical data on the effectiveness of antiviral treatment of H7NX LPAIV infections, and most cases of LPAIV infection with mild to moderate illness were confirmed after resolution of the illness without antiviral treatment.

Human infections with H9N2 LPAIVs

A total of 15 human H9N2 LPAI virus infections have been reported, with the first five having onset of ILI in July and August 1998 in mainland China [253]. This report included a sixth pediatric Chinese case who became ill in November 1999. The H9N2 LPAIVs isolated from the 1998 human infections were genetically related to the G9 lineage H9N2 viruses circulating in chickens, while the 1999 virus isolate was a G1 and G9 lineage reassortant virus [253]. In 1999, two unrelated children in Hong Kong SAR, China presented with mild, self-limiting illness and were admitted to different hospitals [160]. The H9N2 viruses isolated from these two cases were antigenically related to G1 lineage viruses that had been isolated from quail in Hong Kong in 1997 [91, 130]. A follow-up investigation of close contacts of these two cases found no evidence of human-to-human transmission [202]. In November 2003, an H9N2 virus was isolated from a 5-year-old child in Hong Kong who reportedly had no contact with poultry [21]; this virus was similar to Y280-like viruses circulating in poultry in the area. Similarly, no information was available about poultry exposure for several other human H9N2 cases that have been reported [64, 90, 253]. In March 2007 in China, the isolation of H9N2 from a 9-month-old girl who experienced mild respiratory illness was reported [229]. Two additional cases of H9N2 LPAIV infection were identified during 2008 and 2009 in immunocompromised female patients in Hong Kong [43]. One case occurred in a 3-month-old girl with acute lymphoblastic leukemia, and the second case was in an adult with post-bone-marrow-transplant graft-versus-host disease; both patients survived. Genetic analysis of viruses from these two patients revealed that one belonged to the G1 lineage and the other belonged to the Y280 lineage [43]. A single H9N2 human case has been reported from Bangladesh, where a 4-year-old girl with onset of respiratory illness in February 2011 presented for medical care in Dhaka [99]. A nasal pharyngeal wash specimen from this patient yielded a G1-lineage H9N2 virus. The child recovered uneventfully and there was no evidence of transmission. In 2013, two more human infections with H9N2 LPAIVs were detected in Hong Kong, where a 7-year-old boy had clinical onset in November and an 86-year-old man reported clinical onset in December [85, 86].

Demographic information was available for five of the 15 H9N2 cases. Two were adults, including a 47-year-old woman and an 86-year-old man. The ages of seven of the eight children were provided, and ranged from 3 months to 7 years. Five of the

six children for whom gender information was provided were female. Although some of the cases had underlying medical conditions, all recovered completely from the infection, and there was no evidence of transmission of H9N2 LPAIVs to other members of the household or to healthcare workers. In some instances the cases had had contact with live or fresh killed poultry or had visited a live poultry market prior to the onset of symptoms.

H9N2 LPAIVs are of particular interest because they have become very widely distributed in wild waterfowl and domestic poultry in China, South-East Asia, India, and the Middle East, and have been circulating since at least 1994 [89]. The success of H9N2 viruses is further illustrated by the fact that the six internal genes of H9N2 viruses have been transferred to H5N1 HPAIVs, and H7N9 and H10N8 LPAIVs, through genetic reassortment [100]. Interestingly, H9N2 LPAIVs isolated from poultry in Southern China between 2009 and 2013 have been shown to preferentially bind to human-like (α2,6-linked sialic acid) receptors, and some are capable of transmission in ferrets through respiratory droplets [127]. Furthermore, H9N2 LPAIVs have also infected swine. Four H9N2 LPAIVs were isolated from samples collected in 1999 from swine at an abattoir in Hong Kong, and two were similar to Y280-lineage viruses circulating in poultry and waterfowl at the same time. The other two viruses were more closely related to viruses recovered from chickens in 1994, indicating separate introductions from avian species into the swine population [158]. This ability to infect and spread among swine is consistent with the ability to bind to human-like virus receptors, and taken together with the capability for transmission in ferrets would indicate that H9N2 LPAIVs pose an ongoing pandemic threat to humans.

Human infections with H10NX LPAIVs

One of the 10 different subtypes of AIVs used to challenge human volunteers in an early study was an H10N7 LPAIV. Interestingly, 15 volunteers were challenged with the A/turkey/Minnesota/3/79 H10N7 LPAIV, and nasal wash specimens collected from six participants 3 to 4 days after challenge yielded virus isolates. Eight volunteers had mild or very mild clinical disease after viral challenge, while none demonstrated a fourfold or greater rise in HI antibodies to the virus [11].

In 2004, H10N7 LPAIVs were isolated from two children, both under 1 year of age and residing in Egypt [155], and it was noted that H10N7 virus had been isolated from wild ducks in the same town. There was no evidence of additional human infections in the area. In early 2010, evidence was obtained for human infections with an H10N7 LPAIV in Australia during a March outbreak of the same virus in a commercial poultry operation where birds exhibited a notable drop in egg production [7]. Prior to processing, birds from the infected farm tested negative for the virus and exhibited no signs of illness. However, within 7 days after the birds were processed, five workers at the abattoir exhibited symptoms of conjunctivitis; one worker reported conjunctivitis with rhinorrhea, and a third reported conjunctivitis, rhinorrhea, and a sore throat. Although virus isolation was not successful, partial virus genome sequencing from samples taken from two of the symptomatic workers identified an H10N7 virus with a partial genome sequence identical to that found in viruses from the infected flock. None of the workers tested demonstrated a rise in antibody titer to H10 using HI or MN assays [7].

The first human case of H10N8 was a 73-year-old woman who was hospitalized in China [41, 196] with febrile respiratory illness in November 2013, and subsequently died. A second hospitalized case of H10N8 LPAIV infection was reported in January 2014 in a 55-year-old Chinese woman who survived severe illness. The third H10N8 case was a 75-year-old man with illness onset in February of that year, who succumbed to H10N8 LPAIV infection after hospital admission [78, 219, 225]. All three cases were reported to have underlying medical conditions, and two cases reported that they had visited a live poultry market a few days before the onset of illness [254]. There was no evidence of human-to-human transmission. The H10N8 LPAIVs that caused these human infections were reassortant viruses with HA and NA genes from H10N8 viruses and the other six gene segments from H9N2 viruses.

LPAI H10 viruses were isolated previously from live poultry markets in Guangxi Province sampled in 2009–2011 [161]. In January 2012, an H10N8 LPAIV was isolated from a duck sampled in a live poultry market in Guangdong Province [104]. A more recent study has shown that the prevalence

of H10N8 LPAIVs has increased in live poultry markets in China [246]. Furthermore, a study of receptor binding of H10 IAVs has shown that these viruses possess high avidity for human-like receptors [203], consistent with successful experimental human infection by an H10N7 LPAIV reported in a much earlier study [11].

Human infections with H6N1 LPAIV

Of 11 human volunteers who were challenged with the H6N1 A/duck/Pennsylvania/486/69 virus in an early study, three exhibited mild or very mild discomfort, and none of the volunteers demonstrated signs consistent with ILI [11]. Viruses were isolated from nasal wash specimens collected 3 to 4 days post inoculation from two of the infected volunteers. However, HI antibodies were not detected in any of the study participants [11].

The first human case of H6N1 was detected in May 2013 in Taiwan [218]. The patient, a 20-year-old woman, presented with ILI, and was hospitalized and treated with oseltamivir starting 3 days after illness onset. She denied having had contact with poultry prior to the onset of illness. Follow-up of 17 close contacts and 19 healthcare workers identified six who developed fever or respiratory illness after contact with the index case. Swabs collected from those reporting illness were all negative for evidence of H6 virus infection [218], although it should be noted that swabs were collected from these ill individuals after they had recovered. No additional confirmed H6N1 cases were identified, and the patient recovered uneventfully.

LPAIVs of the H6N1 subtype have been isolated from domestic poultry in Taiwan since 1972 [123, 211]. Avian H6NX viruses have also been isolated from asymptomatic wild birds and domestic birds in many countries elsewhere in the world [98, 212, 216]. It is noteworthy that several of the H6N1 LPAIVs circulating in Taiwanese poultry since 2005 have a G228S substitution in the HA which was also present in the isolate obtained from the first human case [218]. It is not known whether this residue enhances binding of H6 viruses to human-like receptors. However, characterization of the receptor-binding specificity of over 250 H6NX viruses isolated from live poultry markets in Southern China revealed that approximately 20% of them bound to α2,6-linked sialic acid, and that a few of these viruses were transmitted to contact guinea pigs in a transmission experiment, indicating that these viruses might also have human pandemic potential after further adaptation [212].

Human infections with HPAIVs

HPAIVs of different H5 (H5N1, H5N6) and H7 (H7N3, H7N7) subtypes have caused a wide spectrum of human illnesses (Table 5.3). Most human cases of infection with H5 HPAIVs have been associated with poultry exposure. The widespread ongoing epizootic of H5N1 HPAIVs has resulted in approximately 700 human cases with high mortality in 16 countries and regions since 1997 (Figure 5.1) As H5N1 HPAIVs continue to circulate, evolve, and reassort with other AIVs among poultry in many countries, further human H5N1 and H5NX HPAIV infections are expected. For example, active influenza surveillance in poultry in Egypt has shown that H5N1 HPAIV and H9N2 LPAIV co-infections were detected relatively often [110]. Furthermore, two human infections with reassortant H5N6 HPAIVs were reported in China during 2014 [230, 233]. Both infections, which were in adults, resulted in severe illness and death, and both cases had contact with poultry. In addition, human infections with H7N3 and H7N7 HPAIVs have been well documented, and additional cases are expected in the future during outbreaks in poultry.

Human infections with H7NX HPAIVs

Human illnesses caused by H7 HPAIVs have ranged from relatively mild (H7N3 and H7N7) to severe and fatal disease (H7N7). The first known case, identified in 1959, was a 46-year-old man who, after traveling in Asia, the Middle East, and Europe, was diagnosed with hepatitis. An H7N7 HPAIV was isolated from a blood specimen collected more than 1 month after he returned to the USA [63]. H7N7 antibodies were not detected in convalescent serum from this patient, and the relationship of the H7N7 HPAIV to his disease is unclear.

In 2003, during a widespread outbreak of H7N7 HPAI among poultry at commercial farms in the Netherlands, 89 human H7N7 cases were identified

Table 5.3 Infections with HPAIV subtypes reported to cause human illness (from data up to December 2014).

Subtype	Patient characteristics	Clinical illness	Illness severity	Countries	Year(s)
H5N1	All ages, primarily children and young adults	Influenza-like illness, lower respiratory tract disease, multi-organ failure	Mild to critical illness, majority with severe to critical illness with mortality of around 60%	Hong Kong SAR, China, Vietnam, Thailand, Cambodia, Indonesia, China, Turkey, Iraq, Azerbaijan, Egypt, Djibouti, Nigeria, Laos PDR, Pakistan, Myanmar, Bangladesh, Canada (imported from China)	1997, 2003–2014
H5N6	Adult	Severe pneumonia, respiratory failure, multi-organ failure	Critical illness, fatal outcome in one adult	China	2014
H7N3	Adults	Conjunctivitis	Mild	Canada, UK, Mexico, Italy	2004, 2006, 2012, 2013
H7N7	All ages	Hepatitis, conjunctivitis, influenza-like illness, severe pneumonia, and respiratory failure	Mild to critical illness with fatal outcome in one adult, majority with conjunctivitis	UK, Netherlands	1959, 1996, 2003

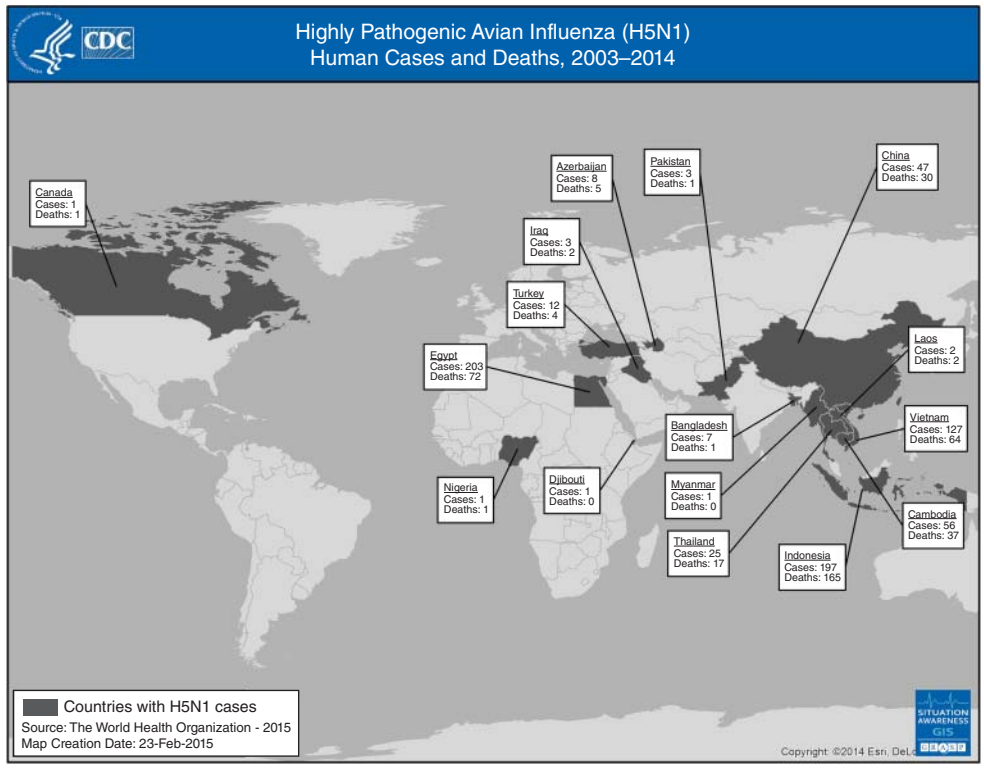

Figure 5.1 Geographic distribution of human H5N1 HPAI cases reported to the World Health Organization between November 2003 and December 2014. Source: World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC). *See Plate section for color representation of this figure.*

[117]. The majority of these H7N7 cases (88%) had conjunctivitis only, while five had ILI and conjunctivitis, two had ILI only, and four had other symptoms. Cullers and veterinarians involved in this outbreak had the highest estimated attack rates for H7N7 HPAIV infection. Only one of the confirmed H7N7 cases had been taking antiviral chemoprophylaxis with oseltamivir, and only three cases received oseltamivir treatment [117]. All of the cases recovered fully except for a previously healthy 57-year-old male veterinarian who developed high fever and severe headache without respiratory symptoms 2 days after visiting a farm with H7N7 HPAIV-infected chickens. Nine days after exposure and 7 days after illness onset, he was hospitalized with pneumonia, after which his condition deteriorated and he died 13 days after illness onset [74, 117]. The H7N7 HPAIV isolated from this case was shown to be distinct from the H7N7 viruses from other patients, but similar to viruses isolated from birds in the same area [74, 141]. A total of 86 cases of primary H7N7 HPAIV infection were identified in an estimated 4500 people who were exposed to H7N7 virus-infected poultry [117]. Presumably these individuals were infected with H7N7 HPAIV through direct or close contact with infected poultry or contaminated material. In addition, three secondary H7N7 cases were identified in family members with no history of poultry exposure, but who were in contact with primary H7N7 cases, suggesting limited human-to-human transmission of H7N7 HPAIVs [117]. During this outbreak, it was confirmed that two family members of a male poultry worker with H7N7 were infected with H7N7 HPAIVs, namely his 13-year-old daughter with conjunctivitis and ILI, and his 37-year-old wife with conjunctivitis [117]. Both received oseltamivir treatment and recovered. In addition, a 44-year-old father of a poultry worker with H7N7 conjunctivitis developed conjunctivitis 1 day after the onset of H7N7-associated conjunctivitis in his son.

Very mild illness was reported for H7N3 HPAIV infections in two individuals involved in culling activities during a large poultry outbreak in British Columbia, Canada, during 2004 [37, 186]. The first case was a 40-year-old male poultry worker who was not wearing protective goggles during culling operations, and developed unilateral conjunctivitis and coryza 3 days after contact with dead poultry [198]. An H7N3 virus was isolated from a nasal specimen from this individual. Although this virus was expected to be an HPAIV, detailed analyses demonstrated that the poultry worker was in fact infected with an H7N3 LPAIV, indicating that both H7N3 LPAIV and HPAIV were co-circulating during this poultry outbreak [186]. The second case was a 45-year-old poultry worker who was wearing glasses that did not prevent direct eye contact with feathers, and who developed unilateral conjunctivitis and headache 1 day after exposure. An H7N3 HPAIV was isolated from a conjunctival swab from this individual [198]. Both 2004 H7N3 cases were treated with oseltamivir and recovered fully. Most recently, H7N3 HPAIVs were identified in conjunctival swabs collected from two poultry workers with conjunctivitis during widespread H7N3 HPAI outbreaks in poultry in Mexico during 2012 [134]. In 2013, during an outbreak of H7N7 HPAIV among poultry in Italy, three poultry workers were identified with conjunctivitis without respiratory symptoms [163]. H7N7 HPAIV was detected in conjunctival swabs from all three individuals.

Serological diagnosis of acute H7 HPAIV infection appears to be limited by the lack of detectable antibody response to this subtype after local infection (conjunctivitis). For example, no H7 antibody could be detected by HI or MN assays using serum collected more than 21 days after illness in both confirmed H7N3 cases in Canada [198]. In the Netherlands, one household cohort study of 62 non-poultry-exposed family members of 25 poultry worker index H7N7 cases found that eight reported conjunctivitis or ILI, and four out of five had detectable HI antibodies to H7 virus. However, this study considered a positive H7 HI antibody titer to be $\geq$ 1:10, and 33 of 56 participants had detectable H7 antibodies, although most had no health complaints, and none of the participants had evidence of neutralizing antibodies to H7N7 virus when tested by means of the MN assay [68]. Since most H7 HPAIV infections have been clinically mild, resulting in conjunctivitis or ILI, it is likely that H7 HPAIV infections have been under-detected, even during recognized H7 HPAI poultry outbreaks.

H7NX HPAIVs are quickly stamped out in birds by agricultural authorities after detection in most countries, but the continuing circulation of H7N3 HPAIVs in Mexico is an indication that the necessary resources are not available everywhere. Like their LPAIV counterparts, H7NX HPAIVs pose a potential pandemic threat for two main reasons. First, H7 LPAIVs are widespread in nature and can become highly pathogenic during replication and passage in poultry. Second, some H7 viruses are able to bind to human-like receptors and spread to contact ferrets in transmission experiments [13].

Human infections with HPAI H5N1 and H5N6 viruses

Although an H5N1 HPAIV was first isolated from poultry in 1959, human infections with this influenza subtype were not recognized until 1997, when human H5N1 HPAI cases were detected in association with poultry die-offs in live poultry markets in Hong Kong [40]. During the 1997 Hong Kong outbreak, 18 human cases with 6 deaths were identified. No additional human cases were reported until early in 2003, when two H5N1 HPAI cases were reported in Hong Kong residents who had traveled to Fujian Province, Southern China [159]. By mid to late 2003, H5N1 HPAIVs had spread in birds from Southern China to South-East Asia, and caused widespread poultry outbreaks in several Asian countries, with associated human H5N1 cases [217, 229]. Since late 2005, H5N1 HPAIVs have spread among wild birds and poultry to Europe, the Middle East, and Africa. Die-offs of multiple wild bird species and poultry were detected in more than 60 countries by 2007. Between 1997 and 2014, nearly 700 human cases of H5N1 HPAIV infection were reported in the following 16 countries and regions (arranged in chronological order): Hong Kong SAR, China; Vietnam; Thailand; Cambodia; Indonesia; People's Republic of China; Turkey; Iraq; Azerbaijan; Egypt; Djibouti; Nigeria; Lao PDR; Pakistan; Myanmar; and Canada (Figure 5.1). Only one imported case has been reported in North America, in a traveler who visited China, returned to Canada in

late December 2013, and died in January 2014 after being hospitalized. H5N1 HPAIV was isolated from a bronchoalveolar lavage specimen from this patient [154]. Most human H5N1 cases to date have experienced severe disease, with a case fatality rate of approximately 60%.

Although the median age was 9.5 years in the 1997 Hong Kong outbreak, human H5N1 cases have occurred across a wide age range (1–60 years), and nearly all cases were previously healthy. Among the 18 Hong Kong cases, 6 fatal cases occurred, including 2 children and 4 adults. The most significant risk factor for H5N1 HPAIV infection was visiting a live poultry market in the week prior to illness onset [140]. No further human H5N1 cases were identified after the Hong Kong government implemented a widespread cull of approximately 1.4 million poultry, temporarily stopped importation of poultry from mainland China, and enacted measures to improve biosecurity in the live poultry markets [40]. An epidemiological study conducted among healthcare workers who cared for H5N1 patients identified two individuals who had a fourfold rise in H5N1 HPAIV-neutralizing antibodies in paired sera, suggesting that nosocomial transmission of H5N1 HPAIV had occurred [22]. A seroepidemiological study of poultry workers and cullers reported an estimated seroprevalence of H5N1 HPAIV-neutralizing antibodies of 10% among 1525 participants, suggesting that asymptomatic and mild H5N1 LPAIV or HPAIV infections had also occurred following exposure to H5N1 HPAIV-infected poultry in Hong Kong [18].

In February 2003, two Hong Kong residents, a 33-year-old man and his 9-year-old son, were hospitalized and H5N1 HPAIVs were isolated from respiratory specimens from both cases [159]. These two confirmed H5N1 cases occurred among five family members who traveled in late January 2003 from Hong Kong to Fujian Province, China. During their visit, the 7-year-old daughter of the adult case developed pneumonia and died, but no laboratory testing was performed. The remaining four surviving family members subsequently returned to Hong Kong, where the father and son became ill and were hospitalized. The father developed severe pulmonary disease and acute respiratory distress syndrome (ARDS), and died, while the son survived [159].

Widespread outbreaks of H5N1 HPAI among domestic poultry occurred in Vietnam and Thailand in late 2003 and early 2004, and were associated with human H5N1 cases in these countries in what has been referred to as the "first wave" (from November 2003 to March 2004), with 68% mortality. During this period, Vietnam reported 22 cases with 15 deaths, and Thailand reported 12 cases with 8 deaths. It should be noted that a fatal H5N1 case in a 24-year-old man who was initially suspected to be a SARS case, and who died of respiratory failure in November 2003 in Beijing, China, was retrospectively confirmed as H5N1 HPAIV infection [261]. The "second wave" of human H5N1 cases in Vietnam (4 cases with 4 deaths) and Thailand (5 cases with 4 deaths) occurred between August and October 2004, and was associated with poultry outbreaks. The "third wave" of human H5N1 cases began in December 2004 and lasted until mid-2005, with most cases reported in Vietnam and Cambodia. It appears that a "fourth wave" of H5N1 cases began in June and July 2005, with the first H5N1 cases identified in Indonesia, and H5N1 cases identified in new countries associated with the spread of clade 2 H5N1 HPAIVs among birds from Asia to Eastern Europe (Azerbaijan and Turkey), the Middle East (Iraq and Egypt), and Africa during the second half of 2005 and throughout 2006. In 2007, the first human H5N1 cases in Nigeria and Laos were reported, and cases continued to occur in Egypt and Indonesia [229].

In 2011, the United Nations Food and Agriculture Organization (FAO) declared six countries to be endemic for H5N1 HPAIVs circulating among poultry, namely Bangladesh, China, Egypt, India, Indonesia, and Vietnam [71]. Periodic outbreaks of H5N1 HPAI among poultry also occur in adjacent countries, such as Cambodia and Laos, with sporadic transmission to humans. Since 2007, the annual numbers of human cases of H5N1 HPAIV infection have generally declined, but sporadic cases have continued to occur in Bangladesh, Cambodia, China, Egypt, Indonesia, and Vietnam. Human cases of H5N1 HPAIV infection exhibit seasonality, with peaks occurring during the cooler and less humid winter months, when there are also seasonal increases in H5N1 HPAI outbreaks in poultry [136] (Figure 5.2).

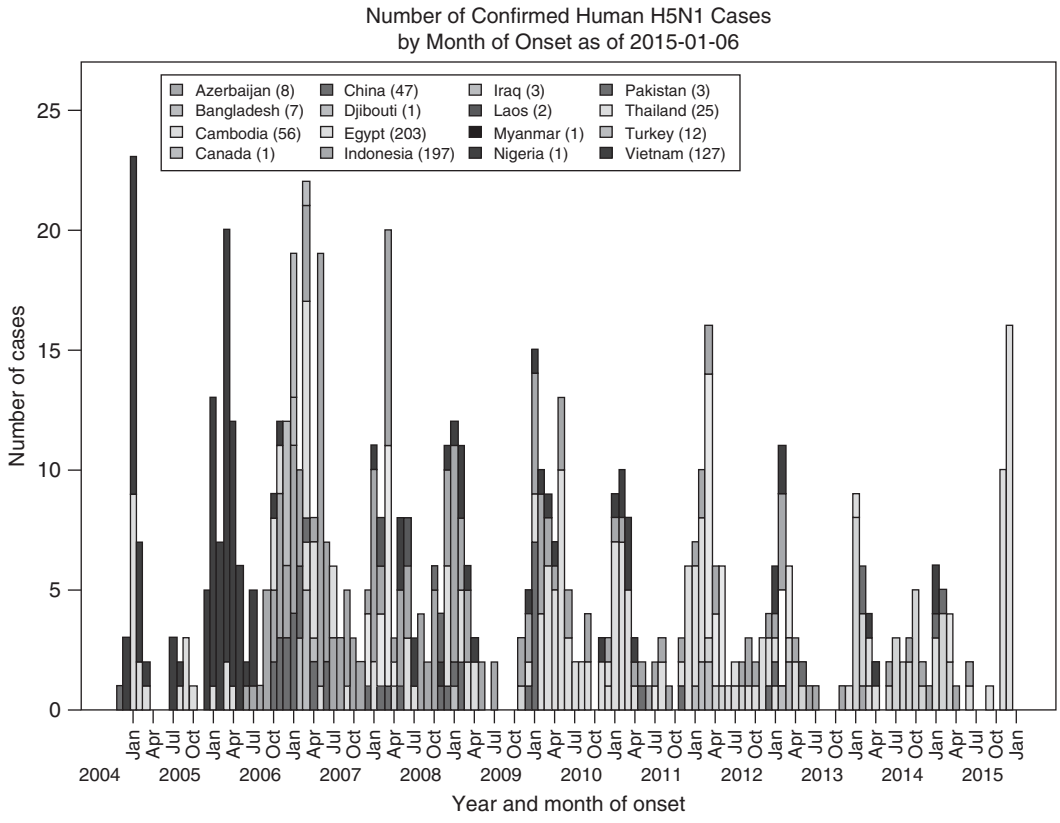

Figure 5.2 Epidemic curve of human H5N1 HPAI cases, by onset date and country, from November 2003 to December 2014. Source: Centers for Disease Control and Prevention (CDC).

The descriptive epidemiology of H5N1 cases since 2003 indicates that children and young adults have been disproportionately affected. The median age of 617 human cases of H5N1 HPAIV infection reported up to April 2013 was 18 years, the same as an earlier analysis of 256 cases from 10 countries [156, 236]. Most cases (89%) were younger than 40 years. There were no statistically significant differences by gender across age groups. The overall mortality was approximately 60%, with the case fatality rate being highest in cases aged 10–19 years (76%) and lowest in cases aged 50 years or over (40%). For fatal cases, the median duration from illness onset to death was 9 days (range 2–31 days). In Indonesia, the median age of 54 H5N1 cases was 18.5 years (range 18 months to 45 years); of these cases, 96.3% were younger than 40 years, 53% were younger than 20 years, and 24% were children under 10 years of age [178]. The overall mortality in these 54 Indonesian H5N1 cases was 76%, and mortality was higher in female cases than in male cases.

Three analytical studies have confirmed the observation from case investigations that direct contact with sick or dead poultry is the primary risk factor for infection, and that H5N1 is primarily a zoonotic disease [6, 66, 257]. One case–control study in China also reported that other independent risk factors for H5N1 HPAIV infection included indirect exposure to sick or dead poultry, and visiting a live poultry market [257]. Many cases have had direct contact with sick or dead backyard poultry, primarily chickens. One observational study in Azerbaijan attributed transmission of H5N1 virus to direct contact with dead wild swans [82]. An observational study in China found that six H5N1 cases in urban areas had no known contact with sick or dead poultry, but had visited a live poultry market prior to illness onset, suggesting that environmental exposures associated with visiting

live poultry markets may be risk factors for H5N1 HPAIV infection [251]. For example, fomite contact or inhalation of aerosolized fecal matter or material on poultry feathers contaminated with H5N1 HPAIVs could occur during visits to live poultry markets. A study of 54 H5N1 cases in Indonesia reported that a source of infection or exposure to H5N1 HPAIV could not be identified for 24% of cases [178].

Consumption of uncooked coagulated duck blood or undercooked poultry has also been implicated as a possible route of infection by H5N1 HPAIV [12]. Contact with fertilizer containing fresh poultry feces, surfaces contaminated with poultry or other animal feces, and self-inoculation of the respiratory tract are also plausible transmission risks. Although H5N1 HPAIV infections of many non-avian species have been documented, including pigs [44, 125], dogs [189, 191], cats [120, 124, 169, 188, 248], stone martens [224], Owsten's civets [171], tigers, and leopards [111], no human H5N1 cases have been linked to exposure to these mammalian species. Drinking, bathing, or swimming in H5N1 HPAIV-contaminated water is likely to pose low but unknown risks of H5N1 virus transmission to humans. The role of multiple exposures or dose response in transmission of H5N1 HPAIVs to humans is also unknown.

Limited, non-sustained human-to-human transmission of H5N1 HPAIVs has been observed rarely, or could not be excluded in some cases in which very close, prolonged contact occurred with a severely ill case at home or in hospital. This has occurred primarily, but not exclusively, among blood-related family members. A seroepidemiological study of healthcare workers in Hong Kong identified two individuals who had contact with H5N1 patients, but denied contact with poultry, and had serological evidence of H5N1 virus infection in 1997 [22]. Nosocomial transmission of H5N1 HPAIV from an H5N1 case to a nurse was reported in Vietnam [12]. Limited, non-sustained human-to-human transmission of H5N1 HPAIV could not be excluded in at least two clusters in Indonesia in 2005 [107]. Probable nosocomial transmission of H5N1 HPAIV from an 11-year-old girl to her 26-year-old mother and 32-year-old aunt probably occurred through very close unprotected bedside contact while the girl was severely ill [200]. Limited, non-sustained human-to-human-to-human H5N1 HPAIV transmission is also believed to have taken place in a family cluster of eight H5N1 HPAI cases with seven deaths in North Sumatra, Indonesia, during 2006 [28]. Transmission of H5N1 HPAIV is believed to have occurred from the index case to six blood-related family members through very close unprotected contact at the case's home while she was ill, and with subsequent transmission from one case to his son during very close unprotected contact in a hospital. Limited, non-sustained nosocomial transmission of H5N1 HPAIV from a severely ill patient to his son probably occurred through close unprotected exposure in China in 2007 [213]. Seroepidemiological studies conducted among healthcare workers exposed to H5N1 patients during 2004 reported no evidence of patient-to-healthcare worker transmission of H5N1 HPAIV [4, 129, 176].

Sporadic clusters of human H5N1 cases with at least two epidemiologically linked confirmed cases have been identified in several countries, with clusters accounting for 20–25% of H5N1 HPAI cases in some instances. The earliest evidence of H5N1 case clusters occurred in Hong Kong during the 1997 outbreak, when two pediatric H5N1 cases were identified among first cousins who played together but did not live in the same household [40]. The next cluster was among family members who had traveled to Fujian Province, China, in 2003, in which two confirmed H5N1 cases and one probable H5N1 case were identified [159]. The majority of H5N1 cluster cases to date are believed to have resulted from avian-to-human transmission after common types of exposure (e.g. to sick or dead poultry or to dead wild birds) [82, 107, 150]. Although most clusters have involved two or three cases, the largest cluster to date was identified in Indonesia with eight cases (seven confirmed and one probable) and seven deaths [28].

More than 90% of H5N1 cluster cases have occurred among blood-related family members, suggesting possible genetic susceptibility, although exposure, age, immunologic, or other factors may influence susceptibility to H5N1 HPAIV infection [105]. It is highly likely that the incidence and size of some clusters have been under-detected because specimens were not available for H5N1 testing from some individuals who were classified as probable H5N1 cases [178]. Clusters are significant

because the first signs that H5N1 HPAIV strains have changed to transmit more easily among people might be an increase in the size of family clusters, an increase in the frequency of clusters, or an increase in cases among close non-blood-related family members. Understanding of the epidemiology, clinical characteristics, and virological findings in such case clusters is critical to facilitating a rapid response.

Data from seroprevalence studies conducted since 1997 to assess the risk of human infection with H5N1 HPAIVs among people exposed to poultry suggest that the risk of avian-to-human transmission is very low. A cluster serosurvey found no evidence of H5N1 virus-neutralizing antibodies among 351 participants from 93 households in a rural Cambodian village where H5N1 HPAI poultry outbreaks and a human H5N1 case had occurred [208]. The serosurvey was conducted approximately 2 months after the poultry H5N1 outbreaks had occurred and the human H5N1 case had been identified in 2005. Another serosurvey that was conducted in rural Cambodia approximately 7 weeks after two human H5N1 cases had occurred in 2006 reported that 1% (7 of 674) villagers were seropositive for H5N1 HPAIV-neutralizing antibodies [209]. All of the seropositive individuals were aged 18 years or younger. A similar study conducted among rural Cambodian villagers in 2007 reported a seroprevalence of 2.6% (18 of 700) in participants who were sampled 9 weeks after a human H5N1 case was identified [24]. In the 2006 and 2007 village serosurveys, individuals who were seropositive for H5N1 HPAIV-neutralizing antibodies were significantly more likely than matched seronegative controls to report bathing or swimming in household ponds [24, 209]. A seroepidemiological study of 901 participants from four rural Thai villages where at least one human H5N1 case was identified in 2005 found no evidence of H5N1 HPAIV-neutralizing antibodies [62]. A serosurvey of 110 poultry-market workers in Guangdong, China, found only one person with evidence of H5N1 HPAIV-neutralizing antibodies [214], and a similar study of 295 poultry workers in northern Nigeria found no evidence of H5N1 HPAIV-neutralizing antibodies [151]. A serosurvey of poultry workers at farms and live poultry markets in Bangladesh in 2009 found no evidence of H5N1 HPAIV-neutralizing antibodies in 212 participants [143]. In Egypt, a serosurvey conducted among individuals exposed to poultry reported a seroprevalence of 2% (15 of 750 participants) with H5N1-neutralizing antibodies [84]. All 15 seropositive participants raised backyard poultry. These limited-cross sectional seroprevalence studies suggest that human infection with H5N1 HPAIVs is rare even among individuals who have unprotected direct contact with sick and dead poultry. Given the likelihood that many millions of people have been in direct contact with sick and dead poultry infected with H5N1 HPAIVs in many countries, avian-to-human transmission of H5N1 HPAIVs is clearly a rare event. However, it must be noted that there is no internationally accepted standardized serological assay for detection of H5N1 HPAIV antibodies or agreement on the definition of a seropositive result, and some serosurveys have reported results for detection of H5 hemagglutinin-inhibition antibodies rather than neutralizing antibodies. Furthermore, our understanding of the natural history of the immune response is incomplete for both severe and clinically mild cases of confirmed H5N1 HPAIV infection, and not all infected individuals develop detectable antibodies. Limited data on the kinetics of the neutralizing antibody response in confirmed H5N1 cases suggest that in people with evidence of asymptomatic infection or clinically mild illness, antibody titers decline after 6–12 months, but that antibodies persist at higher titers for much longer in survivors of severe illness [115]. Therefore sampling people many months or even a year after exposure to H5N1 HPAIV and potential infection may yield an underestimate of asymptomatic infections or mild illness. Collection of serial serum specimens from surviving H5N1 cases would help to define the kinetics of the immune response to H5N1 HPAIV infection over time and interpret the results of these H5N1 HPAIV antibody seroprevalence studies.

Clinical data for patients with H5N1 HPAIV infection have been published in case reports, case series, and comprehensive reviews [201, 244]. The estimated incubation period for H5N1 cases appears to be approximately 2–5 days, and generally 1 week or less following exposure to sick or dead poultry, but it may be longer in cases who visited a live poultry market [12, 45, 50, 93, 97, 150, 250]. For situations where limited, non-sustained human-to-human transmission of H5N1 HPAIV

is believed to have occurred, the estimated incubation period was 4–9 days for cases in Thailand and 4–5 days for cases in China [200, 213]. Early illness is characterized by high fever with signs and symptoms of lower respiratory tract disease, including cough, shortness of breath, dyspnea, and tachypnea, occurring within 1–4 days post onset. Other symptoms in the early stages of H5N1 disease include headache, sore throat, diarrhea, vomiting, abdominal pain, myalgia, and rhinorrhea.

Although nearly all H5N1 cases have presented to hospital with fever, pneumonia, and hypoxia, atypical presentations have also been reported, such as fever with diarrhea, nausea, and vomiting [5], and fever with diarrhea, and seizures progressing to coma with a clinical diagnosis of encephalitis [59]. The median duration from illness onset to hospital admission in 194 H5N1 cases was 4 days (range 0–18 days) [237]. Common laboratory findings at admission in H5N1 cases include leukopenia, lymphopenia, mild to moderate thrombocytopenia, and elevated transaminases [12, 201, 244], and hypoalbuminemia has been reported [105]. Chest radiographic findings in H5N1 patients include diffuse, multifocal, or patchy infiltrates, interstitial infiltrates, and multisegmental and lobular consolidation (Figures 5.3 and 5.4) [12]. Progression to bilateral pneumonia and respiratory failure requiring invasive mechanical ventilation is common. Complications in H5N1 patients include ARDS, multi-organ dysfunction with renal and cardiac disease, and disseminated intravascular coagulation (DIC) and a septic-like shock syndrome. DIC and multi-organ failure were reported in an H5N1 case in a woman in the fourth month of pregnancy in China [183].

Prolonged shedding of H5N1 HPAIV for up to 16 days in the respiratory tract has been reported, and most H5N1 patients are likely to be contagious for at least 2 weeks [12]. H5N1 viral RNA or isolation of H5N1 virus has been reported from rectal swab and diarrheal stool specimens from fatal cases [20, 59]. H5N1 HPAIV has also been isolated from cerebrospinal fluid [5], serum [5, 183], and plasma [46] from critically ill patients, indicating that viremia occurs in the late stages of H5N1 disease, and that this may contribute to the pathogenesis of H5N1 HPAIV infection. One autopsy study reported finding viral mRNA in intestinal tissue, suggesting that H5N1 HPAI viral replication may be occurring in the gastrointestinal tract [199]. Further studies are needed to understand the significance of detection of H5N1 HPAIV in patients with diarrhea, and to

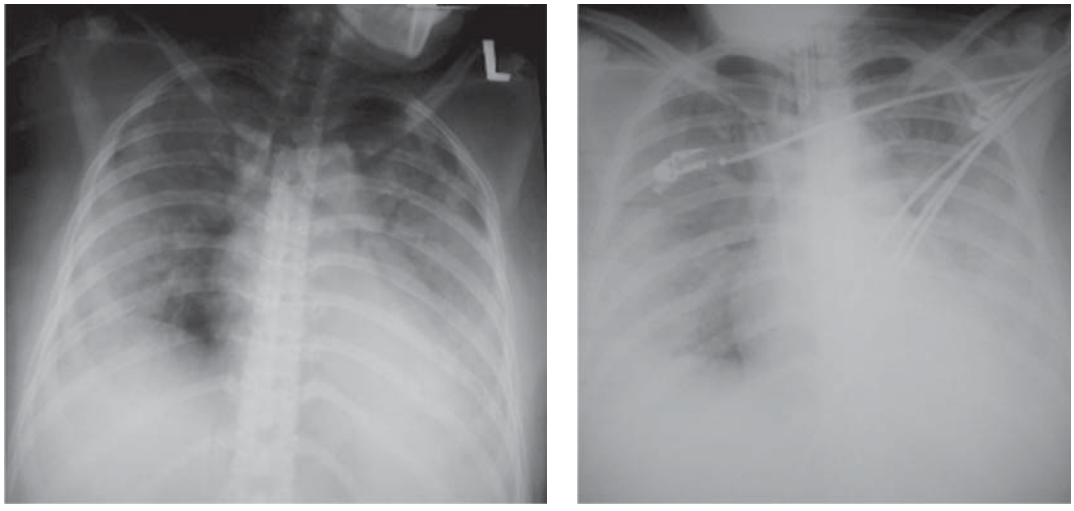

Figure 5.3 Chest radiographic findings in a fatal case of clade 2.1 H5N1 HPAI virus infection in a 37-year-old woman. Bilateral lower lobe consolidation with patchy infiltrates in the upper lung fields were evident at admission on day 7 of the illness. Despite mechanical ventilation, the patient progressed to acute respiratory distress syndrome (ARDS) on day 10, and died on day 11. Source: T. Uyeki, Centers for Disease Control and Prevention (CDC).

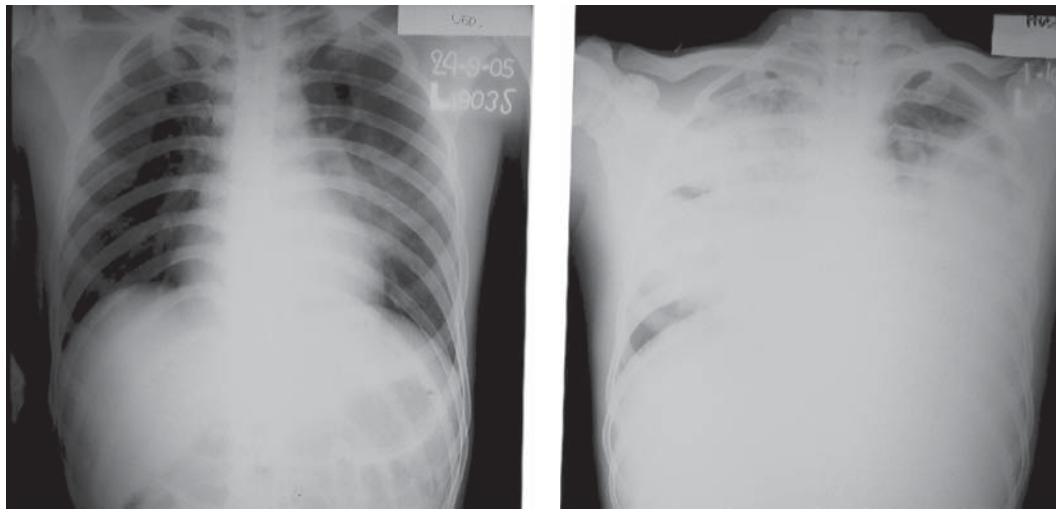

Figure 5.4 Chest radiographic findings in the case of a clade 2.1 H5N1 HPAI virus infection of a 21-year-old man who survived. Infiltrates are present in the left mid-lung field at admission on day 5 of the illness. One week later, consolidation and diffuse infiltrates are present throughout all of the lung fields. The patient made a full recovery without mechanical ventilation. Source: T. Uyeki, Centers for Disease Control and Prevention (CDC).

elucidate the role of the gastrointestinal tract in H5N1 HPAIV pathogenesis.

Although most H5N1 cases have had severe disease, some clinically mild cases have been reported among children. In the 1997 Hong Kong outbreak, seven of 11 confirmed pediatric H5N1 cases had mild uncomplicated influenza, while four were severely ill and two died [40]. The extent and frequency of clinically mild and asymptomatic H5N1 cases are not known, primarily because surveillance has not focused upon people with mild illness. At least four clinically mild H5N1 patients have been identified during field investigations of more severe index cases in Turkey and Indonesia [105, 150]. H5N1 HPAIV infection has been identified in a small number of pediatric patients presenting with ILI through active influenza surveillance in Dhaka, Bangladesh [19, 99]. However, limited cross-sectional serosurveys suggest that mild illness or asymptomatic H5N1 HPAIV infection is uncommon [24, 62, 84, 128, 143, 208, 209, 214]. Studies are needed to investigate whether genetic or other factors, such as those influencing expression of the host inflammatory response, might influence disease severity following H5N1 HPAIV infection.

The WHO has published guidance for investigations of suspected human H5N1 cases [240]. Case finding in most countries has focused on hospitalized patients with severe respiratory disease who had a history of poultry contact. Collection of the appropriate respiratory specimens from suspected cases is critical, because throat swabs have been shown to have a higher yield for detection of H5N1 HPAIV than nasopharyngeal or nasal swabs. Lower respiratory tract specimens have a higher viral load than nasal or throat swabs, and are best for H5N1 diagnosis [49]. Collection of serial respiratory specimens from multiple sites on multiple days from patients with suspected H5N1 will increase the chances of detecting H5N1 HPAIV. Guidance on collection, transportation, and shipping of clinical specimens is available elsewhere [227], and diagnosis of H5N1 HPAIV infections is described in the Diagnosis section. Case definitions for classification of H5N1 cases are available, and the WHO requests that both probable and laboratory-confirmed H5N1 cases be reported [239]. Confirmed H5N1 human cases must be reported to the WHO within 24 hours of diagnosis under the International Health Regulations [238].

Clinical management of suspected and confirmed H5N1 patients should focus on supportive medical care and prompt initiation of antiviral treatment for the patient, and implementation of appropriate infection control procedures. Patients should be isolated immediately and placed in a

separate room. Infection control measures should be implemented promptly, including standard, contact, and droplet precautions. This updated WHO guidance is based upon current understanding that human-to-human transmission of H5N1 HPAIVs most probably occurs via large droplets, and human-to-human transmission has remained a very rare event. Airborne precautions should be followed for aerosol-generating procedures. Personal protective equipment, including disposable gown, gloves, surgical mask, fit-tested N95 or equivalent respirator for aerosol-generating procedures, and eye goggles, should be worn by all healthcare workers and visitors in contact with suspected or confirmed H5N1 patients. The WHO has published updated infection control guidelines [226]. In the USA, it is recommended that all healthcare personnel, in addition to following prevention strategies for seasonal influenza, wear a fit-tested N95 or equivalent respirator and eye protection when providing care for a suspected or confirmed H5N1 patient, whether the procedure is aerosol-generating or not [32, 37]. All respiratory secretions and bodily fluids, including blood and feces of patients with H5N1 HPAIV infection, should be considered potentially infectious.

Since most H5N1 patients have been admitted late in their illness with severe disease, most commonly with pneumonia, supplemental oxygen should be administered along with other supportive measures, such as appropriate fluid management and invasive mechanical ventilation for respiratory failure. Although there are no data from controlled clinical trials, antiviral treatment with oseltamivir is recommended for all cases, and treatment should be initiated as soon as possible [177]. Observational data suggest that oseltamivir treatment can reduce mortality in H5N1 patients when started within 2 days after illness onset, and that starting oseltamivir up to 6–8 days after illness onset can still provide survival benefit [2, 106]. The optimal dose and duration of oseltamivir treatment are not known, and higher doses and longer duration can be considered [223, 236]. One randomized controlled study of double-dose versus standard-dose oseltamivir treatment of 326 hospitalized patients with influenza, including 17 patients with H5N1 HPAIV infection, found no benefit of oseltamivir treatment with a higher dose [190]. Combination treatment with oseltamivir and amantadine can be considered in countries with known or likely amantadine-sensitive H5N1 HPAIVs. Resistance to amantadine and rimantadine has been reported for clade 1 and 2.1 H5N1 viruses. Oseltamivir-resistant H5N1 HPAIVs have been documented in case reports [61, 122]. Use of intravenous neuraminidase inhibitors such as peramivir or zanamivir can be considered. Corticosteroids are not recommended, except for persistent refractory septic shock with suspected adrenal insufficiency [177]. Antibiotic chemoprophylaxis is not recommended, and antibiotic treatment should follow evidence-based guidelines for community-acquired pneumonia, and be guided by microbiological laboratory testing results [177]. A small number of H5N1 patients have received immunotherapy with convalescent plasma collected from a recovered patient or from a participant in an H5N1 vaccine trial [213, 223, 251, 255].

The pathogenesis of H5N1 HPAIV infection appears to be driven by high viral replication and an abnormal host inflammatory response. H5N1 HPAIVs bind preferentially to cells bearing receptors with sialic acid bound to galactose by α-2,3 linkages (SA α-2,3 Gal) that are found predominantly in the lower respiratory tract of humans on bronchiolar and alveolar cells [181, 205]. This may explain why most H5N1 patients develop signs and symptoms of lower respiratory tract disease, and why nearly all H5N1 patients develop severe pulmonary disease. The H5N1 HPAI viral load is higher in lower respiratory tract specimens than in upper respiratory tract specimens [60]. However, H5N1 HPAIV has been isolated from upper respiratory tract specimens from some cases, usually in the late stages of illness, and H5N1 HPAIV has also been shown to infect upper respiratory tract tissue [147]. One observational study found that high pharyngeal H5N1 HPAI viral load was correlated with hypercytokinemia with pro-inflammatory cytokines and chemokines in fatal H5N1 cases [60]. High plasma levels of IL-6, IL-8, IL-10, and γ interferon were found in fatal H5N1 cases compared with non-fatal cases or human influenza patients [60]. This study suggested that high replication of H5N1 HPAIVs may trigger cytokine dysregulation, and that early antiviral treatment may be essential for prevention of hypercytokinemia. Extrapulmonary H5N1 HPAI viral dissemination into the gastrointestinal tract [20, 59], cerebrospinal fluid [5], and blood

[5, 46, 183] has been documented, and may be a factor in multi-organ dysfunction. Most of the pathogenesis with H5N1 HPAIV infection appears to be due to viral damage to cells and/or a virus-induced abnormal host inflammatory response. Hemophagocytosis has been reported as a complication in some H5N1 patients, and may also be a result of hypercytokinemia [195]. The etiology of the marked lymphopenia observed in most H5N1 cases is not completely understood, but could involve differential apoptosis induced by H5N1 HPAIV. Further understanding of the pathogenesis of H5N1 HPAIV infection may facilitate the development of targeted therapies.

In 2014, the first human case of H5N6 HPAIV infection was reported in a patient who died of severe pneumonia in Sichuan Province, China [232]. H5N6 HPAIV was detected in a respiratory tract specimen from this patient. Since H5N6 HPAIV has been detected in poultry in China, Vietnam, and Lao PDR, there is potential for further cases of H5N6 HPAIV transmission to humans who are exposed to it.

As mentioned earlier, H5N1 HPAIVs are enzootic in birds in at least six countries, with viruses of certain clades of H5 HA being spread by wild birds. In addition, H5N1 viruses have undergone multiple genetic reassortment events in bird populations, resulting in spread of H5N8 HPAIVs to Japan, Korea, several countries in Europe, and to North America from 2013 to 2014. In North America these H5N8 viruses have undergone reassortment with a North American lineage virus bearing an N2 neuraminidase gene, resulting in an H5N2 HPAIV being detected in birds in this region. Although no human H5N2 or H5N8 HPAI cases have yet been detected, the ability of H5N1 HPAIVs to reassort with other AIVs in birds, and to be spread over vast distances by migrating wild birds, highlights the challenges of H5 HPAI control in avian species, which is necessary in order to decrease human exposure and reduce the pandemic risk of these viruses.

Recommended measures for responders to LPAI and HPAI outbreaks

People involved in culling and disinfection activities and poultry workers involved in responding to suspected outbreaks of LPAI or HPAI in poultry should be equipped with appropriate personal protective equipment (PPE) and educated about the signs and symptoms of AIV infection in poultry and in humans. They should also be aware of biosecurity and infection control measures, including being outfitted with PPE (goggles, disposable protective clothing and gloves, disposable fit-tested N95 respirator or equivalent, and boots that can be disinfected), and observe proper PPE donning and removal, disinfection, and hand hygiene.

Antiviral chemoprophylaxis with prescribed oseltamivir may be recommended up to 10 days after the last known exposure to poultry infected with LPAIVs or HPAIVs. Public health or medical personnel should be responsible for active daily monitoring of workers for compliance and adverse events associated with oseltamivir chemoprophylaxis, and for any signs and symptoms of AIV infection, including ILI and conjunctivitis with LPAIVs. Monitoring should occur up to 10 days after the last known exposure to infected poultry. Self-monitoring by workers can be used if resources do not permit active monitoring of all exposed workers by designated public health staff. Public health officials should be informed of any illness, and appropriate clinical specimens (conjunctival, nasal, and throat specimens) collected for rRT-PCR testing for seasonal influenza and suspected AIV subtypes at a qualified laboratory. Paired acute and convalescent sera can also be collected for serological testing. Responders should receive human influenza vaccine annually to decrease the risk of co-infection and possible reassortment with human IAVs and AIVs. Interim guidance for responders is available [32, 65, 152, 242].

Human infections by influenza A variant viruses

Human infections by viruses that normally circulate in swine populations were first detected by virus isolation during the 1970s, with the most famous of these resulting in the 1976 USA swine influenza vaccination campaign, during which about 47 million people were vaccinated [179]. Unfortunately, a significant increase in the number of cases of Guillain–Barré syndrome associated with vaccination necessitated cessation of the campaign.

During the next three decades only one or two sporadic H1N1v human cases were detected annually [118]. However, after the emergence of the swine-origin H1N1 virus in Mexico that caused the 2009 pandemic, there was both a great improvement in the ability of many laboratories worldwide to use molecular methods to detect human cases caused by animal IAVs, and a much greater interest in the early detection and reporting of swine-origin influenza virus infections in humans. As a result, a dozen cases of swine-origin H3N2 virus infections in humans in the USA were detected and reported during 2011, and over 300 cases were reported the following year, further increasing interest in the detection of such cases.

Due to concerns about the previously utilized nomenclature for these viruses, members of the World Health Organization's Global Influenza Surveillance and Response System (GISRS), the Food and Agriculture Organization of the United Nations (FAO), and the World Organisation for Animal Health (OIE) agreed during late 2011 and early 2012 to use standardized terminology when reporting on viruses of swine origin that have infected humans. Initially, the new terminology was adopted to describe swine-origin H3N2 viruses that have infected humans, but this terminology was later applied to swine-origin influenza A H1N1 and H1N2 viruses isolated from humans [235]. Thus when an influenza virus known to be circulating in pigs is recovered from an ill person and is different from those currently circulating in humans, the virus is referred to as a variant influenza virus. Another notation for variant is the use of a lower-case letter "v" after the virus designation (e.g. H3N2v), where "v" indicates "variant." This terminology is useful as IAVs circulating in swine are sometimes easily transmitted to humans who are in close contact with infected pigs. In addition, human influenza A viruses are transmitted to swine and can subsequently become established in and evolve separately in this species [206]. Thus, unlike the situation with AIVs, which are transmitted only from birds to humans, a "two-way street" exists for influenza A viruses circulating in humans and swine, with transmission in both directions. The most dramatic illustration of this bidirectional transmission occurred in 2009, when an H1N1 virus that is believed to have originated in swine caused a global human influenza pandemic that began in Mexico. This H1N1 pandemic virus was subsequently transmitted by humans back to pigs in many parts of the world, and is now endemic in pig populations globally. The distribution of these viruses in swine is discussed in greater detail in Chapter 18 of this book, and is reviewed elsewhere [206, 260].

Human infections with H3N2 variant viruses

Human H3N2 viruses were first isolated from pigs in Taiwan soon after the 1968 pandemic, followed by isolation in Hong Kong between 1976 and 1982, and in Italy in 1977 and 1983 [23, 260]. Subsequent isolates of influenza H3N2 viruses from swine in Italy during the 1985–1989 period possessed human-like HA and NA genes, while the internal genes were of avian origin, demonstrating that human H3N2 viruses that had previously been transmitted to swine had subsequently reassorted with an AIV during co-circulation in pigs [23]. However, it was not until the late 1990s that H3N2 viruses became successfully established in swine herds in North America [258]. Evidence now suggests that there have been distinct temporal introductions of human H3N2 viruses into pigs in North America, with subsequent reassortment events that resulted in incorporation of the HA and NA genes of the human influenza virus into the backbone of triple reassortant viruses that were circulating in pigs. Characteristics of swine viruses with the triple reassortant internal gene (TRIG) cassette are discussed more fully in Chapter 18 of this book.

The first reported isolation of H3N2v viruses from humans occurred in two children in the Netherlands in 1993 [48]. The children lived in different parts of the country, had no epidemiological link, and both had a mild illness and fully recovered. A similar H3N2v virus was isolated from a 10-month-old child who was experiencing mild respiratory illness in Hong Kong in 1999 [88]. The virus isolate from this child was phylogenetically similar to the H3N2 viruses isolated from the two children in the Netherlands, and to H3N2 viruses circulating in European swine [88].

An H3N2v virus was isolated in 2005 from a nasal swab collected from a Canadian farm worker with ILI onset 3 days after noting similar illnesses in the

pigs under his care [149]. The worker was treated with antivirals and recovered. A similar H3N2v virus was isolated during the following year in Canada from a hospitalized 7-month-old child who lived on a communal farm where pigs were raised [172]. Although there was serological evidence of limited community and household transmission and infection among swine on the farm, virus was not recovered from other individuals on the farm or from the pigs.

In 2009 and 2010, seven human infections with H3N2v viruses were reported in the USA, with two cases having clinical onset in 2009. All seven cases fully recovered [51, 182]. An investigation suggested that within-household transmission may have occurred in one 2010 case. Viruses isolated from each of these seven cases were characterized as triple reassortant or TRIG viruses with HAs that were phylogenetically most closely related to HA genes of human IAVs that circulated during the early 1990s [182]. Before the onset of clinical illness, six of the seven cases had had direct or indirect contact with swine in settings that included live markets, swine exhibitions, and farms. Pigs were reported to appear ill in only one of these instances. Sampling of pigs at fairs in Ohio in an overlapping time period during the 2009–2011 exhibition seasons indicated that H3N2 viruses isolated from swine in 2010 and 2011 were antigenically similar to the H3N2v viruses isolated from humans during 2009 and 2010 [73].

An additional 12 H3N2v cases were reported during 2011 from five states in the USA, with July being the earliest onset date [34]. Eleven of these cases were less than 10 years of age, and all fully recovered. Virus isolates from these cases were distinct from previously analyzed H3N2v viruses in that their M genes were derived from the 2009 pandemic H1N1 virus, whereas the other seven gene segments originated from the TRIG IAVs from pigs [131] (Table 5.4). Possible limited human-to-human transmission may have occurred in two households and three cases in children [34, 243]. Subsequent studies evaluating cross-reactive antibodies to an H3N2v virus with the pandemic matrix gene were conducted in Norway, Canada, and the USA [25, 184, 185, 210]. These studies used HI and MN assays, and revealed that little or no cross-reactive antibody to H3N2v viruses could be detected among children under 10 years of age, indicating that this group was at highest risk for infection. In contrast, these studies showed that 35–70% of those aged 20–40 years had antibody titers considered protective against this virus, probably greatly limiting infections in this age group.

During the following year, the US Centers for Disease Control and Prevention received reports of 309 cases of H3N2v from 10 US states [26]. Of these, 306 reported clinical onset between 9 July and 7 September 2012 [102], and most cases reported direct or indirect exposure to swine at agricultural exhibits, such as county and state fairs, prior to onset, with reported cases occurring mainly in Ohio (n = 107) and Indiana (n = 138). Cases typically presented with signs and symptoms of human seasonal influenza, such as fever, cough, and fatigue, and most recovered uneventfully, but 16 cases required hospitalization. Ohio reported 11 of these 16 hospitalizations, including one death [31]. The median age of these 306 cases was 7 years; 283 individuals (92.5%) were less than 18 years old, and 50% were aged 5–11 years [102]. Over 90% of the cases reported direct or indirect exposure to swine within 4 days of illness onset, with examples of indirect exposure including attending an event where swine were being exhibited, or being within 2 meters of a pig without touching it or having other direct swine contact. Evidence suggested limited person-to-person transmission in 15 instances [102].

Concurrent with these H3N2v outbreaks in people, respiratory sampling of swine at some of the implicated fairs was also taking place. In 2012, at one fair in Indiana, four H3N2v cases were identified and 12 pigs were sampled; all of the sampled pigs were positive for H3N2 virus [35]. Genetic analysis of the viruses recovered from people and pigs indicated that they were very closely related genetically and that they contained the pandemic H1N1 matrix gene (Table 5.4), similar to the H3N2v viruses isolated in 2011 [35, 131]. Further evidence for zoonotic transmission of H3N2 viruses from swine to humans was reported in Ohio, where swine exhibited at seven fairs that were associated with human cases also had H3N2 virus recovered. Gene sequences of viruses from swine and humans at these fairs had more than 99% homology [16], indicating that the viruses had been transmitted from swine to people. Virus-positive pigs were also

Table 5.4 Gene composition of influenza A H3N2v viruses, 2011–2014.

Genome	North American swine H3N2 (TRIG)[a]	North American H3N2v (2011–2013)	North American H3N2v (2013)	Iowa H3N2v (2014)	Ohio and Wisconsin H3N2v (2014)	Influenza A (H1N1) pdm09
Hemagglutinin	H	H	H	H	H	*H*
Neuraminidase	H	H	H	H	H	*E*
Matrix	C	*E*	*E*	*E*	*E*	*E*
Polymerase basic 1	A	A	*A*	*A*	A	*A*
Polymerase basic 2	H	H	H	H	H	*H*
Polymerase acidic	A	A	A	A	A	*A*
Nucleoprotein	C	C	C	*C*	C	*C*
Non-structural	C	C	C	C	C	*C*

[a]Triple reassortant.

C = classical swine H1N1, H = influenza A(H3N2) human seasonal, A = avian-origin North American lineage; E = Eurasian swine. Italicized gray-tinted boxes indicate that the genomic component is more similar to the H1N1pdm09 virus than to the TRIG.

identified at three additional fairs in Ohio, none of which had human cases associated with them [16], and in most instances H3N2 virus-positive pigs were asymptomatic [17].

In 2013 there were a total of 19 cases of H3N2v virus infection in the USA, of which 18 cases were under 18 years of age, which was consistent with previous observations [26]. An additional three cases of H3N2v virus infection were reported in the USA during 2014, all of which were children who reported direct contact with swine prior to the onset of clinical illness [15]. The various genotypes of H3N2v viruses detected between 2011 and 2014 are shown in Table 5.4.

Human infections with H1NX variant viruses

The first human case of infection with an H1N1 variant virus, which was reported in 1976, involved a 16-year-old boy who had been diagnosed with Hodgkin's disease in 1971, and died of respiratory distress in 1974 after being infected by a swine-origin influenza virus [187]. The patient lived on a farm where he helped to care for swine, and where two adult pigs had HI antibody titers of $\geq$ 1:640 to the virus isolated from the patient [187].

During January and February 1976, H1N1v viruses were isolated from five military recruits at Fort Dix, New Jersey. All of the recruits were hospitalized for acute respiratory illness, and one of them died [81]. The clinical onset of acute respiratory illness for these patients was between 26 January and 3 February [83], and laboratory analysis of isolated viruses demonstrated that they were similar to viruses that had been circulating in swine since 1937, and that they had the greatest antigenic similarity to a swine influenza virus isolated in 1975 [112]. Serological testing identified an additional 8 individuals with HI titers of $\geq$ 1:20, all of whom were hospitalized for acute respiratory disease [80]. Although laboratory testing could not confirm additional cases, an estimate that approximately 230 individuals stationed at Fort Dix might have been infected was based on clinical presentations, cohorts of military recruits who were in training at specific times, and serological testing of single sera from subsets of individuals from each cohort [94]. Responses to interviews indicated that none of the cases had been in contact with swine within 6 months prior to becoming ill, and there were no swine on the military base [80]. Additional studies indicated that there was limited spread on the training base, with no evidence for cases after 14 February, or for cases in the community [83, 94]. It is still not known how the virus was first introduced into Fort Dix and why it did not transmit more readily from person to person [197].

Two additional human cases of infection by H1N1v virus were reported in 1976 from Wisconsin. One was a 22-year-old man with onset of

mild ILI in November, and in this instance samples taken from six of eight ill pigs on the farm where he worked yielded the same influenza virus [30]. In December, the second case was identified in a 13-year-old boy who resided on a farm with pigs that were also clinically ill at the same time as respiratory illness onset in the patient [29, 67]. Although these cases did not share an epidemiological link, both had direct contact with ill swine prior to the onset of illness.

In subsequent years, sporadic human cases with H1N1v virus infections were reported. Two cases resided in Texas and occurred in 1979 and 1980, respectively. Both exhibited ILI and recovered fully. One case was a college student who had worked at a large swine exhibition shortly before the onset of illness, and the second case was a 6-year-old boy who had visited the swine exhibit at a livestock show 2 days before illness onset [54]. There was no evidence of transmission to other individuals in either of these cases. In February 1982, an H1N1 variant virus similar to the virus isolated at Fort Dix in 1976 was recovered at autopsy from a child who had died of fulminant pneumonia and with acute lymphoblastic leukemia in remission [157]. An investigation of family members and contacts of this child indicated that there was no evidence of exposure to swine, nor was there any evidence of transmission within the community [157].

During 1986, three H1N1v virus infections were reported in Europe, with one case in the Netherlands and two in Switzerland [57, 58]. All three cases recovered, and there was no evidence of transmission in humans. However, the virus appeared to be spreading rapidly among pig populations in these countries. Prior to this report, three other cases of H1N1v infection were reported from the Asian area of Russia in 1983 [47].

During September 1988, an apparently healthy 32-year-old woman who was 36 weeks pregnant became ill with respiratory symptoms 4 days after she had visited the swine exhibit at a local county fair in Wisconsin. She was hospitalized with respiratory distress, and subsequently died [137, 173]. Swine at the exhibit that she attended had reportedly exhibited ILI, but no samples were collected from the pigs at that time. An investigation of serum samples taken from those exhibiting swine at the fair indicated that additional infections may have occurred [220].

During July 1991, a previously healthy 27-year-old man was hospitalized in Maryland for respiratory distress, and an H1N1v virus was isolated from the patient's sputum 4 days after admission [222]. No other pathogens were identified, and the patient subsequently died. An investigation of this case revealed that the patient had been in close physical contact with ill swine in a research setting prior to the onset of illness, but there was no evidence of person-to-person spread. In 1994, two additional laboratory workers became infected with H1N1v virus, apparently while collecting nasal swabs from pigs that had been infected experimentally with A/swine/Indiana/1726/88 [221]. There was no evidence that additional laboratory workers were infected, and both cases made a full recovery.

During the summer of 1993, a 5-year-old girl who had previously been in close contact with pigs on the farm where she lived in the Netherlands was hospitalized with pneumonia, and an H1N1v virus was isolated from a lower respiratory tract specimen taken from this patient. This H1N1v virus isolate was antigenically and genetically similar to avian-like swine IAVs that were circulating in European swine at the time [168]. This study also showed that an older H1N1v virus from a case that occurred in 1986 in the Netherlands was antigenically and genetically similar both to that from the case described above, and to viruses contemporaneously circulating in European swine [168]. In 1995, a 37-year-old woman who worked on a swine farm in Minnesota became ill with acute respiratory distress, was hospitalized, and died 3 days after hospital admission [114, 142]. An additional patient in the USA from whom an H1N1v virus was isolated in 1998 was also reported [142].

In February 2002, a 50-year-old Swiss farmer whose pigs had previously exhibited respiratory disease developed a typical influenza-like respiratory illness. An H1N1v virus most closely related to contemporary avian-like H1N1 viruses circulating in European swine was isolated from a respiratory specimen taken from this patient [87]. He made an uneventful recovery, and no evidence of human-to-human transmission was reported.

The first human case of H1N1v TRIG virus infection was reported during 2005 in a 17-year-old boy in Wisconsin who presented with symptoms of ILI [145]. Prior to illness onset the case had been in direct contact with pigs while assisting with their

processing for slaughter. The patient recovered uneventfully within 4 days, and there was no evidence of human-to-human transmission. An additional case involved the reported isolation of H1N1v from a swine farmer in Iowa in 2005 [142].

Between 2005 and 2009, 10 additional cases of H1N1v TRIG virus infections were reported in the USA [180], with two of the 2007 cases linked to a single county fair. In 2007, an outbreak of respiratory illness occurred among swine and people gathered for a county fair in Ohio, where influenza H1N1 viruses were recovered from nasal swabs from seven clinically ill swine and two ill people [113]. The two human cases (a father and daughter) had both handled exhibition pigs at the fair [113], and both recovered uneventfully. Sequence and PCR results indicated that the viruses isolated from these cases were identical TRIG viruses [113], and further comparison of these viruses indicated that they were closely related to IAVs contemporaneously circulating in the swine population [207].

An additional case of H1N1v was reported during 2008 from a 19-year-old man from South Dakota who became ill with symptoms consistent with ILI, and who recovered uneventfully [55]. Prior to the onset of illness he had participated in a judging event involving 10 swine that appeared to be clinically normal, but no samples were collected from the pigs. Two other students who were also exposed to swine at the exhibition were seropositive for H1N1v antibodies, indicating that other transmission events had probably occurred [55].

In Spain the isolation of an H1N1v virus from a 50-year-old woman who became ill in November 2008 was reported. This H1N1v virus was antigenically and genetically similar to those circulating in European swine [1]. The woman worked on a farm with swine, and although none of the pigs in her care appeared to be ill, she was in close contact with them prior to the onset of her illness.

Although relatively rare, H1N2v virus infections have also been reported, and these have been reviewed elsewhere [116]. The majority of these cases have occurred in children with a clinical presentation similar to that of human seasonal influenza. In addition, the HAs of H1N2v viruses are similar to those of human H1N1 viruses, making detection of these human infections challenging, as laboratory testing may not distinguish them from seasonal H1N1pdm09 influenza. Although single cases are very likely to be under-detected, cluster events such as an outbreak of ILI among exhibitors at swine exhibitions or similar events are more likely to be detected and further investigated, as was the case for four H1N2v infections among individuals who were in contact with swine at a Minnesota fair in 2012 [162].

Swine exhibitions create unique environments that bring together swine and young people in a casual, often hands-on interface. Recent joint animal health and public health investigations of human cases caused by variant IAVs at the animal–human interface have added to our understanding of how these infections occur, and have provided insight into practical prevention opportunities. As a result, veterinary and human health experts in the USA jointly produced a working document specifically on the prevention of human variant virus infections in swine exhibition settings [144]. Development of this collaborative document was driven by the increase in the number of H3N2v infections detected in the USA during 2012. It is hoped that guidance put forward in this and similar documents developed elsewhere will have an impact on the transmission of influenza between swine and humans in settings where they are in close contact.

Conclusions

A variety of subtypes of LPAIVs, HPAIVs, and swine IAVs have been transmitted to humans, primarily through direct or indirect contact with infected birds or swine. Transmission to humans of IAVs resident in animal populations will occur for the foreseeable future, due to current animal husbandry practices and the growing populations of people, pigs, and poultry on the planet. Therefore we must respond to the ongoing threats that these viruses present to public health. Improved virological surveillance in people, pigs, and poultry will help both to identify the emergence of influenza A virus subtypes with pandemic potential, and to facilitate the antigenic and genetic characterization of the causative viruses. Such characterization, along with antiviral resistance testing, will help to identify changes in the level of threat that these viruses pose to public health. Although gaps in

influenza surveillance are greatest in developing countries, the strengthening of surveillance and response capacity for avian and swine IAVs is needed worldwide.

Illness severity in humans has varied greatly for infections by AIVs, but it is clear that H5N1 HPAIV infections are typically more severe than those caused by other animal IAVs. For this reason, attention and pandemic preparedness efforts focused primarily on the widespread H5N1 HPAI epizootic from late 2003 until after the 2009 H1N1 pandemic, when some resources were allocated to influenza surveillance in swine, particularly in the USA and certain countries in Europe. More than a decade of heightened attention to human H5N1 cases has resulted in advances in our understanding of the epidemiology, virology, and clinical aspects of human infections with H5N1 HPAIVs. The striking lethality of H5N1 HPAIV infections for a variety of species, and the spread of the H5N1 HPAIV to multiple continents, have also provided a unique window in which to view how a pandemic virus might evolve from an AIV that transmits rarely to humans to one that might transmit more readily and might eventually cause a pandemic. Although the number of countries experiencing endemic or frequent outbreaks of H5N1 HPAI among poultry has declined since the peak in 2006–2007, human infections continue to occur in South Asia (Bangladesh and India), South-East Asia (Cambodia, Vietnam, and Indonesia), East Asia (China), and the Middle East (Egypt), posing an ongoing threat to public health as H5N1 HPAIVs continue to evolve through mutation and reassortment.

Although over the last 15 years the focus has primarily been on H5N1 HPAIVs, the public health impact of LPAIVs must not be minimized. Two influenza pandemics of the twentieth century were caused by IAVs that arose through genetic reassortment between LPAIVs and the human IAVs that were circulating in 1957 and 1968 for the Asian and Hong Kong pandemics, respectively. The importance of LPAIV infections of humans has been further highlighted by the recent ongoing H7N9 LPAI outbreak in humans in China, where most cases have had severe respiratory illness. Furthermore, the sudden emergence of this new reassortant H7N9 LPAIV that causes no apparent disease in poultry, but which causes severe disease in humans, is a reminder that we must always expect the unexpected where influenza is concerned. In addition, severe respiratory infections in humans have also been caused by H6N1 and H10N8 LPAIVs. The different levels of pathogenicity of certain LPAIV subtypes (e.g. H7N9) for birds compared with humans have puzzled researchers, but a recent study indicates that the higher virulence in humans might be attributable to a mammalian virulence factor encoded by avian hemagglutinin genes of the H1, H6, H7, and H10 subtypes [164]. Another study revealed similar transcriptomic signatures in mice infected with the 1918 virus, the H7N9 LPAIV, and the H7N7 and H5N1 HPAIVs. In this study, high levels of pathogenicity were associated with increased transcription of cytokines and decreased transcription of lipid metabolism and coagulation signaling genes [139]. Furthermore, the continuing evolution of and reassortment among and between LPAIVs and HPAIVs circulating among domestic poultry and wild birds dictates that surveillance of AIVs that infect poultry, wild birds, and humans must be further enhanced in order to identify the ongoing emergence of new AIVs that pose a pandemic threat.

The most significant scientific advances have been made in our understanding of the molecular characteristics of AIVs that infect humans. However, there are still major gaps in our understanding both of the epidemiology and clinical aspects of human infections, and of how interactions at the animal–human interface influence the risk of transmission of AIVs to humans who are in close contact with birds in a variety of settings. More research is needed to improve our understanding of human infections with AIVs, especially how H5N1 HPAI and H7N9 LPAI virus infections of the respiratory tract are initiated. Unanswered questions also remain about the immune response to human infection with AIVs, including the most appropriate serological testing methods for detecting human infections with LPAIVs and HPAIVs. Furthermore, the pathogenesis of both LPAIV and HPAIV infections of humans is not well understood. In particular, we need to determine whether there are genetic or other biological factors that can influence infection and/or disease severity following infection. Finally, since there are no proven therapies for severe disease caused by H5N1 HPAIV or H7N9 LPAIV infections, development of new

treatments is urgently needed. For example, the potential benefits of a combination of antiviral treatment and immunotherapy for patients with severe disease caused by H5N1 HPAIV and H7N9 LPAIV infections must be studied.

Although human disease caused by H1N1v and H3N2v viruses is usually less severe than that caused by AIVs, there have been severe and fatal cases, usually among individuals with underlying health conditions that put them at greater risk for complications. The fact that the 2009 pandemic was caused by a novel H1N1 IAV reassortant with all 8 gene segments from swine IAVs highlights the need to remain vigilant for human infections caused by swine-origin viruses. Importantly, human populations now have relatively high levels of antibodies to classical H1N1 and to H3N2 viruses circulating in swine, resulting in a much lower likelihood that these viruses might spread widely in humans. Nevertheless, because swine and human IAVs share host-cell-receptor specificity and are readily transmitted bidirectionally, we must expect that H3N2v, H1N1v, and H1N2v virus infections will continue to occur. It should be noted that the human population has little or no detectable antibody to the avian-like H1N1 IAVs circulating in pigs in Europe and Asia, and that these swine IAVs pose a unique threat for this reason.

Between 2000 and 2014 there was a dramatic increase both in the number of zoonotic IAV infections and in the number of different influenza A subtypes causing them, compared with previous periods [101]. These increases necessitated the development of a systematic, transparent method of evaluating the risk associated with each different virus subtype so that resources could be allocated to those with the greatest potential to cause an influenza pandemic. In response to the increase in the number of influenza A virus subtypes causing zoonotic influenza cases, an Influenza Risk Assessment Tool (IRAT) was recently developed collaboratively with input from subject matter experts in the fields of influenza epidemiology, virology, human and veterinary medicine, animal ecology, and risk assessment [53]. The IRAT has been used to guide preparation of pre-pandemic candidate vaccine viruses (CVVs), since influenza vaccines are the cornerstone of prevention and control efforts. This tool has also been used to guide decisions about which CVVs should be provided to vaccine manufacturers for production of seed lots, production of clinical trial lots, and production of pre-pandemic H5N1 and H7N9 vaccines for the USA stockpile [53]. Timely influenza risk assessment of newly emerging viruses relies on rapid sharing of viruses and data from zoonotic IAV infections along with rapid diagnostic test development and distribution so that additional human cases can be identified quickly. These measures must be complemented by studies on the antigenic and antiviral susceptibility properties of these viruses, along with their ability to cause disease and be transmitted among mammals. Knowledge about the level of immunity in humans and the geographic and species distribution in animals further enhances the information required for influenza risk assessment [53]. Clearly, a "One Health" approach is essential for assembling the information utilized by the IRAT so that work done on IAVs with pandemic potential can be prioritized appropriately.

Although pandemic preparedness is an essential public health activity, the key to reducing the public health risk and to preventing human infections with avian and swine IAVs is prevention and control of animal outbreaks through improved biosecurity, vaccination, and rapid response to outbreaks. It is also important to protect people at risk of exposure during poultry-culling operations and swine exhibits. Of note, it is especially challenging to prevent and control human infections caused by H7N9 viruses and variant viruses, because these infections in birds and pigs, respectively, are very often asymptomatic. Reducing the number of human cases requires improved collaboration, communication, and coordination between veterinary health and public health experts worldwide, by adopting a "One Health" approach. Public health and animal health authorities must also work closely during outbreak investigations and in the development and implementation of measures that will reduce human exposure to infected birds and swine. An excellent example of a multidisciplinary and inter-institution response to an avian influenza outbreak of concern to both veterinary and public health authorities occurred recently in Cambodia [170]. The establishment of similar joint activities to address questions and to fill gaps in avian and/or swine IAV surveillance in other countries is also needed. Furthermore, a long-term "One

Health" perspective will develop and strengthen global epidemiological and laboratory capacity for influenza A viruses to the benefit of both veterinary health and public health. In addition, improvements in biosecurity along with the development and exercising of national and local capacity to respond rapidly to avian and variant virus outbreaks in humans would enhance global pandemic preparedness.

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SECTION II

Influenza in animals of the class aves

6 The innate and adaptive immune response to avian influenza virus

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Overview of immunity

The major function of an immune response is to recognize and eliminate infection. The immune system of vertebrates is made up of two functional elements – the innate and the adaptive – which differ in their time of response and mechanisms of pathogen recognition [83, 84]. The early reactions of the innate immune system use germline-encoded receptors, known as pattern recognition receptors (PRRs), which recognize evolutionarily conserved molecular markers of infectious microbes, known as pathogen-associated molecular patterns (PAMPs) [40, 83, 85]. The later adaptive immune responses use highly specific antigen receptors on T- (cellular immunity) and B-lymphocytes (humoral immunity) that are generated through random processes by gene rearrangement [40, 58]. The innate immune response stimulates the adaptive immune response and influences the nature of the response. That influence is predicated on the type of cytokine response generated. A T-helper (Th) 1 response profile includes interferon (IFN)-γ, interleukin (IL)-2, IL-7, IL-12, IL-15, and IL-18, and is associated with vigorous $CD8^+$ T-cell antigen-specific responses. In contrast, a Th2 cytokine response profile includes IL-4, IL-5, and IL-10 to stimulate antigen-specific antibody production. Thus we can determine the mechanism of antigen processing based on the Th cytokine profile following infection or vaccination. Typically, vaccinating birds against avian influenza (AI) with inactivated virus drives a Th2 response, whereas a natural infection may stimulate a balanced Th1/2 response.

The innate immune response depends on factors that exist prior to the advent of infection, and that are capable of a rapid response to microbes. Innate immunity has four primary components: (i) physical and chemical barriers, such as skin, epithelia, and production of mucus; (ii) innate cells, including macrophages, heterophils (neutrophil equivalent in avian species), and natural killer (NK) cells; (iii) complement proteins and mediators of inflammation; and (iv) cytokines. In terms of viral infections, our understanding of how the innate immune system responds and initiates an appropriate response has been increased by the discovery and characterization of the Toll-like receptor (TLR) family [127]. TLRs are well recognized as PRRs that detect PAMPs. Engagement of TLRs by PAMPs on cells, such as macrophages and heterophils, drives innate immune effector function, such as production of pro-inflammatory cytokines, while stimulation of TLRs on dendritic cells induces T-cell activation cytokines (e.g. IL-12) [98, 111]. Although our knowledge of TLRs is primarily derived from mammalian systems, it is apparent that the immune system of avian species is close enough to use mammalian immunology as a model for studying avian immunology. Several TLRs have been identified that recognize viral PAMPs. These include, but are not limited to, TLR2 (cytomegalovirus proteins), TLR3 (double-stranded RNA, reovirus), TLR4 (RSV fusion-protein), TLR7 (single-stranded RNA, influenza), TLR8 (single-stranded RNA, HIV), and TLR9 (DNA, herpes simplex virus, CpG motifs) (for a review, see [19]). Homologues of the TLRs presented above have been described in avian species, with the notable exception of TLR21, which

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birds use to respond to CpG motifs (mammalian TLR9 equivalent) [21, 48, 70]. With regard to specific information on the innate immune response in birds, the preliminary phylogenetic characterization of TLR7 from different avian species was determined and compared with sequences to TLR7 from mammalian sources which have been associated with recognition of single-stranded RNA and influenza virus [62, 78, 101]. Preliminary results indicate that there is 98–99% similarity between chicken species, but only 93% and 85% similarity to turkey and duck, respectively. Differences in the ligand-binding domain were observed between chicken and duck species. Between avian and mammalian species, only 64% and 70% similarity was found between chicken and human or murine species, respectively. Whereas mammalian TLR7 and TLR8 have been reported to respond to AI virus (AIV) infection, gallinaceous bird species appear to contain an insertion element at the TLR8 genomic locus which results in a lack of TLR8 expression [101]. The relevance of genetic differences in TLR7 and the lack of TLR8, in terms of receptor binding and downstream cytokine signaling, have yet to be determined, but may play a role in innate immunity against AIV and disease resistance or susceptibility.

Recognition of PAMPs by PRRs, either alone or in heterodimerization with other PRRs (TLRs, nucleotide-binding oligomerization domain (NOD) proteins, RNA helicases, such as retinoic acid-inducible gene 1 (RIG-I) or MDA5, and C-type lectins), induces intracellular signals that are responsible for the activation of genes that encode pro-inflammatory cytokines, anti-apoptotic factors, and antimicrobial peptides [24, 72, 133].

Although a certain degree of redundancy exists between signals induced by various PRRs, in general no single PRR is likely to be the sole mediator of activation of the innate immune response. Therefore a variety of pathogens, each containing different PAMPs, can interact with a certain combination of PRRs on or in a host cell. The variety of PRR complexes triggers specific intracellular signal transduction pathways that will induce specific gene expression profiles, particularly cytokine/chemokine expression, representing the host's best attempt to control a particular pathogen [25, 39, 44, 86, 116, 130]. The identification of these signaling pathways and their resultant cytokine profiles in cells of the innate immune system following infections with AI represents one component of this proposal. Furthermore, the induction of cytokine mRNA transcripts is regulated by "molecular bridges" known as transcription factors. The activation of transcription factors, such as NF-kB, activation protein-1 (AP-1), and interferon regulatory factors (IRF) 3, 5, and 7, represents required steps in intracellular signaling that result in changes in gene expression.

Adaptive immunity, including humoral and cellular pathways, provides pathogen-specific detection and requires more time for development than the innate response. For example, infection with low pathogenic AIV (LPAIV) results in the production of virus-neutralizing immunoglobulin (Ig) Y (IgG equivalent in avian species) antibodies against the virus that block viral attachment and uncoating. However, antibody protection is only specific to a particular subtype of field virus. Humoral immunity is also affected by rapid mutation of AIV. One of the major biological results of vaccine-induced immune pressure is rapid antigenic changes and the emergence of immunological escape mutants. In practical terms this means that vaccine seed strains must be continually updated in order to maintain adequate efficacy. Current selection of seed strains relies to some extent on protein sequence analysis between the vaccine strain and target field virus. However, protein sequence and antigenic matches are not always correlated. Antigenic mapping using data obtained by antigenic cartography has been used to map human seasonal influenza and swine influenza, and in recent years has been used by the World Health Organization to help to select the seasonal influenza vaccine strains. Antigenic cartography has also been used to screen H5 and H7 poultry vaccines against outbreak viruses for potential protection or resistance [1, 126].

Cell-mediated immunity is specific immunity mediated by T-lymphocytes, and has been suggested to be an important factor in the development of protection against viral diseases in vaccinated animals. Since intracellular replication is necessary for antigen processing, the protective antigens do not have to be localized to the surface of the virus. The subsets of T-lymphocytes, namely $CD4^+$ T-helper cells and $CD8^+$ T-cytotoxic cells, constitute the principal cells of the CMI response.

A number of studies have demonstrated the importance of $CD4^+$ and $CD8^+$ T-cells against respiratory viruses, including AIV [63, 119]. In particular, $CD8^+$ T-cells contribute to protection by detecting and lysing virus-infected host cells. The benefits of a secondary cellular response have been shown to result in decreased duration and amount of viral shedding, thereby reducing transmission potential to susceptible cohorts and decreasing the severity of disease.

Innate immune responses to avian influenza virus

Many studies have compared the innate responses in whole tissues, and these findings cannot be related to a specific cell population. The gene expression profiles and protein expression during influenza infection are dependent on the tissue analyzed (lung, gut, spleen, PBMC, brain), the time point, and infection status, and profound differences have been described between virus strains within the same species. Our understanding of the mechanisms of disease development is still incomplete, despite numerous pathological and clinical descriptions. However, in general an early and substantial innate response characterized by increased expression of pro-inflammatory cytokines, interferons, chemokines, PRRs, and acute-phase proteins (APPs) in infected pigs, chickens, turkeys, and ducks is described, and the intensity and duration of the response are dependent on the susceptibility of the animal and the viral load. However, substantial differences between virus strains within a species have been described. There is growing evidence that the so-called "early cytokine" responses are the cause of many of the clinical signs. These early cytokines and chemokines are produced by non-immune and immune cells at the site of infection, and have an effect not only on the local responses but also on the systemic responses. In most species, type I IFNs (IFN-α and IFN-β), TNF-α (not present in avian species), and IL-1 are involved in the early cascade of responses, followed by IL-8 (two biologically different forms in avian species), IL-6, and IFN-γ. Whether some are more important than others has yet to be established, especially if one relies on measurement of mRNA levels, because it is not yet technically possible to measure biological activity at the protein level. Most importantly, it should be noted that these cytokines which are involved in exacerbating inflammation, cytopathic effects, and attracting immune cells to the site of infection in the respiratory tract are also involved in antiviral responses and resolution of the infection.

Interferon

The IFN response to virus infection is relatively well understood (for a review, see [42]), although new proteins involved in this pathway continue to be identified [43, 64]. The antiviral response is rapid, occurring within minutes, and is typically induced by double-stranded RNA by-products of IAV replication. Double-stranded RNA is recognized by pathogen recognition TLR3 or cytoplasmic RNA sensors, retinoic acid-inducible gene-I (RIG-I), and melanoma differentiation associated protein-5 (MDA-5), which contain N-terminal caspase recruitment domains (CARDs) and C-terminal DExD box RNA helicase domains [14]. Binding of double-stranded RNA to the helicase domain triggers a second interaction with CARD-containing protein IPS-1 (MAVS/VISA/Cardif), resulting in activation of IKK-related kinases TBK-1 and IKKe, which phosphorylate IRF3 [52]. IRF3 is one of a family of nine IRF transcription factors, and is required for IFN expression [57]. IRF3 phosphorylated at its C-terminal transactivation domain dimerizes and translocates to the nucleus, where it binds the IFN-β promoter in cooperation with transcriptional co-activators. Because chickens lack RIG-I, the level of IFN-β expressed following infection may not be sufficient to control the disease [79].

Induction of IFN transcription occurs through assembly of transcription factors NFKB, ATF2/c-Jun, and IRF3 on the positive regulatory domain enhancer element of the IFNB promoter and the interferon-stimulated response element (ISRE) in promoters of a subset of IFN-stimulated genes (ISG) [53, 67, 73]. Secreted IFNs bind to cell surface type I IFN receptors and activate the JAK/STAT pathway, resulting in the formation of IFN-stimulated gene factor-3 (ISGF3). ISGF3 is a heterotrimeric complex consisting of STAT1 (signal transducers and activators of transcription), STAT2, and IRF9 [129]. ISGF3 translocates to the

nucleus and induces transcription of IFN-α and numerous ISGs, thus amplifying the response through a positive feedback mechanism. It is these steps of IFN induction, amplification, and effector function that are frequently targets of inhibition by viral proteins. Previous research has demonstrated that AIVs are sensitive to the antiviral effects of type I IFN [11, 45, 103, 117]. Previous studies with cells and mice deficient in either RIG-I or MDA-5 suggest that only RIG-I is essential for induction of IFN in response to IAV infection [66, 76, 91, 142]. Our previous studies demonstrated that chicken cells pre-exposed to IFN reduced AI infection by more than 100-fold [60]. Thus the timing and level of expression of IFN are critical to its outcome. Because infection of chickens with highly pathogenic avian influenza virus (HPAIV) may result in death within 2–6 days, the adaptive immune response contributes little to protection from disease in unvaccinated birds. In other bird species, including ducks, the innate and adaptive immune response is able to protect the bird against disease.

RIG-I is a cytosolic RNA sensor, and triggering by nascent RNA transcripts generated during influenza virus replication leads to the production of IFN-β, and expression of downstream ISGs. Interferons initiate an antiviral program in neighboring cells, limiting the spread of the virus, and decreasing viral titers. Interference in the expression of RIG-I is a hallmark of lethal influenza infection, as demonstrated by the infection of macaques with the regenerated 1918 "Spanish influenza" strain [68]. RNA viruses are more virulent and replicate to higher levels in mice that lack RIG-I [66]. Furthermore, the complement of genes activated by RIG-I and IFN-β is not redundant, with the genes activated by other influenza receptors leading to production of IFN-α (i.e. TLR3, NRLP3).

With regard to birds, ducks have a functional RIG-I but chickens do not [10]. Ducks survive HPAIV infection due to a rapid innate immune response. In contrast, chickens die within a few days of HPAIV infection, even in the presence of an innate immune response. Humans infected with H5N1 HPAIV strains also succumb to the virus within this early time frame. RIG-I signaling leads to the production of IFN-β and downstream ISGs. This critical difference between avian species defines their differential susceptibility to influenza in the first few days post-infection, although it is probably not the only difference.

Recent studies suggest that duck RIG-I functions in chicken cells. Duck RIG-I was transfected into DF-1 cells (a spontaneously immortalized cell line derived from chicken embryonic fibroblasts), which lack RIG-I. Chickens have the (MDA5) receptor, which shares the downstream pathway with RIG-I. It was demonstrated that duck RIG-I is functional in chicken DF-1 cells and confers detection of RIG-I ligand [32]. RIG-I detection of influenza in ducks but not in chickens provides a simple explanation as to why ducks are resistant to strains that would kill chickens within a few days. Another protective mechanism was more recently noted, when it was observed that a much higher proportion of duck primary cells undergo rapid apoptotic death when infected with influenza than is the case for chicken cells [33]. These early apoptotic events may limit early replication and release of viral particles throughout the host, thereby allowing the interferon and adaptive time to prevent lethal damage from being caused by the virus. Signaling through mitochondria for induction of apoptosis may also be influenced by RIG-I.

Duck RIG-I initiates an antiviral program in transfected chicken cells. In humans and mice, RIG-I detection of intracellular accumulation of viral RNA triggers IFN-α, which up-regulates over 100 interferon-responsive genes, and initiates an antiviral program and inhibits viral replication. Duck RIG-I expressed in chicken DF-1 cells complemented the missing chicken RIG-I in the pathway. Detection of RIG-I ligand triggered IFN-α in chicken cells, and expression of interferon response genes Mx and PKR. The presence of RIG-I in transfected cells, infected with either LPAIV or HPAIV, effectively reduces the viral titer by 50% compared with vector alone [9]. This raises the question of which regulatory nodes are controlling the downstream interferon stimulated genes and, more importantly, whether any of these pathways suggest alternative therapeutic targets that can be manipulated to protect humans or animals.

Duck RIG-I is up-regulated by HPAIV but not by LPAIV. It has been demonstrated that RIG-I is up-regulated in tissues of infected ducks at 1 day post infection, which suggests that duck RIG-I is activated immediately upon influenza infection [28]. In comparison with

mock-infected animals, a recombinant version of an HPAIV derived from a fatal H5N1 HPAIV human isolate – A/Vietnam/1203/2004 (VN1203) – greatly up-regulated RIG-I gene expression, while an H5N2 environmental LPAIV – A/British Columbia/500/2005 (BC500) – did not [9]. This is despite observing a higher titer of virus in swabs from the ducks infected with LPAIV. This raises the question of how the RIG-I gene (DDX58) is regulated. It is known that RIG-I is induced by interferon, but many unanswered questions remain. The transcriptional regulation of RIG-I has not been examined in any species. It was further demonstrated that RIG-I was up-regulated 200-fold in lung tissue infected with VN1203, but less than 10-fold by infection with BC500 in intestine. It is not known whether cell- and tissue-specific factors contribute to the regulation of RIG-I. RIG-I expression also increases with the age of the animal, and the factors that contribute to this are not known.

Inducible antimicrobial components

The acute-phase response is an early response accompanied by a large number of local, systemic, and metabolic changes that are also referred to as inflammation. A variety of proteins are involved in the active responses induced after entry of IAV. These innate inhibitors belong to families of proteins that are highly conserved in evolution, and include plasma proteins called acute-phase proteins (APPs), C-reactive protein, serum amyloid A, collagenous lectins (e.g. collectins, surfactants, and ficolins), pentraxins, alpha-macroglobulin families, and antimicrobial peptides (e.g. defensins and cathelicidins).

The alpha-macroglobulin family of proteins has long been known to have potent antiviral activity. Alpha-2-macroglobulin is a major neutralizing inhibitor of influenza A virus in pig and horse serum [104, 114]. Collectins express carbohydrate recognition domains (CRDs) that bind to mannose-rich glycans on the viral hemagglutinin (HA), and in some cases to the neuraminidase (NA) [47, 128], to mediate a range of anti-IAV activities including inhibition of IAV hemagglutination and NA enzyme function, neutralization of virus infectivity, virus aggregation, increased IAV uptake by neutrophils, and opsonization of virus to enhance neutrophil respiratory burst responses to IAV [50, 93, 108]. Porcine surfactant D has very potent anti-influenza activity compared with that from humans and rodents [135]. Although strictly not a collectin but a C-type lectin due to its lack of a collagenous domain, chicken lung lectin has moderate activity to IAV [54].

In contrast to collectins, members of the pentraxin family – long pentraxin PTX3, short pentraxin, and serum amyloid P component – provide sialylated ligands that mimic the structure of the cellular receptors used by IAVs, thereby blocking the receptor-binding site of HA. PTX3 is stored in neutrophils, whereas DC and macrophages produce PTX3 *de novo* upon inflammatory stimulation (for a review, see [17]). PTX3 has recently been described in pigs, and expression in serum is increased during experimental influenza infection [30]. Pulmonary innate defenses may be particularly efficient against IAV in pigs, and represent an important barrier limiting disease severity. Restriction of IAV infection by porcine innate defenses might also limit the induction of antibody-mediated adaptive immune responses in pigs that are required to drive antigenic drift.

Antigen-presenting cells

The induction of innate immunity is a crucial step in the onset and steering of subsequent adaptive immune responses. Antigen-presenting cells in particular, including macrophages (MΦ) and dendritic cells (DC), play a central role as regulators of adaptive immune responses by interacting with T-lymphocytes and B-lymphocytes. Although the avian respiratory tract differs significantly from the mammalian one in terms of morphology and airflow, in both mammals and avian species the respiratory tract has a network of macrophages and DC that are situated in immediate proximity to the respiratory epithelial cells [32, 110]. Respiratory macrophages and DC are among the first cells to detect and respond to IAV, and are essential for control of the innate and adaptive immune responses.

Several studies have reported that IAV replicates productively in human and mouse MΦ and DC, while others have described these infections as "abortive" or "dead end" (for a review, see [121]). Differences in virus strain and subset of macrophages and DC (derived from lung and from

blood of bone marrow) are likely to influence the outcome of infection. Infection with most seasonal IAVs and LPAIV is abortive and contributes to the effective host defense. Some HPAIV strains can infect MΦ and DC productively, which is likely to have consequences with regard to viral dissemination, amplification, and therefore pathogenicity and immunogenicity. In mammals, DC have been associated with the virus-induced cytokine deregulation or a "cytokine storm" characterized by the presence of elevated levels of pro-inflammatory cytokines and IFNs [12, 33]. Cytokine storms in pigs and chickens are less obvious, and appear to be dependent on the virus strain [8, 65, 89, 109]. Great care must be taken when interpreting cytokine data, especially mRNA expression levels, because it is not clear what levels of the different cytokines will exert deleterious biological effects. For example, if chickens are infected with other pathogens, the cytokine responses also increase to levels found in chickens infected with HPAIV, but in contrast to HPAIV there is no mortality, which suggests that other factors play a role in the extremely high mortality rates that occur during HPAIV infections.

More recently, the interaction of DC with IAV in pigs, horses, and chickens has been investigated in more detail. *In-vitro* cultured porcine bone marrow-derived DC (BM-DC) and plasmacytoid DC (pDC), equine blood-derived DC and chicken BM-DC can be successfully infected with LPAIV, although limited replication was detected [13, 16, 90, 97, 137]. Horse DC but not chicken DC produced type I IFN upon infection, and no or only small changes in cell surface activation markers (CD80, CD86, and MHC II) were induced after infection [13, 16, 137]. In pig alveolar macrophages and newborn pig trachea cells infected with SIV H3N2, both IFN-β and type III IFN (IFNλ1) but not IFN-α were up-regulated [34].

Comparison of LPAIV and HPAIV infection responses in chickens indicated that in contrast to LPAIV, infection of chicken BM-DC with H7N1 or H5N2 HPAIV resulted in increased viral load and a significant increase in IL-8 (CXCLi2), IFN-α, and IFN-γ mRNA expression [137].

DC express a variety of PRRs on their cell surface, including TLRs that recognize PAMPs, resulting in activation of macrophages and DC. Infection of chicken DC with LPAIV and HPAIV strains H7N1 or H5N2 induced differential up-regulation in particular of TLR1, TLR3, and TLR21 mRNA expression, which may relate to the differences in cytokine responses induced by these HPAIV and LPAIV strains. In pig alveolar macrophages, RIG-I, TLR3, TLR7, and TLR8 mRNA expression was rapidly up-regulated by infection with H3N2 swine IAV. In contrast, only RIG-I was up-regulated in newborn pig trachea cells [35].

As well as having a crucial role in the induction of adaptive immune responses, DC may also play a role in the enhancement of infection and spread of viruses. Although the primary attachment receptor for IAV is sialic acid (SA), attachment and entry of IAV into cells can occur independently of SA [125]. SA may enhance binding to the cell surface to increase subsequent and/or simultaneous interaction with secondary and/or co-receptors that are required for virus entry. Recent evidence suggests that specialized receptors on macrophages and DCs, namely CLRs, can act as capture and/or entry receptors for many viral pathogens, including IAV. In mouse it was shown that the macrophage mannose receptor, macrophage galactose-type lectin 1 and DC-SIGN/L-SIGN, can bind IAV to facilitate uptake and possibly destruction of the virus [75, 107, 134]. Although this has not been shown for all CLRs, MGL [92] and DC-SIGN [51] can act as endocytic receptors for IAV and support infection, but for other receptors additional receptors and/or co-receptors may be required for virus entry. An understanding of the specific mechanisms by which MΦ and DC recognize and internalize IAV may provide important information that is relevant to the tropism of different IAVs for particular airway cells. and therefore pathogenesis. The mRNA expression of CLRs in chicken DC is affected by AIV infection [31], but receptor-binding studies in farm animals and natural hosts have not yet been published.

Natural killer (NK) cells

NK cells are innate lymphocytes that provide early protection against numerous intracellular pathogens. They elicit antiviral responses by killing virus-infected cells without prior sensitization in a non-adaptive non-MHC-restricted way. NK cells express both activating and inhibitory receptors, and the balance between these signals determines NK-cell activation (for a review, see [46]). In

human patients with severe influenza infection, reduced frequencies of NK cells are observed in the blood [36, 49], and pulmonary NK cells are lacking [139]. *In vivo* studies in mice have shown that NK cells [94, 124] are required for the clearance of IAV. Recently enhanced activation of NK cells in the lungs of chickens was described after infection with H9N2 virus. In contrast, infection with H5N1 HPAIVs resulted in decreased activation of lung NK cells, indicating that decreased NK-cell activation may be one of the mechanisms that contributes to the pathogenicity of H5N1 HPAIVs [59]. In lungs of piglets infected with 2009 pandemic H1N1, increased numbers of NK cells were associated with the areas where IAV nucleoprotein (NP) was detected [41]. NK-cell responses in horses have not been described, although mathematical modeling of immune responses in horses shows that the rapid and substantial viral decline (around 2–4 logs within 1 day) can be explained by the killing of infected cells that is mediated by interferon-activated cells, such as NK cells, during the innate immune response [99].

Polymorphonuclear leukocytes

Polymorphonuclear leukocytes or granulocytes are important host defense cells during the phase of innate immunity. Activated granulocytes engulf micro-organisms by phagocytosis, and kill ingested micro-organisms by the production of a combination of toxic oxygen radicals, proteolytic enzymes, myeloperoxidase, defensins, and other bactericidal peptides. The degree of activation, the release of granule proteins, and generation of reactive oxygen species (ROS) all play a key role in pathogen clearance. Although granulocytes are traditionally associated with the fighting of bacterial infections, these cells can also be involved in virally induced pathology [105, 136].

Viruses can activate granulocytes either by direct binding or by binding through antiviral antibodies mediating antibody-dependent cytotoxicity. Uncomplicated influenza A viral replication in the respiratory epithelium results in inflammatory infiltrates mainly consisting of mononuclear leukocytes and lower number of polymorphonuclear leukocytes. In contrast, infection with highly virulent IAV strains, such as the lethal H1N1 1918 HA/NA:Tx/91 in a mouse model, produced pathological changes in alveolar macrophages and neutrophil migration correlated with lung inflammation. Depletion of neutrophils before a sublethal infection with 1918 HA/NA:Tx/91 virus resulted in uncontrolled virus growth and mortality in mice. In addition, the depletion was associated with decreased expression of cytokines and chemokines [132].

More recently it has been shown that matrix metalloprotease (MMP) 9 mediates excessive neutrophil migration into the respiratory tract in response to influenza virus replication [20]. MMPs are a family of proteolytic enzymes that are involved in remodeling the extracellular matrix (ECM) under both physiological and pathological conditions. They can be produced by a range of cells in the respiratory tract, where they mediate wound healing, airway remodeling, and cell trafficking. As such, MMPs play an important role in immunity, and their proteolytic activity can also directly dampen the inflammatory potential by down-regulating cytokine and chemokine function. However, excessive responses such as are found during HPAIV infection contribute to pathology. On the other hand, the antiviral role of neutrophils has been demonstrated in mice [38]. IL-6 was necessary for the resolution of influenza infection by protecting neutrophils from virus-induced death in the lung, and by promoting neutrophil-mediated viral clearance. Ultimately the balance between cytokine production, cell activation, amount of cell death, and duration of responses will determine the pathological changes and outcome of infection.

The generation of ROS or inducible nitric oxide synthases (iNOS), a family of enzymes that catalyze the production of nitric oxide (NO) from L-arginine, has been studied during AIV infection in chickens and ducks. A striking difference in iNOS mRNA levels was detected between ducks and chickens infected with H7N1 HPAIV. Infected ducks showed a rapid increase (8 hours post infection) in iNOS expression in the lung, whereas chickens showed a delayed increase (2 days post infection) [28]. Interestingly, at 1 day post infection with H5N1 HPAIV, iNOS mRNA levels in lung of chickens were higher than those in ducks, as were the NO levels in serum [22]. Understanding the beneficial and detrimental roles of NO and iNOS during HPAIV infection may provide insights into the underlying

mechanisms and differences observed in disease severity, but as with many other parameters these responses appear to be variable and dependent on the virus strain.

Concluding remarks on innate immunity

The innate immune system forms the first line of defense against IAVs. It consists of a plethora of components and responses that aim to prevent the infection of epithelial cells and underlying tissue. Innate immune cells, such as macrophages, granulocytes, and NK cells, are recruited to the site of infection in order to control virus replication and prevent further dissemination. The major differences in susceptibility that are found between chickens and waterfowl are likely to be caused by these early innate immune responses. Interestingly, several immune genes involved in the innate responses seem to be missing in avian genomes. Chickens appear to lack functional TLR8 and RIG-I in the genome, which affects the recognition of intracellular bacteria and RNA viruses, including IAV, and may be associated with the high mortality rates observed after infection with HPAIV. In contrast, ducks only lack TLR8, and survive infections with HPAIV.

Because of the abundance of responses and of components involved in the responses against influenza, no single innate component, cell, or pathway is responsible for the outcome of the infection. Future research into the innate responses will not only improve our basic understanding of the disease resistance, but will also enable intervention strategies to enhance the early defense against virus entry and dissemination, and to improve the onset and magnitude of the adaptive immune responses.

Immunological basis for vaccination of poultry

Introduction

This section will provide a general overview of the basic immunology involved in response to AI. Various factors, including age of bird, type, dose, and inoculation site following vaccination will affect the presentation and processing of antigen by host immune cells. Although many different cell types are involved in establishing an immune response, vaccination may or may not result in protection following challenge with AI virus (AIV). Numerous factors can contribute to vaccine failure, including a lack of antigenic similarity to the field strain, an overwhelming dose of challenge, or insufficient antigenic load to induce a protective immunological response. Although many poultry vaccines contain live viruses to establish protective immunity by the introduction of a non-lethal infection, the limitations of using live AIV in commercial poultry present unique obstacles to the generation of vaccine-induced protection of poultry against AI.

Following the administration of vaccine, the host immune system directs uptake and processing of the antigen with responses in both the innate and adaptive immune systems. The responses in the innate immune system occur first, and have been described earlier in this chapter. The later adaptive immune responses use highly specific antigen receptors on bursa-derived B-lymphocytes (humoral immunity) and thymus-derived T-lymphocytes (cellular immunity) that are generated randomly by gene rearrangement. It is the adaptive immune responses to vaccination that produce virus-neutralizing antibodies, and AIV-specific cytotoxic lymphocytes that are responsible for virus recognition and clearance in the host following exposure. Following vaccination, these two immunological systems work in concert to establish protection of a host against disease.

Immunology of antigen recognition and processing

The avian immune system appears to be similar in many ways to the mammalian immune system [18, 61, 106, 120]. Traditionally, the immune response to a pathogen has been divided into the humoral and cell-mediated immune responses. Protective humoral immunity of poultry against AI viruses is primarily the result of an antibody response directed against the hemagglutinin (HA), of which there are 16 different HA subtypes. Antibodies produced against the HA are neutralizing, and thus prevent attachment of the virus to host cells. When bursectomized chickens (i.e. unable to

produce antibody responses) were vaccinated and challenged, no protection was provided, indicating the role of antibodies in protection against AI. AI vaccines are generally custom-made against the specific HA subtype and/or NA subtypes as the current field virus. Because protection is provided through an immune response to the HA, the more efficacious vaccines target the specific phylogenetic lineages of the virus within an HA subtype. Cell-mediated immunity (CMI) is specific immunity mediated by T-lymphocytes, and has been suggested to be an important factor in the development of protection in chickens vaccinated against viral diseases [119, 120]. The subsets of T-lymphocytes, namely $CD4^+$ helper cells and $CD8^+$ cytotoxic cells, constitute the principal cells of the CMI response. The major histocompatibility complex (MHC) plays a central role in the presentation of antigens by antigen-presenting cells to cytotoxic T- lymphocytes (CTLs), and optimal activation of CTLs depends on identical MHC class I antigens. During an immune response, vaccine antigen must reach secondary lymphoid tissues. Antigen-presenting cells, such as dendritic cells, macrophages, and B cells, process antigen and display epitopes to T cells in MHC class II molecules and provide other signals needed to initiate immunity. Immunity to different infectious diseases requires distinct types of immune reactions, which have to be evoked by differently designed vaccines. Criteria for efficacious AI vaccines include an early onset and long duration of immunity. Because infected birds can succumb to HPAI before CMI responses are mobilized, the establishment of neutralizing antibodies is the key immunological parameter of relevance to AI vaccine-induced immunity. Most antibody responses that aim to evoke specific immunoglobulin G (IgG) of high affinity are dependent on assistance from helper T cells, which receive their activation signals from antigen-presenting cells.

Anatomy and physiology of the avian immune system

With regard to physiology, many differences exist between avian and mammalian immune systems, including the structure and distribution of lymphoid tissue. One of the major differences is the use of the lymphatic system for migration of lymphocytes to and from sites of vaccination, and challenge differs between the species. Mammalian systems contain lymph nodes – highly organized sites for interactions between B cells, T cells, macrophages, and other antigen-presenting cells that are important for activation of adaptive immunity. However, lymph nodes are absent in most avian species, including chickens [100, 140]. Instead of lymph nodes, avian species have concentrations of lymphoid tissue around the organs that are unencapsulated and contain small lymphocytes [100]. Like lymph nodes, these small lymphocyte aggregates form germinal centers in response to antigen [131]. Avian species do possess lymphatic vessels, which are believed to be involved in trafficking of mesenchymal stem cells to central lymphoid organs [100]. In chickens, lymphoid accumulations have been observed along the posterior tibial, popliteal, and lower femoral veins [96]. In contrast to chickens, ducks do possess lymph nodes, which are formed as a swelling of the lymphatic duct, and contain both efferent and afferent lymphatic vessels [140].

A number of immunologically relevant tissues used for antigen processing have been identified in chickens. Intestinal avian lymphoid tissue includes the bursa of Fabricius (cloacal bursa), cecal tonsils, Meckel's diverticulum, Peyer's patches, and diffuse mucosal lymphoid infiltrates. The bursa is the main organ responsible for B-cell production and differentiation, and is located dorsal to the distal end of the cloaca. The cecal tonsil is the most concentrated tissue in the intestine, and can be observed as two oval areas on the facing walls of the ceca. Two types of germinal centers are found in the cecal tonsil [95]. In the deep tissue they are incompletely capsulated, whereas closer to the surface of the organ they are fully capsulated. Peyer's patches are primarily located along the distal ileum, and consist of germinal centers and diffuse lymphoid tissue [23]. Intestinal antigens are absorbed and processed by germinal-center macrophages and epithelial cells. Meckel's diverticulum contains epithelial secretory cells in the germinal center that produce large amounts of plasma cells [96].

Several accumulations of lymphoid tissue have been described in the paranasal area. The most important of these is the Harderian gland, which is considered to be a secondary lymphoid organ [7]. In chickens, the Harderian gland is the major site

of antiviral IgA–antibody-forming cells, whereas free IgA is found in bile and mucosal washes and can exist in both monomeric and multimeric forms [56]. The Harderian gland is located directly behind the eye orbit, and is heavily infiltrated by plasma cells [141]. This gland is believed to be critical for local immune responses of the eye, nasal turbinate, and upper respiratory tract areas. The production of germinal centers in the Harderian gland can be observed by 3 to 4 weeks of age [3]. Vaccinations of poultry via the eyedrop method are believed to stimulate mucosal immune responses through the Harderian gland.

Despite the physiological differences in structure and organization between mammalian and avian species, the functional aspects of lymphoid cells and peripheral organs are similar. This includes lymphoid cell functions, division, classes, interactions, specificity, and net effect, which are highly similar between mammalian and avian species.

Overview of the immunological response to avian influenza virus

The major function of an immune response is to recognize and eliminate a pathogen through the innate and adaptive immune systems. The innate and adaptive immune responses are mechanisms of an integrated system of host defense in which numerous cells and molecules function cooperatively. A general description of the vertebrate innate immune response was provided in the overview of immunity at the beginning of the chapter, and birds have many of these general features, including PRRs, PAMPs, physical and chemical barriers, phagocytic cells, complement proteins, and mediators of inflammation, cytokines, and the TLR family.

The adaptive immune response is an inducible response that occurs only in vertebrates. The adaptive host defense, mediated by T- (thymus-derived) and B- (bursa-derived) lymphocytes, is adjustable to the antigenic response because of the somatic rearrangement of T-cell-receptor genes and immunoglobulin, respectively. This results in the creation of individual lymphocyte clones that express distinct antigen receptors. The receptors on lymphocytes are generated by somatic mechanisms during the ontogeny of each individual, and thus generate a diverse repertoire of antigen receptors with random specificities on the lymphocytes. For influenza, $CD8^+$ CTLs play a crucial role in controlling infectious virus from the lungs of mice. Previous studies have provided evidence that $CD8^+$ CTLs directed against viral epitopes conserved among influenza A viruses, such as those within the HA and NP, contribute to protection against influenza [4, 5]. It was also determined that influenza virus NP-specific CTLs generated through vaccination or introduced by adoptive transfer led to a more rapid viral clearance and recovery of the host, and protection from death [2, 6]. The major histocompatibility complex (MHC) plays a central role in the presentation of antigens by antigen-presenting cells to CTLs, and optimal activation of CTLs is dependent on identical MHC class I antigens. There are limited data on CTL responses to influenza viruses in poultry, which require the availability of inbred chickens. However, it has been demonstrated that T-lymphocytes or $CD8^+$ cells produced in H9N2-infected chickens can protect against lethal H5N1 challenge when adaptively transferred into naive MHC-matched birds [119].

Classically, MHC class II molecules are used by professional antigen-presenting cells and are recognized by $CD4^+$ T cells. Following recovery from infection, the antigen-specific clones remain as memory lymphocytes (both T and B) that provide a more rapid response to secondary exposure to the antigen.

With regard to AIV infection, attachment occurs primarily through interactions of the HA protein with host sialic acid residues found on cells lining the mucosal surface, and results in internalization and infection of the virus within the host. Endosomal enclosure and maturation around the virus result in a drop in pH, which is necessary for uncoating of the virus. During this stage the host innate immune response is stimulated through interactions of the endosomally located TLRs with the single-stranded viral RNA genome TLR7. Activation of TLR7 with the RNA agonist results in a cascade of cytokine and interferon production designed to limit or suppress viral replication and recruit effector cells for stimulation of adaptive immunity. Interestingly, activation of TLRs in endosomal compartments also appears to require endosomal maturation with a subsequent pH change, as pretreatment of cells with chloroquine

results in decreased or no detection of cytokine induction following AIV infection [37, 77]. As the virus replicates in the cytoplasm, viral proteins are processed and expressed by MHC class I molecules for presentation to $CD8^+$ lymphocytes, resulting in the development of cytotoxic T-lymphocytes that are capable of lysing virus-infected cells. At the same time, newly released virions are taken up by professional antigen-presenting cells, including macrophages, dendritic cells, and B cells. The processed viral peptides are expressed through MHC class II molecules that are recognized by $CD4^+$ T-helper lymphocytes. These activated $CD4^+$ lymphocytes present viral antigen to B cells, resulting in antibody production.

Mucosal immunity

Mucosal surfaces found in respiratory and gastrointestinal tracts serve as portals of entry for many infectious agents that affect poultry [88, 143]. Mucosal immunity is the first line of defense for the host, and extensive efforts are currently in progress in rodent animal models and humans to design vaccines that are able to confer protection at the mucosal site [123]. In mammals, both mucosal sites can respond individually to antigenic stimulation, and the induction of an immune response in one region results in subsequent immunity at other mucosal sites – a phenomenon known as the "common mucosal immune system" [80, 82]. A similar immune mechanism has been described in chickens [88]. It has been demonstrated that immune lymphocytes can migrate from one mucosal site to repopulate and provide protective immunity at distant mucosal sites [55]. Because respiratory pathogens, such as Al virus, invade at mucosal surfaces, vaccines that can induce strong mucosal immunity would be superior to other types. Current parenterally administered Al vaccines are poor inducers of mucosal immunity, and therefore organisms can invade the host before the systemic immunity can impede the infection [29]. Mucosal immunization has the major advantage of inducing mucosal and systemic immunity [123]. Al viruses that infect poultry invade two primary mucosal regions – the respiratory tract and the gastrointestinal tract. Early research in mucosal immunology indicated that a vigorous T-cell response could be observed in the respiratory tract after immunization of the respiratory tract, suggesting that a local response was capable of being generated at the site of immunization – a critical element for mucosal immunization [26, 138]. The importance of the specificity for local immune protection is highlighted by recent observations that exposure of the lung to aerosol formulations for protection against influenza was more effective than either intranasal or parenteral vaccination [122].

The concept of mucosal priming at one site providing sensitized cells to other mucosal sites indicates the potential for an orally administered vaccine to provide protection against a respiratory challenge [27]. This phenomenon was also suggested by a report that the adaptive transfer of influenza-specific T-cell clones could migrate to mucosal sites [15]. More recently, as already mentioned, it was further demonstrated that immune lymphocytes can migrate from one mucosal site to repopulate and provide protective immunity at distant mucosal sites [55]. This "mucosal trafficking" has been demonstrated with B and T cells, and as a result the mechanisms of immune protection at mucosal sites may involve both humoral and cell-mediated components [81]. Taken together, these observations are consistent with the known circulation pathway of lymphoblasts from lymph nodes through the thoracic lymph to distant mucosal surfaces.

The primary antibody that mediates protection at mucosal surfaces is immunoglobulin A (IgA), although IgG and IgM can also be found [115]. Resistance to influenza infection in rodent models and humans correlates with the induction of IgA antibody in the respiratory tract [71]. As mentioned earlier, IgA is found in bile, crop, and mucosal washes, and can exist in both monomeric and multimeric forms [74, 102, 118]. Similarly to its mammalian counterpart, avian IgA possesses a J chain and secretory component [87]. Although IgA does not fix complement, the immunoglobulin does have a number of effector functions, including viral neutralization, inhibition of bacterial adherence, and acting as an opsonin for mucosal phagocytes [69, 81]. Mucosal IgA antibody has also been shown in some cases to possess more cross-reactivity than serum IgG antibody, and thus may contribute to the cross-protection that is observed in mucosal-vaccinated animals [112, 113].

Concluding remarks on adaptive immunity

AI vaccines provide protection by stimulating host immunity, which is largely based on antibody production against the HA glycoprotein. These antibodies are capable of neutralizing the virus, thereby preventing infection, and reducing disease and virus transmission. Although many AI vaccine constructs have been described, only two are licensed for use in the field, namely inactivated AI virus and recombinant vectored vaccines. Recombinant vaccines have been demonstrated to induce cellular immune responses, and thus provide a greater immune benefit for the host. However, the HA insert must still be matched to provide the best possible immune response match to the field virus.

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7 Wild bird infections and the ecology of avian influenza viruses

David E. Stallknecht and Justin D. Brown

Introduction

Over 50 years have passed since the first isolation of an avian influenza virus (AIV) was reported from common terns (*Sterna hirundo*) in South Africa [14]. Despite their historically limited scale, field studies conducted from the 1970s through to the 1990s greatly advanced our understanding of the natural history of AIV in wild bird reservoirs. Interest in this subject accelerated in 2002, in response to the detection of H5N1 highly pathogenic (HP) AIV in wild birds in Hong Kong, and its subsequent spread to avian populations in Eurasia and Africa. Consequently, the number of publications related to wild bird surveillance and ecology has increased dramatically [100]. This most recent burst in surveillance and research activity has resulted in a much improved global perspective, and a more detailed understanding of host range, wild bird reservoirs, AIV genetic diversity within these populations, spatial and temporal patterns of infection, transmission and maintenance mechanisms, and the risks associated with viruses such as H5N1 HPAIV that can be shared between domestic and wild avian populations. The goals of this chapter are to provide an overview of our current understanding of the natural history and epidemiology of AIV in wild bird reservoirs, and to identify gaps in this understanding that need to be addressed in future research.

Host range

There are several published reviews that provide detailed information on the host range of AIV in wild birds [157, 206, 211]. Because the number of species that are included on these lists has broadened due to recent surveillance and research, an updated species list is presented in Table 7.1. The results listed in this table underestimate the true host range, as they are based on reported virus isolations. There are numerous additional reports of polymerase chain reaction (PCR)-positive results from species in which AIVs have yet to be isolated, and there are still many species that have never been tested.

Host range is only partially defined by species susceptibility. The reason for this relates to the genetic, geographic, and behavioral differences that are represented in the more than 9000 species of wild birds that exist globally [64]. Although the number and diversity of birds from which AIVs have been isolated are extensive (Table 7.1), there are some taxonomic and behavioral characteristics that define the majority of these positive species. Most are associated with aquatic habitats, and most species belong to one of two avian orders, the Anseriformes (ducks, geese, and swans) and the Charadriiformes (gulls, terns, and shorebirds). Even within these orders, variation in the probability of testing positive for AIV is apparent. Within the Anseriformes, for example, most AIV isolations have been reported from the subfamily Anatinae (dabbling and diving ducks). Variation is also present within the Anatinae, with different isolation rates reported from the species in this subfamily. In general, more isolations are reported from dabbling ducks than from diving ducks and geese [82, 148, 206, 243], and within the dabbling ducks, most isolations have been reported from mallard (*Anas platyrhynchos*) [155].

Animal Influenza, Second Edition. Edited by David E. Swayne.

Table 7.1 Free-living species from which low-pathogenic avian influenza viruses have been isolated.

Taxononomic group	Species	References
Anseriformes Anatidae Anatinae	American black duck *(Anas rubripes)*, American wigeon *(Anas americana)*, Blue-winged teal *(Anas discors)*, Bufflehead *(Bucephala albeola)*, Canvasback (*Aythya valisineria*), Cinnamon teal (*Anas cyanoptera*), Common merganser (*Mergus merganser*), Common teal (*Anas crecca*), Eurasian wigeon (*Anas penelope*), Falcated teal (*Anas falcata*), Gadwall (*Anas strepera*), Garganey (*Anas querquedula*), Harlequin duck (*Histrionicus histrionicus*), Hooded merganser (*Lophodytes cucullatus*), Long-tailed duck (*Clangula hyemalis*), King eider (*Somateria spectabilis*), Mallard (*Anas platyrhynchos*), Mottled duck (*Anas fulvigula*), Northern pintail (*Anas acuta*), Northern shoveler (*Anas clypeata*), Pacific black duck (*Anas superciliosa*), Redhead (*Aythya americana*), Ring-billed duck (*Anas erythrorhyncha*), Ring-necked duck (*Athya collaris*), Ruddy duck (*Oxyura jamaicensis*), Spot-billed duck (*Anas poecilorhyncha*), Steller's eider (*Polysticta stelleri*), Sunda teal (*Anas gibberifrons*), Tufted duck (*Aythya fuligula*), Velvet scoter (*Melanita nigra*), White-winged scoter (*Melanitta fusca*), Wood duck (*Aix sponsa*), Yellow-billed duck (*Anas undulata*)	[2, 6, 10, 11, 16, 28, 37, 39, 41, 52, 53, 59, 60, 67, 69, 70, 78–81, 88, 90, 91, 93–96, 102, 105, 107, 114, 117, 124, 136, 140, 143, 146, 153, 158, 161, 162, 165, 166, 170, 172, 175, 182, 184–186, 195, 197–199, 201, 203, 204, 209, 214, 215, 217, 219, 220, 226, 228–230, 238, 240, 242, 247, 248]
Anseriformes Anatidae Anserinae	Bar-headed goose (*Anser indicus*), Brent goose (*Branta bernicla*), Canada goose *(Branta canadensis),* Egyptian goose (*Alopochen aegyptiacus*), Greylag goose (*Anser anser*), Greater white-fronted goose (*Anser albifrons*), Lesser snow goose (*Chen caerulescens*), Mute swan (*Cygnus olor*), Tundra swan (*Cygnus columbianus*), Whooper swan (*Cygnus cygnus*)	[16, 28, 37, 53, 60, 69, 94, 159, 160, 170, 172, 184, 195, 197, 204, 217, 228]
Anseriformes Anatidae Tadorinae	Australian shelduck (*Tadorna tardornoides*), Common shelduck (*Tadorna tadorna*), Ruddy shelduck (*Tadorna ferruginea*), Spur-winged goose (*Plectropterus gambensis*), South African shelduck (*Tadorna cana*)	[77, 78, 143, 170, 204]
Anseriformes Anatidae Dendrocygninae	Fulvous whistling duck (*Dendrocygna bicolor*), White-faced whistling duck (*Dendrocygna viduata*)	[52, 59]
Charadriiformes Scolopacidae	Dunlin (*Calidris alpina*), Eurasian woodcock (*Scolopax rusticola*), Least sandpiper (*Calidris minutilla*), Red knot (*Calidris canutus*), Ruddy turnstone (*Arenaria interpres*), Sanderling (*Calidris alba*), Semipalmated sandpiper (*Calidris pusilla*), Spotted redshank (*Tringa erythropus*), Spur-winged lapwing (*Vanellus spinosus*), Temminck's stint (*Calidris temminckii*), Wood sandpiper (*Tringa glareola*)	[62, 80, 98, 107, 110, 142, 149, 189, 249]
Charadriiformes Charadriidae	Pacific golden plover (*Pluvialis fulva*)	[175]
Charadriiformes Laridae	Arctic tern (*Sterna paradisaea*), Black-backed gull (*Larus fuscus*), Black-headed gull (*Larus ridibundus*), Black-legged kittiwake (*Rissa tridactyla*), Black-tailed gull (*Larus crassirostris*), Common tern (*Sterna hirundo*), Franklin's gull (*Larus pipixcan*), Glaucous gull (*Larus hyperboreus*), Great black-backed gull (*Larus marinus*), Herring gull (*Larus argentatus*), Kelp gull (*Larus dominicanus*), Laughing gull (*Larus atricilla*), Lesser noddy (*Anous tenuirostris*), Mediterranean gull (*Larus melanocephalus*), Mew gull (*Larus canus*), Ring-billed gull (*Larus delawarensis*), Sabine's gull (*Xema sabini*), Sandwich tern (*Sterna sandvicensis*), Silver gull (*Chroicocephalus novaehollandiae*), Slaty-backed gull (*Larus schistisagus*), Slender-billed gull (*Larus genei*), Sooty tern (*Sterna fuscata*), Vega gull (*Larus vegae*), Whiskered tern (*Chlidonias hybrida*), White-winged tern (*Chlidonias leucoptera*), Yellow-legged gull (*Larus michahellis*)	[8, 14, 28, 29, 53, 54, 69, 85, 91, 95, 134, 139, 140, 143, 153, 172, 185, 195, 204, 225, 228, 233, 234]

(*continued*)

Table 7.1 *(Continued)*

Taxononomic group	Species	References
Charadriiformes Alcidae	Common murre (*Uria aalge*), Guillemot (*Cepphus* spp.), Thick-billed murre (*Uria lomvia*)	[53, 172, 191]
Ciconiiformes	Glossy ibis (*Plegadis falcinellus*), Gray heron (*Ardea cinerea*), Hadeda ibis (*Bostrychia hagedash*), Squacco heron (*Ardeola ralloides*), White stork (*Ciconia ciconia*)	[105, 166, 170, 185, 186]
Columbiformes	Collared dove (*Streptopelia decaocto*)	[181]
Galliformes	Ring-necked pheasant (*Phasianus colchicus*), Rock partridge (*Alectoris graeca*)	[136, 182]
Gaviiformes	Arctic loon (*Gavia arctica*), Red-throated loon (*Gavia stellata*)	[105, 249]
Gruiformes	American coot (*Fulica americana*), Eurasian coot (*Fulica atra*)	[16, 134, 136, 140, 162, 182, 218]
Passeriformes	American redstart (*Setophaga ruticilla*), Barn swallow (*Hirundo rustica*), Black-faced bunting (*Emberiza spodocephala*), Carrion crow (*Corvus corone*), Common jackdaw (*Corvus monedula*), Common redstart (*Phoenicurus phoenicurus*), Common whitethroat (*Sylvia communis*), Dark-eyed junco (*Junco hyemalis*), European starling (*Sturnus vulgaris*), Garden warbler (*Sylvia borin*), Hermit thrush (*Catharus guttatus*), House sparrow (*Passer domesticus*), Icterine warbler (*Hippolais icterina*), Purple finch (*Carpodacus purpureus*), Red-backed shrike (*Lanius collurio*), Song sparrow (*Melospiza melodia*), Spotted flycatcher (*Musicapa striata*), Swainson's thrush (*Catharus ustulatus*), Tennessee warbler (*Vermivora peregrina*), Willow flycatcher (*Empidonax traillii*), Willow warbler (*Phylloscopus trochilus*), Yellow vented bulbul (*Pycnonotus goiavier personata*), Yellow wagtail (*Motacilla flava*), Yellow warbler (*Dendroica petechia*), Yellow-breasted bunting (*Emberiza aureola*), Yellow-rumped warbler (*Dendroica coronata*), Yellow-throated warbler (*Dendroica dominica*)	[7, 16, 104, 107, 135, 183, 186]
Pelecaniformes	Great cormorant (*Phalacrocorax carbo*), American white pelican (*Pelacanus occidentalis*)	[105, 127, 218]
Piciformes	Great-spotted woodpecker (*Dendrocopos major*)	[185]
Podicipediformes	Pied-billed grebe (*Podilymbus podiceps*), Red-necked grebe (*Podiceps grisegena*)	[16, 130]
Procellariiformes	Wedge-tailed shearwater (*Puffinus pacificus*)	[44, 45, 140]

These differences primarily reflect species-related behavioral differences (habitat preference, feeding strategies, and migration patterns) that affect transmission, but some of the differences may be related to sampling efforts that can be biased towards abundant and easily sampled species that historically have a high prevalence of AIV.

An even more restrictive pattern occurs within the Charadriiformes. Although AIVs have been isolated from species in at least four families (Scolopacidae, Charadriidae, Laridae, and Alcidae), gulls (Laridae) have been the most consistent source of positive results. In contrast, reports of virus isolation from other birds within this family, such as terns, are relatively few. Species-related variation is also apparent within the shorebirds (Scolopacidae and Charadriidae), and globally, confirmed infections with AIV have only been reported from a limited number of species (mostly in the Scolopacidae), and even when detected, the prevalence is often very low [38, 61, 148].

Isolations have been reported from other species that utilize aquatic habitats, including birds in the orders Ciconiiformes, Gaviiformes, Gruiformes, Pelecaniformes, Podicipediformes, and Procellariiformes. Collectively, these include relatively few positive species (Table 7.1). It is probable that many of these positive results are associated with

spillover of viruses from ducks and gulls on shared aquatic habitat. This was the case with isolates recently reported from American white pelicans (*Pelacanus occidentalis*) [127] and red-necked grebes (*Podiceps grisegena*) [130] that were sampled on waterfowl habitats in Minnesota. In both cases, viruses isolated from these species clearly reflected a duck or combined duck and gull origin.

There are reports of AIV from species in the orders Columbiformes, Piciformes, and Passeriformes that are commonly associated with terrestrial habitats, but there are few reported isolations. In a recent review of AIV in passerines [200] that included reported positive results for virus isolation, PCR, and serological testing, it was concluded that there is little evidence that these birds are involved in the maintenance and transmission of AIV under natural conditions. However, under conditions where peridomestic birds have contact with AIV-infected domestic birds, these terrestrial birds may become infected and may play a limited role in transmission [200].

These host relationships have been supported by recent large-scale serological studies [24]. However, serological testing has also provided some insight and questions related to host range determinants that are based on virus detection alone. For example, serological results provided the first evidence that ducks were involved in the epidemiology of AIV [9]. More recent studies have shown that antibody prevalence can also be high in some species of birds from which virus isolations are rarely reported or in which the prevalence of infection is consistently low. This has been observed with red knot (*Calidris canutus*) at Delaware Bay [145], Canada goose (*Branta canadensis*) [82, 115], and pink-footed goose (*Anser brachyrhynchus*) [101]. These contrasting results suggest that many of these species are infected during their lifetime, but do not contribute to the high prevalence events annually observed in ducks during the late summer and autumn or in shorebirds at Delaware Bay during the spring. As many of these species are long-lived, this may relate to the age structure of the population and resulting population immunity. It may also relate to behavior (e.g. grazing feeding behavior) and a reduced viral shedding time.

Species susceptibility

Species susceptibility has been evaluated in experimental trials, but the available information is limited. Experimentally, it is possible to infect a broad diversity of taxonomic groups with AIV, but species-related differences in the ability to infect, the duration of viral shedding, and the predominant route of shedding exist for individual AIV strains [4, 5, 26, 97, 110, 196, 246], avian species [35, 151], and age [33]. Existing experimental work is supported by numerous reported AIV isolations from species in taxonomic groups, such as Passeriformes, Psittaciformes, and Galliformes. However, in almost all cases these AIV-positive birds have direct or potential contact with infected poultry [3, 4, 193, 200]. A similar situation has been reported with H5N1 HPAIV under experimental conditions [167–169]. The recognition that these viruses have the potential to infect diverse avian species is important from the standpoint of preventing introduction into domestic animal populations, especially in view of the fact that potential transmission may not directly involve a species that represents a known AIV host or reservoir under natural conditions. At present the variation in species response to an AIV infection is not well understood, but it does not appear to be directly related to sialic-acid-receptor distribution [56].

Wild bird reservoirs

It is well established that all AIVs that infect avian and mammalian hosts historically originate from wild bird reservoirs. A recent exception to this may be the bat influenza viruses (H17N10 and H18N11) that have recently been reported, but to date these have not been associated with birds or with spillover to other hosts [223, 224]. In defining AIV reservoirs it is important to understand that these represent multispecies systems that provide for the maintenance of these viruses. It is also important to recognize that reservoirs can change as these viruses move to and evolve within new host populations. A reservoir is defined as "any animate or inanimate object or any combination of these serving as a habitat of a pathogen that reproduces itself in such a way as to be transmitted to a susceptible host" [223]. As previously stated, the collective

information on AIV in wild birds clearly identifies two broad taxonomic groups that represent overlapping and in some cases unique AIV reservoirs, namely the Anseriformes and the Charadriiformes. This is not to say that all species within these groups contribute equally to maintaining these viruses. In some cases, individual species may contribute to viral maintenance in unique ways. For example, although mallards are an important component of the wild duck AIV reservoir and are often identified as the most important species associated with AIV maintenance, this species often cohabits with many other dabbling duck species. In addition, in many wintering areas in North America, such as the coastal marshes surrounding the Gulf of Mexico, mallards do not represent the predominant duck species, and other species, such as the blue-winged teal (*Anas discors*) or green winged-teal (*Anas crecca*), may represent the primary species in which these viruses are seasonally maintained [207]. Species such as the northern shoveler (*Anas clypeata*), that have unique feeding habits, may also make a unique contribution to AIV maintenance by virtue of a feeding strategy that increases contact with sediments potentially contaminated with AIV [85]. In addition, several studies have suggested that the maintenance of these viruses in ducks may be dependent on the interactions between subpopulations of resident, local, and long-range migrants even within a single species [52, 86, 87, 207, 232, 235].

With regard to the Charadriiformes, most isolated AIVs have been reported from species in two families, the Scolopacidae and Laridae. Within the Laridae, there is evidence that two subtypes (H13 and H16) are maintained in gull populations [8, 51, 91]. These viruses are only occasionally reported from ducks and waders, but are most prevalent in gull populations in breeding colonies [234]. The H13 viruses can be isolated from gulls at a low prevalence throughout the year, which suggests that they are maintained annually within these populations [69, 134]. This has not been demonstrated with the H16 viruses, but data for this subtype are currently limited. In contrast, within the Scolopacidae, molecular studies involving numerous AIV genes have not shown genetic differences between shorebird and duck AIVs [202]. In addition, a very low prevalence of infection has been documented globally from species in this family [38, 61], and most AIV isolations worldwide have been associated with one species, the ruddy turnstone (*Arenaria interpres*), at one site (Delaware Bay) and at one time of year (May and June) [145, 213]. Based on these surveillance results alone, it is difficult to determine whether this group (the Scolopacidae), or even the ruddy turnstone, are significant contributors to the overall AIV reservoir, and it is possible that isolations from this group may represent localized spillover hosts for viruses that are maintained in ducks and gulls [145]. In contrast, annual amplification of AIVs in ruddy turnstone at Delaware Bay may represent an important component in the maintenance and northern movement of these viruses during spring migration [119].

As previously stated, host range is only partially defined by host susceptibility. Likewise, documentation of both infection with and susceptibility to AIV does not determine whether a species is important as a reservoir. At present there appear to be a limited number of bird species that contribute to the maintenance and transmission of these viruses, but this can change rapidly if there are alterations in natural or man-made conditions that enhance the potential for maintenance and transmission.

Spatial and temporal variation in AIV infection

Spatial and temporal variations in prevalence are relatively consistent in duck, gull, and shorebird populations on a continental basis, but patterns can vary globally. This variation can be attributed to differences associated with breeding and wintering areas, local species composition and behavior, and the presence of different environmental drivers affecting the distribution of birds. In North American ducks, the prevalence of AIV peaks in late summer and early autumn, and is associated with concentration of susceptible hatching-year birds during pre-migration staging [93]. During this time, AIV infection can exceed 30% in this age group, and consequently AIV surveillance can be greatly enhanced by concentrating on juvenile birds [243]. The temporal patterns observed in ducks correspond to consistent spatial patterns, with the highest AIV prevalence in North America observed in waterfowl on breeding and staging areas in

Canada and the northern USA. As birds migrate south, the AIV prevalence rapidly decreases, and on wintering areas it is often lower than 1–2% [206]. In Europe and Asia a similar pattern is evident, but in parts of northern Europe the period of high prevalence appears to extend into late autumn [238]. The reason for this is not understood, but it may relate to differences in migration timing or to more northerly (ice-free) wintering areas. As in North America, AIV prevalence estimates for wintering ducks in Italy [39, 40], France [126], Spain [166], Portugal [220], and Georgia [134] are generally low. A peak in prevalence associated with spring migration has been reported from ducks in both Europe and North America [81, 237]; however, the prevalence (approximately 5–10%) is relatively low compared with autumn migration. Temporal patterns are less pronounced for ducks in southern South America, Africa, and Australia [59, 62, 140, 156, 165]. It has been suggested that influenza cycles in Africa result from seasonal rainfall patterns [37], and that the seasonality may be less extreme due to a more gradual recruitment rate of juveniles as a result of an extended breeding season [62]. Unlike Africa, which hosts both resident and migrant birds, duck populations in southern South American and Australia have little or no overlap between northern-latitude migrants and resident species [177, 227]. Spatial and temporal patterns of infection in other waterfowl, such as geese and swans, are poorly described, partially due to consistently poor isolation results. However, in greater white-fronted geese (*Anser albifrons*) in Europe, short-duration viral shedding, primarily from the respiratory tract, was consistently detected during the winter [116].

In gulls, a clear connection between the prevalence of H13 and H16 viruses and breeding season has been reported, and, as in ducks, this peak is associated with the recruitment of juvenile birds [134, 233]. For gulls, finer-scale temporal relationships have been described, including AIV infection associated with specific juvenile age classes in the breeding colony, and differences in the timing of prevalence peaks associated with the H13 and H16 viruses [234]. Outside of the breeding period, AIVs including H13 and H16 viruses can be isolated, but their prevalence is very low [69].

Primarily based on results from Delaware Bay, the main AIV prevalence peak in Charadriiformes is reported to occur in the spring, with a lesser peak occurring in the autumn [110]. Although this may be the case for gulls, seasonal and spatial patterns of AIV in other families within this order have not been described, and in general the prevalence of infection globally is consistently low [38, 53, 61, 80, 210]. For Delaware Bay, which is the only site worldwide where consistent AIV isolations from shorebirds have been reported, the reason for the high prevalence of AIV in spring-migrating ruddy turnstones is not understood [80, 110, 119, 121].

Susceptibility to subsequent infections

An understanding of avian reservoirs for AIV and the spatiotemporal patterns of infection in these hosts can only be understood with some knowledge of population immunity. This is particularly important in wild avian populations, as many of these species are long-lived and in some cases repeatedly challenged with a diversity of AIV subtypes.

There is currently very limited information available relating to the primary immune response of wild birds, and consequently our understanding of long-term immune protection or the response to multiple infections is incomplete. On the basis of field data, population immunity appears to be a very important component of the annual cycle. For example, it has long been accepted that the high prevalence of AIV in juvenile ducks during pre-migration staging is related to the concentration of naive birds [92]. This same relationship appears to exist with gulls infected with H13 and H16 LPAI viruses in breeding colonies [234]. With shorebirds at Delaware Bay, the observed prevalence of AIV at the species level correlates with the prevalence of pre-existing antibodies in ruddy turnstones and red knot [145]. The duration of viral shedding in naturally infected mallards has also been shown to be reduced with time, suggesting that acquired immunity reduces viral shedding [122].

Experimental studies have demonstrated the potential effects of existing immunity on clinical outcome and viral shedding using both low pathogenic (LP) AIV and HPAIV [34, 36, 51, 109]. Based on re-isolation histories from sentinel or

recaptured birds, similar results are suggested by field studies [123, 221]. The findings of these studies support the development of complete or partial immunity to homologous and heterologous challenges, respectively. Although these studies have been primarily restricted to mallards, similar results have been reported for Canada geese [15]. Controlled challenge experiments, reflecting long-term exposures and multiple infections, as would occur under field conditions, are currently lacking. In view of the longevity of wild avian species, and the annual and perhaps year-round exposure of these species to AIV, the potential effects and significance of population immunity represent one of the most important and yet least studied areas related to AIV transmission and maintenance.

Subtype diversity

All 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes of AIV, and most combinations of these, have been reported from wild birds [158]. However, these subtypes are not equally represented among wild bird populations, and variation can occur between hosts, locations, seasons, and years. In North American ducks, the H3, H4, and H6 subtypes represent the predominant reported HA subtypes [119, 194, 206]. Certain HA subtypes (e.g. H11) are frequently isolated, and others (e.g. H8 and H9) are uncommon [199, 206]. These trends in HA predominance exist both in Eurasia and in North America [32, 124, 148, 243] (Table 7.2). In gulls, subtype diversity is not as well defined; however, the H13 and H16 viruses consistently predominate worldwide [8, 110, 119, 157]. Other subtypes, including H1, H6, H10, and H11, are regularly isolated from gulls but at a relatively low prevalence [8, 69]. With regard to shorebirds, our knowledge of subtype diversity is limited in scope, with most isolates recovered from ruddy turnstone at Delaware Bay, USA. Nine HA subtypes of AIV have been reported to occur more often in Charadriiformes than in ducks, including H5, H7, and H9 AIVs [119]. At Delaware Bay, H6, H7, H10, H11, and H12 AIVs are over-represented

Table 7.2 Predominant HAs and HA/NA combinations reported from ducks, gulls, and shorebirds. The three predominant HA subtypes detected in each study are highlighted.

HA	Alberta [194][a]	Northern Europe [148]	Minnesota [243]	Sweden [124]	Taiwan [32]	IRD[b] (Ducks) [205]	IRD (Gulls) [205]	IRD (Shorebirds) [205]
Total isolates	2839	332	575	1081	237	5581	234	808
H1	106[c] (H1N1)	27 (H1N1)	24 (H1N1)	141 (H1N1)	14 (H1N1)	319 (H1N1)	12 (H1N3)	102 (H1N1)
H2	24 (H2N3)	26 (H2N3)	17 (H2N3)	96 (H2N3)	4 (H2N3)	253 (H2N3)	21 (H2N7)	6 ND[d]
H3	886 (H3N8)	32 (H3N8)	211 (H3N8)	74 (H3N8)	29 (H3N8)	1696 (H3N8)	2 (H3N6)	48 (H3N8)
H4	510 (H4N6)	52 (H4N6)	152 (H4N6)	291 (H4N6)	105 (H4N6)	1667 (H4N6)	3 ND	40 (H4N6)
H5	6 (H5N2)	19 (H5N2)	16 (H5N2)	99 (H5N2)	3 (H5N2)	210 (H5N2)	5 (H5N1)	21 (H5N2)
H6	1199 (H6N2)	59 (H6N1)	65 (H6N1)	105 (H6N2)	10 (H6N1)	567 (H6N1)	12 (H6N8)	58 (H6N1)
H7	21 (H7N3)	37 (H7N7)	13 (H7N3)	41 (H7N7)	19 (H7N1)	200 (H7N3)	2 (H7N3)	34 (H7N3)
H8	10 (H8N4)	7 (H8N4)	16 (H8N4)	19 (H8N4)	3 (H8N4)	64 (H8N4)	0	4 (H8N4)
H9	7 (H9N1)	5 (H9N2)	0	9 (H9N2)	2 ND	27 (H9N2)	3 ND	33 (H9N7)
H10	23 (H10N7)	16 (H10N7)	44 (H10N7)	63 (H10N4)	37 (H10N3)	289 (H10N7)	10 (H10N2)	201 (H10N7)
H11	23 (H11N9)	29 (H11N9)	14 (H11N9)	118 (H11N9)	8 (H11N9)	217 (H11N9)	16 (H11N1)	76 (H11N9)
H12	12 (H12N5)	7 (H12N5)	2 (H12N5)	14 (H12N9)	1 (H12N2)	54 (H12N5)	3 (H12N5)	71 (H12N5)
H13	1 (H13N6)	6 (H13N8)	0	0	0	2 (H13N6)	105 (H13N6)	3 (H13N6)
H14	0	ND	0	0	2 (H14N7)	7 (H14N6)	0	0
H15	0	ND	0	0	0	0	0	0
H16	0	4 (H16N3)	0	0	0	1 (H16N3)	40 (H16N3))	0

[a] Reference.

[b] Influenza Research Database.

[c] Number of isolates for each HA type (predominant subtype combination reported for each HA).

[d] ND = predominant subtype combination not determined.

in shorebirds; they are either present almost every year, or they are the dominant subtype in a single year [213]. As in gulls, the H1, H6, H10, and H11 subtypes are well represented; however, the H13 and H16 AIVs are not (Table 7.2).

The effects of location and season on subtype diversity have not been adequately investigated, but season appears to be the more important of the two. In North American ducks, the H3, H4, and H6 viruses are common during autumn staging and migration; however, they are poorly represented during spring migration, when the H7 and H10 subtypes predominate [81, 178]. Because prevalence is generally low outside of Delaware Bay, seasonal variation in subtype diversity has not been adequately investigated in shorebirds. In gulls, only the relationship of H13 and H16 with spring/summer breeding is apparent.

Subtype diversity can vary from one year to another. In ducks sampled in the autumn, subtype predominance shifts annually between H3 and H4 AIV [119, 194, 243]. The reason for this is not understood, but may relate to population immunity. The greatest amount of annual variation has been reported from shorebirds at Delaware Bay, where the predominant HA subtypes in a given year between 2000 and 2009 have included H1, H3, H4, H7, H9, H10, and H12 [119, 213]. This variation may be the result of limited virus introductions in a given year, as well as population immunity. In some years, the overall subtype diversity at Delaware Bay is very low, with few subtypes represented [13]. With the exception of H13 and H16 annual infections in gulls, there is little evidence for the presence or absence of annual subtype diversity patterns.

It is not yet understood why or how such subtype diversity is maintained in wild bird populations, or why certain HA subtypes predominate in a given avian population or season. In addition, we currently do not know why certain HA/NA subtype combinations (H1N1, H2N3, H3N8, H4N6, H5N2, H6N1, H7N3, H8N4, H9N2, H10N7, H11N9, H12N5, H13N6, and H16N3) are over-represented in reported isolates from ducks. This is a consistent observation in ducks sampled in Eurasia and North America (Table 7.2). These patterns may relate to structural advantages associated with the overall fitness of certain HA/NA combinations, population immunity, or both. These same subtype combination patterns are apparent for AIVs isolated from shorebirds (predominantly isolates from Delaware Bay) (Table 7.2), but possibly due to limited isolates outside of the H13 and H16 viruses are not consistent with gull isolates (Table 7.2).

Genetic diversity

A diverse genetic population of AIVs is maintained in wild birds. Contrary to earlier beliefs that these viruses were in a state of evolutionary stasis [68], more recent studies have revealed a more complicated picture with a high degree of genetic diversity and the simultaneous circulation of multiple genetic lineages within a given subtype [202]. The AIVs freely reassort within the wild bird reservoirs [46, 83, 102, 245], and genetic studies have clearly demonstrated distinct North American, Eurasian, and southern South American lineages or sub-lineages [43, 67, 165, 192, 202]. These broad-scale geographic differences suggest global isolation with infrequent mixing of viruses [141, 202]. This pattern is somewhat unexpected in view of the migratory behaviors of many avian species that can be infected with AIV and annually move between these continents.

The presence of a variety of Eurasian AIV genes in North American isolates originating from ducks, gulls, and shorebirds has been reported, and areas have been identified in the north Pacific and Atlantic where mixing of Eurasian and North American AIVs is more likely to occur [47, 58, 74, 118, 132, 164, 173, 174, 244]. Although these studies support gene flow between continents, there are no instances to date where a complete "foreign" AIV has been detected in these populations. Even if introduced, such transmission events may have limited persistence within these new populations [179]. In addition, there is little indication of individual exotic genes persisting in the "new" populations once they have been introduced [120]. A notable exception relates to the Eurasian H6 gene that displaced the original North American H6 [12]. This appears to have been a long-term process, and the mechanisms underlying this displacement and the potential consequences of this introduction have not been determined. A recent example of "new" viral genes being incorporated

into the North American AIV gene pool involves the H14 viruses. The H14 virus was first detected in North America in 2010, at which time it was of mixed Eurasian and North American lineage. Sequencing of subsequent isolates revealed extensive reassortment with North American genes, and only the H14 HA gene has persisted [17, 57, 176]. A successful introduction of a North American H10 HA gene to Australia has also been reported [236].

From the collective literature it appears that intercontinental AIV gene movements do occur, and that although rare, they can be successful. In contrast, with the possible exception of recent events associated with H5N8 HPAIV, there is a lack of evidence of Eurasian or North American virus introductions across continents via migrating wild birds.

Mechanisms for AIV maintenance and transmission

The transmission cycle

Transmission of AIV in wild bird populations mainly occurs via a fecal/oral route [89, 190, 195]. In ducks, replication occurs primarily in the intestinal tract [197], and high concentrations of infectious virus are shed in feces [91, 241]. It has been suggested that contaminated water or sediments are a possible source of infection. This view is supported by a host range that includes numerous water birds, and the isolation of these viruses from surface water utilized by these birds [75, 90, 108, 213]. In addition, mallards can be infected via various routes (intranasal, intratracheal, intraocular, intracloacal, and intra-ingluvial) that could involve contact with infective water [55]. Virus-contaminated surface and ground water have both been suggested as long- and short-term sources of AIV that is introduced into domestic poultry populations [76].

Transmission mechanisms associated with other avian groups, such as the shorebirds and gulls, are less well understood, but, as with ducks, shedding is associated with both the cloacal route and the oropharyngeal route, and virus can be isolated from feces.

Host factors that affect susceptibility and viral shedding

There are numerous host factors that drive the potential for infection, viral shedding, and contact with the environment (Figure 7.1). As previously stated, many species of wild birds are susceptible to AIV, but the predominant route of shedding, the duration of shedding, and the amount of infectious virus that is excreted vary between species. In ducks, prolonged viral shedding for more than 28 days has been reported [44]. Although such extended shedding is possible, it is probably exceptional, and in experimental infections of mallards the duration of shedding is generally less than 14 days, with most virus being excreted 2–6 days post infection [27, 33, 84]. In the field, however, such estimates may not be applicable to birds where immunity from recurrent infections is common. This may partially explain why estimates based on field data indicate a much shorter shedding period for mallards, with mean minimum and maximum

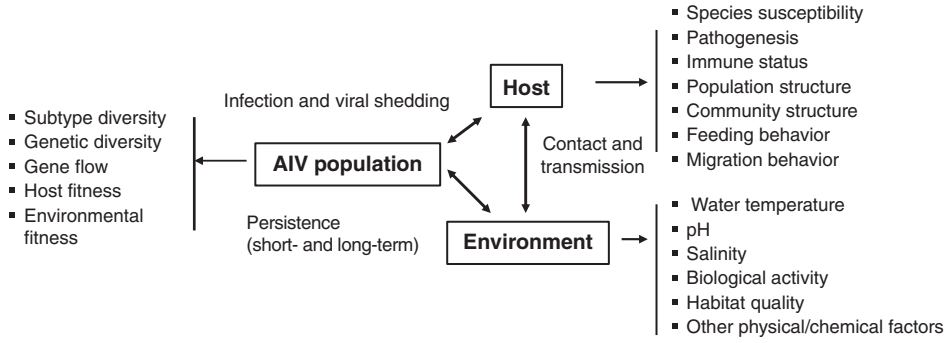

Figure 7.1 Variables that affect the host, agent, and environmental components of the AIV maintenance cycle in wild bird populations.

estimates ranging from 3.1 to 8.3 days [122]. These effects on susceptibility and shedding potentially extend to the population level, especially with regard to long-lived species such as geese. In the case of Canada geese, few infected birds are detected, but antibody prevalence is generally high [115]. This suggests that although most birds are infected during their lifetime, they may contribute little to annual transmission.

Environmental factors that affect viral infectivity

The importance of environmental persistence of AIV in the transmission and maintenance of these viruses is still unclear [106, 187, 212]. The initial investigation of environmental persistence of AIV [242] demonstrated that an AIV could remain infective in feces or water for extended periods of time. Subsequent research has confirmed this finding with many other AIV strains and subtypes [20, 25, 138, 208, 209], as well as demonstrating that decreased water temperature, neutral pH, and low salinity, within naturally occurring ranges, are important factors that enhance infectivity [25, 112, 150, 209]. Fluctuations in temperature [128], freeze–thaw cycles [212], and ammonia concentrations [113] also reduce the duration of infectivity. Certain biological factors in aquatic habitats have been shown to affect the duration of AIV infectivity in water, including filtration and inactivation by clams [49] and intact biologically active water [42], and possible bioaccumulation in tadpoles [99] and water fleas [1]. Feathers in contact with feces or contaminated water can also serve as indirect fomites for these viruses [129]. These studies highlight the complexity of the reasons for the persistence of AIV in the environment, our understanding of which is currently incomplete.

The AIV maintenance cycle

It is not known how AIVs and the subtype diversity present in these virus populations are maintained. Although characterization of these mechanisms is an extremely complex and challenging task, it is critical to understanding viral evolution and the risks associated with emergence of important viruses such as the HPAIV Eurasian H5N1. It is probable that the maintenance cycle is dependent on (i) the combined contributions of continual bird-to-bird transmission through multi-host and spatio-temporally disconnected avian populations, (ii) migratory behaviors that mix these hosts, and (iii) environmental persistence in aquatic habitats enabling AIVs to be maintained on a short-term and possibly long-term basis in the absence of a susceptible host. This proposed cycle is based on a requirement for naive birds that can be infected throughout the year, with connectivity provided by migration and shared habitats. Transmission on breeding, staging, and wintering areas is well established, as is the movement of AIV southward and northward during autumn and spring migrations, respectively. A potentially interesting and poorly understood portion of this proposed cycle relates to individual species contributions, which may be related to migratory or other specific behaviors. This has been suggested for blue-winged teal (*Anas discors*), which are early migrants that are not present in northern areas when AIV prevalence rates peak in other duck species [207], and for their ecological equivalent, the garganey (*Anas querquedula*), in Europe and Africa [40]. This migratory behavior may provide a susceptible population for virus maintenance on wintering grounds. These same multispecies relationships may exist with gulls and shorebirds, and may partially link these reservoirs with the duck reservoir.

As for environmental reservoirs, the isolation of numerous subtypes of AIVs from natural waters and sediments [187] and the demonstration of very long-term persistence in water under experimental conditions provide the basis for this hypothesis. Although it is possible that these viruses are maintained in waterfowl breeding habitats from year to year, and that such persistence is needed for successful maintenance [18, 180], it is more probable that environmental persistence serves as a short-term source of residual virus that links departed and arriving birds during migration. This potential contributory role in the AIV maintenance cycle requires further study, and may be important in defining how viruses move between migratory and locally mobile species as well as populations that may have limited direct contact or that are temporally disconnected [86]. It may also be extremely important in elucidating how these viruses move between wild and domestic hosts.

Viruses shared between poultry and wild birds: an H5N1 HPAI case study

Transmission of LPAIV from wild birds to domestic poultry is well documented, and such exchanges of H5 and H7 LPAIVs set the stage for the evolution of HPAIV [147]. Prior to 2002, there was only one reported isolation of an HPAIV from free-living wild birds that were not known to be associated with infected domestic fowl. This was the 1961 report of H5N3-infected common terns in South Africa [14]. This situation changed dramatically as a result of the H5N1 HPAIV outbreak in Eurasia. In 2002 and 2003, H5N1 HPAIV was isolated from both captive and free-living birds in Hong Kong [48]. During and after 2005, when these viruses apparently spread via migratory birds, isolations of H5N1 HPAIV from more than 50 species of wild birds were reported in Asia, Africa, and Europe [231]. In Europe, H5N1 HPAIV was detected in wild birds until 2009 [50]; in Asia such reports have continued.

Although the suggestion has repeatedly been made, there is no evidence that the H5N1 HPAIV spillover into wild birds resulted in a wild bird reservoir for these viruses. Once introduced into these populations, however, there is abundant evidence that some HPAIV introductions were associated with wild bird migration and local movement [188].

The wild bird species from which H5N1 HPAIV was isolated included species in the Anseriformes, Charadriiformes, Ciconiiformes (herons and storks), Columbiformes (pigeons), Falconiformes (raptors), Galliformes (quail and pheasants), Gruiformes (coots and moorhens), Passeriformes (perching birds), Pelecaniformes (cormorants), and Podicipediformes (grebes) [31, 48, 132, 137, 144, 188, 211, 231]. These positive species fell into three broad categories. The first group included aquatic birds, and this group was primarily composed of Anseriformes. The second group included raptors, and other species such as crows which potentially either predate or scavenge other birds (wild or domestic). The final group included numerous passerine species that often exist in peridomestic settings. The involvement of water birds, especially ducks and gulls, was consistent with the known natural history of AIV. However, affected species included a disproportionate number of swans, geese, and diving ducks. These waterfowl groups are under-represented with regard to reported LPAIV isolations. Wild raptors and scavengers are not generally associated with naturally occurring LPAIVs, and this may be related to the lack of morbidity and mortality associated with LPAIV-infected birds that serve as potential food for these species. With regard to passerines, which are not normally associated with LPAIVs, most infections probably resulted from captive birds entering markets or being directly exposed to infected poultry flocks [200].

There have been numerous experimental infections of wild bird species with H5N1 HPAIV [19, 21–23, 71–73, 111, 131, 152, 163, 167–169, 216], and these studies have consistently shown a broad range of susceptibility and viral shedding that is primarily associated with respiratory rather than alimentary tract infections. The clinical response in both ducks and gulls is related to the lineage of the H5N1 HPAIV [19, 168, 216], and the clinical outcomes can vary between closely taxonomically related species. This was demonstrated both in North American ducks, including mallard, blue-winged teal, redhead (*Aythya Americana*), wood duck (*Aix sponsa*) and Northern pintail (*Anas acuta*) [19], and in Eurasian ducks, including tufted duck (*Aythya fuligula*), common pochard (*Aythya ferina*), mallard, common teal, Eurasian widgeon (*Anas Penelope*), and gadwall (*Anas strepera*) [111], as well as in geese and swans [22]. Variation in the extent and duration of viral shedding was also highly variable between species, but viral titers (especially in cloacal swabs) were generally low compared with LPAIV. Observed viral titers were related to clinical response, with the highest titers associated with birds with the most severe clinical disease [19, 111, 216]. In swans, variation between species related to the time of onset of clinical disease and the duration and extent of viral shedding was also apparent [22]. There has been some limited research on the effects of pre-exposure on H5N1 HPAIV infections [15, 36], and the results demonstrated that pre-exposure with a homologous and heterologous LPAIV can reduce both clinical severity and viral shedding.

As with LPAIV, the risk of infection and the potential for viral maintenance in avian reservoirs are dependent on both susceptibility and potential

contact, and the latter can be greatly influenced by species behavior. With "new" viruses such as H5N1 HPAIV, the risk of virus movement within individuals and populations needs to be understood, and in this case host range, population size, and migratory behavior are very important [50, 63, 175]. Many studies related to H5N1 HPAIV have either been ornithology based or have incorporated extensive ornithology data and techniques. The use of satellite telemetry is a good example. Numerous studies have used telemetry [66], sometimes in concert with influenza testing, to determine points of potential contact with infected poultry flocks [218], to connect or disconnect areas where outbreaks have occurred in poultry [60, 171], and to determine the ability of a specific host species to move these viruses [154].

Collectively, the H5N1 HPAIV experimental and field studies clearly indicate that these viruses can move with some migrating bird populations, and contribute to local spread [65], and that peridomestic birds may infected on a local scale. These studies do not support the establishment of a wildlife reservoir for these viruses, and it is most likely that domestic rather than wild ducks represent the true reservoir [30, 103]. The failure of these viruses to establish in wild bird reservoirs most probably relates to a reduced potential for virus shedding and transmission associated with their adaptation to domestic hosts and their environments, migration timing [125], and possibly to dilution or population immunity, as these wild populations are already home to numerous LPAIVs. That said, it is likely that the involvement of wild birds in the epidemiology of these viruses would be negligible if contact with infected domestic poultry was eliminated.

This is not the first HPAIV to have been capable of replicating in wild bird species, and with more recent viruses, such as H5N8 HPAIV, which has been isolated from both wild and domestic birds in Asia [133], Europe, and North America, and the H7N9 LPAIVs that have infected humans in China [239], questions relating to wild bird involvement with such viruses will continue to arise.

Future directions

At present our understanding of AIV in natural avian reservoirs is extensive but in no way complete. As shown in Figure 7.1, there are numerous variables that potentially affect and possibly regulate these viruses within the AIV population, avian hosts, and the environment. An understanding of these interactive associations is needed in order to answer basic questions related to viral maintenance in wild bird populations associated with both natural and man-made systems, to understand subtype diversity, to assess the potential for successful AIV invasion of new hosts or new geographic areas, and to understand the risks associated with the movement of these viruses to domestic animal hosts or humans.

Among the host factors listed, population immunity is the least well understood and merits further study. Potential effects associated with community structure, population structure, and migration are also important, and have been insufficiently studied to date. These effects are critically important for understanding the epidemiology of viruses that can be shared between wild and domestic populations.

The AIV population is well defined, and the significance of subtype is understood in relation to pathogenesis (H5/H7), host restriction (H13/H16), or predominance (H3/H4). However, it is not yet known why these subtypes evolved and how they are individually maintained. Wild birds, especially ducks and gulls, offer a unique opportunity to understand how diverse AIV subtypes interact and compete within a population. From a more applied perspective, an understanding of other factors such as subtype seasonality and host associations could greatly improve the efficiency of future surveillance efforts. The genetic variability present in the wild bird AIV gene pool is also well documented, and numerous studies have demonstrated intercontinental movement of viral genes as well as the successful introduction of such genes. However, as the factors associated with these successful introductions are not known, we do not yet have a complete understanding of the risks associated with the movement of specific viruses such as the Eurasian H5N1.

The precise roles of environmental persistence and potential environmental reservoirs are still speculative but nevertheless important. If such

persistence is long term, this "behavior" could influence both the genetic structure of the AIV population and the spatial and temporal distribution of these viruses in the host populations. If environmental persistence is limited to the short term, it may be a necessary component of the maintenance cycles of both LPAIV and HPAIV. Relatively few studies have addressed the potential abiotic and biotic factors that affect the environmental persistence of these viruses. In addition, field-based studies or more complex experimental systems that better reflect natural systems are needed in order to better understand this subject.

Finally, due to the rapidly changing environments and populations associated with both wild and domestic birds worldwide, our knowledge of the epidemiology of AIV will continue to evolve and challenge our understanding of these complex systems and interactions. This is especially true in areas where there is increasing contact between wild and domestic populations.

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8 The global nature of avian influenza

David E. Swayne

Introduction

Avian influenza virus (AIV) is a global virus that knows no geographic boundaries, has no political agenda, and can infect commercial and non-commercial poultry, indoor- and outdoor-reared poultry, pet birds, captive-bred and feral wild birds, birds in zoological collections and reserves, and a variety of other avian and non-avian species. AIVs have been isolated from poultry, captive birds, and wild birds in Africa, Asia, Australia, Europe, North and South America, and most recently, in Adele penguins in Antarctica [70, 94, 185]. However, reports of AIV infections and the diseases that they cause in domestic poultry and other birds vary among individual countries, regions, and continents. The reported frequency of AIV is greatly influenced by the availability of accurate assays and diagnostic capacity, thoroughness of surveillance programs, the type of birds and production sector sampled, the time of year, geographic location, climatic conditions, and other undefined factors. However, failure to conduct adequate surveillance and diagnostics on poultry and other birds should not be construed as evidence to support the absence of AIVs or their associated infections in birds within a country.

For example, diagnostics and surveillance in the USA prior to the 1983–1984 H5N2 high-pathogenicity avian influenza (HPAI) outbreak were undertaken mostly on commercial poultry and the occasional backyard flock using virus isolation in embryonating chicken eggs and serology with the agar gel immunodiffusion (AGID) test on disease cases submitted for diagnosis (i.e. passive surveillance). Therefore between 1964 and 1985 the list of isolates (all low-pathogenicity avian influenza (LPAI) viruses except for the 1983–1984 HPAI viruses) or anti-influenza A antibodies was primarily from range-reared turkeys and of limited number [193]. However, with the identification of LPAI in the live poultry market (LPM) system in the north-eastern USA during 1986, and reports in subsequent years from other non-traditional avian species, such as ratites and game birds, and the elimination of range rearing of turkeys, routine active surveillance has increased and the number of AIV isolations has grown geometrically, with the LPM birds and non-traditional poultry species becoming a major source of LPAI viruses (LPAIVs) [195]. The LPM system is typically supplied with poultry from small, specialized mixed bird farms and not from the integrated commercial poultry production systems, except for spent laying chickens. Movement of personnel from LPM on and off commercial farms to obtain spent layers has been a primary transmission point of H7N2 LPAIVs from LPM to commercial poultry [211].

Active serological surveillance (i.e. monitoring of H5/H7 AI) is conducted annually on over 95% of broiler, layer, and turkey breeder flocks in the USA through the National Poultry Improvement Plan (NPIP) (A. Rhorer, personal communication, March 2, 2007). This program began in 1998, when it was limited to commercial broiler and layer breeder flocks, with a modest expansion in 2000 to include commercial turkey breeders, and a major expansion in September 2006 to include meat chickens and turkeys at slaughter plants, representing coverage of over 90% of meat birds in the USA through the NPIP monitoring program. Since 2007, the numbers of surveillance tests have remained at a high level (Figure 8.1). In addition, the National Chicken Council has a preslaughter testing program that covers the majority of commercial broiler flocks.

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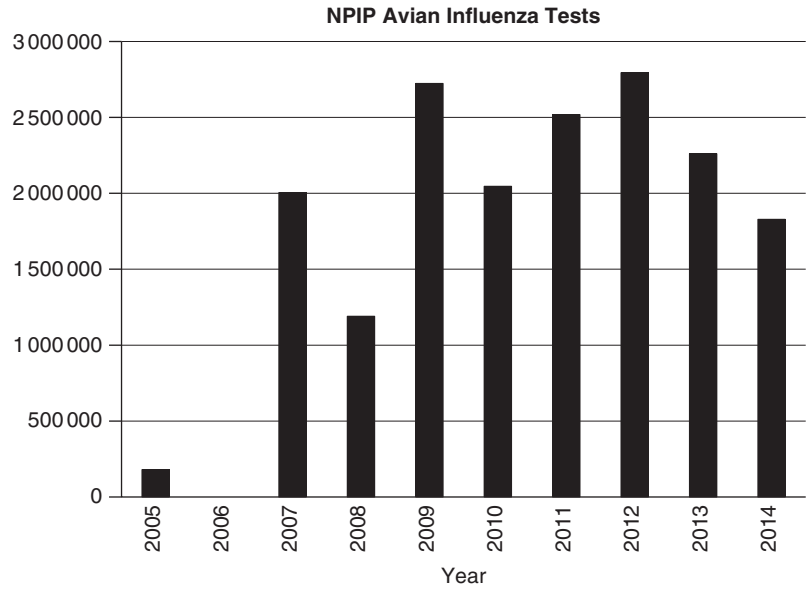

Figure 8.1 Numbers of avian influenza surveillance tests conducted under the National Poultry Improvement Plan in the USA for the years 2005 and 2007–2014.

An example of recent testing in the USA was in 2014, when 1 397 400 AGID serological tests were performed using reference reagents from the National Veterinary Services Laboratories. These yielded 1578 positive sera, of which 97% were from turkeys against H1 or H3 with N1 or N2 antibodies, indicative of vaccination against swine influenza viruses, and the other 3% were antibodies against H2 (NH: chicken; MN: turkey), H4 (MN: turkey), H5N2 (PA: commercial turkey), H6 (PA: chicken; TX: mixed), H6N8 (MN: turkey), and H7 (NY: pet chicken; NV: Sage Grouse) [196]. In the LPM system, a total of 658 virological samples that were examined for AIV yielded 11 LPAIVs [196]. However, these results do not indicate that the USA has a higher frequency of LPAIV than other countries, but only that testing of large numbers of samples from high-risk sectors of poultry yielded AIVs and/or anti-AI antibodies. As surveillance expands in other countries and continents, and as diagnostics improve, new sources of AIVs will be identified.

General history

To understand the global nature of avian influenza, we must recognize the changing definition of avian influenza infections and the diseases that they cause, based on scientific improvements in diagnosis and an increased knowledge and understanding of ecology and epidemiology over the past 125 years. Historically, five major events have changed our definition of avian influenza and thus had an impact on the reported frequency of avian influenza worldwide:

1. early diagnosis of fowl plague in domestic poultry was based primarily on clinical features, lesions, and animal studies
2. recognition of LPAIVs and their infections in domestic poultry was based on serology and virus isolation
3. the discovery of avian influenza in asymptomatic wild bird reservoirs
4. the discovery that HPAIV can arise from mutation of H5/H7 LPAIV
5. the unprecedented global spread of the H5N1 A/goose/Guangdong/1/1996 (Gs/GD)-lineage HPAIV, including reassortment of various genes and episodic periods of wild bird transmission.

Fowl plague in poultry

In historical terms, avian influenza has only relatively recently been described in the poultry health literature, with historical records reporting the first cases as a highly lethal, systemic disease of chickens (i.e. HPAI) in Italy during 1878 [133]. This severe systemic disease has most frequently

been termed "fowl plague" or "fowl pest", but other names have also been used, including peste aviaire, Geflugelpest, typhus exudatious gallinarium, Brunswick bird plague, Brunswick disease, fowl disease, and fowl or bird grippe, among others [75, 163, 164]. The current official terminology, "highly pathogenic avian influenza (HPAI)", was adopted in 1981 [17], and "high pathogenicity" is an equivalent grammatical variant of "highly pathogenic" (i.e. the two terms can be used interchangeably) [185].

From the 1870s to the early 1900s, fowl plague spread from Northern Italy into Europe, with reports in Austria, Belgium, France, Germany, Great Britain, Hungary, The Netherlands, Romania, Russia, and Switzerland [46, 80, 89, 92, 163, 164]. By the mid-twentieth century, fowl plague had been diagnosed in North Africa (Egypt), the Middle East, Asia (China and Japan), South America (Argentina and Brazil), North America (the USA), and much of Europe. Fowl plague was endemic in parts of Europe and Africa into the 1930s [9]. Interestingly, in many situations fowl plague disappeared at the same time as Newcastle disease was recognized as a disease of poultry. For example, fowl plague was reported in Italy into the early 1930s but had disappeared by 1937, when the epidemic of Newcastle disease began [134]. The discovery of Newcastle disease (also known as "pseudofowl pest"), and its similarity to fowl plague in terms of lesions, clinical presentation, and high morbidity and mortality rates, resulted in confusion over the diagnosis of field cases and their respective viral etiologies. In some cases, the HPAI term "fowl pest" was used interchangeably with "Newcastle disease."

It was first ascertained that fowl plague was caused by a filterable virus in 1901, but the virus remained unclassified until 1955, when it was determined to be an influenza A virus [38, 141]. The pre-1959 outbreaks of fowl plague were caused by what are classified today as H7N1 and H7N7 HPAIVs [49]. However, at the time it was generally considered that the fowl plague viruses were all the same, as the antigenic and genetic differences between "individual strains" were unknown (D. Alexander, personal communication, February 27, 2007). As a result, viruses that were exchanged between laboratories may not have kept their original names, or were renamed for the shipping laboratory, and were not maintained as pure passage cultures of the original material. Today, any conclusions concerning the source and date of many historical fowl plague isolates must be interpreted with caution. For example, Petek states that the virus FPV-(fowl plague virus)-Brescia was isolated in 1935 and has been erroneously called Brescia/02, but should be correctly labeled A/chicken/Brescia/35 (H7N1) [134]. However, there are references to FPV-Brescia that pre-date 1935 [33], and some samples obtained before 1935 may have been correctly maintained as A/chicken/Brescia/02 [134] (D. Alexander, personal communication, February 27, 2007).

The early fowl plague cases in chickens and other gallinaceous poultry were diagnosed primarily on the basis of sudden high mortality, the presence of specific lesions (e.g. cyanotic combs, hemorrhage in the ventriculus and proventriculus, and petechiae on the heart), and identification of a filterable virus [75, 183]. Such virus isolates cross-reacted in hemagglutination inhibition (HI) tests using antisera from recovered birds, which led to the conclusion that a positive "H7" HI test indicated a fowl plague virus (HPAI) or infection by such an agent. However, in 1959, 1961, and 1966, clinical disease that was indistinguishable from classic fowl plague (i.e. H7) was identified in chickens, common terns, and turkeys, respectively, but these viruses were not inhibited by antisera from fowl plague-recovered birds in standard HI tests (i.e. the viruses were not H7, but H5). Thus the original fowl plague virus infections in poultry were detected by severe clinical disease and linked serologically to two subtypes, namely H5 and H7 HPAIVs.

These early HPAI (fowl plague) outbreaks have been covered in detail in Chapters 7 and 8 [75, 183] of the first edition of this book [188].

Recognition of low-pathogenicity avian influenza in poultry and other man-made systems

Mild clinical forms of avian influenza (i.e. those that produced respiratory disease and a decrease in egg production) were first recognized in various domestic poultry species in 1949, with occasional reports through to the mid-1960s [46]. These forms have been termed low-pathogenic, pathogenic, mildly pathogenic, and non-highly pathogenic avian influenza. In 2002, at the Fifth International Symposium on Avian Influenza, the

term "low pathogenicity (LP)" was adopted as the official designation for AIVs of low virulence (i.e. AIVs that did not meet the biological or molecular criteria for HPAIVs) [56].

The earliest known LPAIV was the "Dinter" or "N" strain, isolated in 1949 from chickens in Germany (i.e. A/chicken/Germany/49 [H10N7]). However, it was not discovered that this virus was an LPAIV until 1960 (for a review, see [46]). Between 1953 and 1963, LPAIVs were isolated from a series of respiratory disease cases in domestic ducks in Canada, Czechoslovakia, England, and the Ukraine. This was followed by descriptions in turkeys from 1963 to 1965 of LPAIV infections being a cause of respiratory disease and falls in egg production in Canada and the USA. However, additional cases of LPAIV were not described again in chickens until 1966, in Italy, along with the first cases in pheasants and quail. Throughout the latter half of the 1960s, reports of respiratory disease and isolation of LPAIVs were common in turkeys and domestic ducklings. These early LPAIVs were a variety of hemagglutinin (HA) and neuraminidase (HA) subtypes.

Initially, H5 and H7 subtypes of influenza A virus were only associated with fowl plague viruses, but in 1966 and 1968, LPAIVs were isolated from turkeys with low mortality or ill-defined syndromes that were typed as the H5 subtype, and in 1971 an LPAIV that was subtyped as H7 was isolated from a turkey flock in Oregon that experienced mild respiratory disease with diarrhea [8, 25, 46, 158]. Since 1971, numerous H5 and H7 LPAIVs have been isolated and characterized, thus dispelling the myth that subtypes H5 and H7 equate with HPAIV. In fact only a small proportion of the diverse H5 and H7 AIVs are highly pathogenic; most of them are LPAIVs [8, 49, 65]. Furthermore, the development of the AGID serological test in 1970, and its subsequent adoption as the primary international test for identifying AIV-infected chicken and turkey flocks, expanded the identification of LPAIVs in the 1970s and 1980s [23, 24].

Low- and high-pathogenicity avian influenza viruses in wild birds

Although early reports of fowl plague suspected that there was involvement of wild birds in transmission of the disease, the first definitive proof of AIV infection in wild birds was in common terns with high mortality in South Africa during 1961 [26]. In the late 1960s, a survey of migratory waterfowl showed serological evidence of infection by AIVs [45]. However, the first isolates of LPAIVs were not obtained until 1972, from migratory ducks in a Newcastle disease virus surveillance program in California [156] and from a pelagic seabird (shearwater) in Australia [43]. Since then, numerous surveys have been conducted, mostly in North American birds, and have demonstrated asymptomatic infection by AIVs in healthy wild aquatic birds, principally in the orders Anseriformes and Charadriiformes [65, 66, 79, 160–162]. Since the initial reports, extensive surveys have been conducted in Russia, Israel, China, Europe, and other countries [83, 85, 122, 150]. These surveys have yielded tens of thousands of LPAIVs of all 16 HA and 9 NA subtypes from asymptomatic wild birds (Chapter 7, Table 7.2). However, some HPAIVs have been isolated from wild birds: (i) during an epidemic with high mortality in common terns, A/tern/South Africa/61 (H5N3); (ii) single isolations of A/finch/Germany/72 (H7N1), A/gull/Germany/79 (H7N7), and A/peregrine falcon/UAE/2384/98 (H7N3); (iii) during the recent H5N1 (plus reassortant H5N8 and H5N2) HPAI panzootic in Asia, Europe, Africa, and North America, with multiple isolations from over 50 species of wild birds; and (iv) great-tailed grackle (*Quiscalus mexicanus*) and barn swallow (*Hirundo rustica*) with H7N3 in Mexico during 2012 [10, 26, 39, 72, 84, 86, 110, 198]. The ecology of AIVs in wild birds has been covered in Chapter 7 of this book, and will not be discussed further here or in later chapters. Gallinaceous species of birds, both domestic and wild, are not natural reservoirs of AIVs [127, 166].

High-pathogenicity avian influenza viruses arise from mutation of H5 and H7 low-pathogenicity avian influenza viruses

The HPAIVs do not circulate in the primordial reservoir (i.e. migratory aquatic birds) or in poultry as long-term established virus lineages, but all HPAIVs have arisen from mutation of LPAIVs based on

specific changes to the proteolytic cleavage site of the H5 and H7 HA when these LPAIVs are allowed to circulate unchecked in poultry populations [116, 136]. In general, the H5 and H7 LPAIVs have only two non-consecutive basic amino acids at the carboxy-terminal end of the HA1, whereas HPAIVs have specific changes at this site which can include substitutions of non-basic with basic amino acids, insertions of multiple basic amino acids from codons duplicated at the HA cleavage site, short inserts of basic and non-basic amino acids from an unknown source, non-homologous recombination with inserts which lengthen the HA proteolytic cleavage site, but which may or may not contain additional basic amino acids, and the presence or absence of a specific glycosylation site at the amino-terminal end of the HA1 protein that can shield the cleavage site [34, 57, 68, 76, 126–128]. Detailed information about the impact of the HA proteolytic cleavage site on the cellular biology of HPAIVs and LPAIVs has been covered in Chapter 1 of this book.

Global spread of the H5 Guangdong-lineage high-pathogenicity avian influenza virus

The first outbreak of the H5N1 Gs/GD-lineage HPAIV was in domestic geese in China during 1996, and it was quickly followed by outbreaks in the LPM system of Hong Kong during early 1997 [151, 210]. Initially, the viruses were poorly infectious for domestic waterfowl, and failed to cause disease [131, 132]. However, by 2002, H5N1 HPAIV had emerged that infected and caused high mortality in a variety of captive-reared duck species [52]. From late 2003 onward, the H5N1 HPAIV extended into neighboring Asian countries, infecting primarily poultry but also sporadically affecting wild birds and some mammals [153]. Several waves of the H5 Gs/GD lineage of HPAIV infected and were spread by wild birds in 2005, 2010, and 2014 [3]. This led to dispersion to Europe and Africa in 2005–2006, reinfection in Japan during 2010, and re-spread to Europe and, for the first time, to North America in 2014 [3, 72].

The global spread of the H5 Gs/GD -ineage HPAIV is described in detail in Chapter 9 of this book, and also in Chapter 11 of the previous edition [154].

Regulatory aspects

Since the early descriptions of fowl plague, various governments and other entities have practiced eradication as the primary means to deal with HPAI and protect the food supply [197]. Initially, eradication programs focused on identifying HPAIV using *in-vivo* chicken pathogenicity tests (i.e. pathotype) and differentiation of these viruses from LPAIVs. However, in 1994, specific molecular and *in-vitro* criteria were added as alternatives to *in-vivo* testing to define HPAIV [194]. Today, the World Organization for Animal Health (Office International des Epizooties [OIE]), an intergovernmental organization, sets the international sanitary and health standards for animals, including avian influenza, and such codes are used to safeguard international trade in poultry and poultry products. Using the OIE Code and other standards, avian influenza can be divided into three categories:

1. HPAI (formerly HP notifiable AI, i.e. HPNAI), which includes all H5 and H7 HPAI
2. all H5 and H7 LPAI (formerly LP notifiable AI, i.e. LPNAI)
3. all other LPAI that are not notifiable to OIE (H1–4, H6, and H8–16 LPAI), but may be reportable to national and state/provincial authorities [12, 117].

However, based on pathobiological characteristics (e.g. disease, lesions, and signalment), all LPAI are indistinguishable except that some H5 and H7 LPAIVs have shown the ability to change to HPAIVs, which is the reason for their listing as international viruses for control. The definitions of HPAIV and H5/H7 LPAIV are as follows [117]:

1. HPAIVs have an intravenous pathogenicity index (IVPI) in 6-week-old chickens greater than 1.2, or alternatively cause at least 75% mortality in 4- to 8-week-old chickens infected intravenously. H5 and H7 viruses which do not have an IVPI greater than 1.2 or that 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 hemagglutinin molecule (HA0). If the amino acid motif is similar to that observed for other high-pathogenicity avian influenza isolates, the isolate being tested should be considered to be HPAIV.

2 LPAIVs are all influenza A viruses of H5 and H7 subtype that are not HPAIVs.

Although the *in-vivo* pathotyping test is based on testing only chickens, most AIVs give similar *in-vivo* test results when related species of gallinaceous birds are used [4, 130]. By contrast, most AIVs that are highly lethal or HP for chickens have produced no infections or asymptomatic infections in the domestic duck, except for some strains of the Eurasian H5N1 HPAIV which are also highly lethal, depending on the virus strain, for young to adult domestic ducks [5, 73, 123, 131]. Pathogenicity test results are specific for the host used in the test [185].

Terminology

The terminology used to describe avian influenza varies with individual reports, publications, lectures and other media forms used to disseminate information. In the OIE Terrestrial Code, "LPAI" refers only to H5/H7 LPAI. However, throughout this book, the term "LPAI" will be used to indicate any of the 16 HA subtypes of LPAI. Other terms may vary in various publications and in the chapters which follow. For example, the term "outbreak" can be used to mean a single farm, as in Italy, or it could refer to the complete epidemic involving a strain or lineage of virus in a country or region. A "case" typically means a single diagnostic submission from a farm, or an outbreak on a single farm.

Low-pathogenicity avian influenza in poultry and captive birds

There is neither an international mandate nor uniform standards used around the world for LPAI surveillance, and there are no requirements to report LPAI to OIE other than H5/H7 LPAI in poultry. Therefore published reports of LPAI are sporadic and infrequent, most being published in peer-reviewed scientific literature concerning single cases or clusters of cases. However, several national and international organizations have attempted to compile reports on LPAI and LPAIV infections from individual countries, regions, and continents, especially the first to the ninth International Symposia on Avian Influenza (1981–2015) [18, 47, 48, 136, 169–171, 176, 179, 182]. Surveillance for avian influenza and reporting has been most common from the USA, the European Union, Australia, and Canada, with sporadic reports from other countries. Some countries lack the veterinary diagnostic infrastructure or financial resources to conduct adequate diagnostics and surveillance for LPAI, or they give LPAI low priority compared with other animal diseases, while other countries adopt a policy of "Do not look and you do not have AI."

Humans have developed new avian anthropocentric systems through captivity, domestication, rearing of birds at the agriculture–wild bird interface, non-industrial and industrial agriculture, national and international commerce, and non-traditional raising practices [177, 184]. AIV can survive and be perpetuated in a variety of different categories of man-made ecosystem [177, 185], including the following:

1 bird collection, trading, maintenance, and exhibition systems
2 village, backyard, and hobby flocks, especially outdoor rearing and mixing of bird species
3 live poultry market (LPM) systems
4 range- or outdoor-reared commercial poultry
5 integrated indoor commercial poultry.

The frequency of LPAIVs in domestic poultry, captive birds, and wild birds is largely unknown, but in most developed countries, infections are sporadic in poultry, being most frequent in chickens, turkeys, and ducks [8, 10]. However, in the integrated commercial poultry systems in developed countries, avian influenza has been a rare occurrence in view of the 25–30 billion chickens raised each year [192]. Cases have been reported in captive wild birds kept as caged pets, or in quarantine stations, private collections or reserves, and zoological parks [8, 10].

For poultry, the reported frequency is highest in birds raised on small mixed-species farms with outdoor access (village and rural poultry), or those raised for the LPM systems, which typically use few veterinary services, have poor control of bird movement, and lack biosecurity. However, the incidence and distribution vary widely with

geographic region, country, species and age of bird, time of year, and the environmental or agricultural system occupied [185]. A few examples of LPAI in different man-made systems will be discussed next.

Examples of low-pathogenicity avian influenza in man-made systems

Historically, LPAIVs have been reported in range-reared turkeys in Minnesota, USA, following exposure to LPAIV-infected wild waterfowl during the autumn staging and migration south for the winter [61]. However, the number of infected turkey flocks has varied from one year to the next, ranging from only two flocks in 1983 to 141, 178, and 258 flocks in 1978, 1995, and 1988, respectively [62]. To eliminate this problem, the Minnesota turkey industry decided in 1998 to eliminate outdoor raising of turkeys, which resulted in reduced numbers of cases, with only 33 influenza A-infected flocks between 1996 and 2000; most of these were infections with H1N1 swine influenza viruses and not LPAIV infections [63]. This low rate of influenza A virus infection of commercial turkeys in Minnesota continued through 2016. In addition to direct exposure increasing infection rates, turkeys have a greater susceptibility to wild bird influenza A viruses, which have contributed to more cases in turkeys than in chickens [185].

Various subtypes of LPAIVs have been isolated from poultry in the LPM system of the north-eastern USA (from 1986 to the present), but since the implementation of a control program in 2002, the rate of infection has declined from a high of 60% of the markets to below 20%, with less than 1% of the samples from markets being positive for LPAIVs in 2008 [95, 190, 196]. However, these LPMs and the small farms that supply the birds have become a major reservoir of H5/H7 LPAIVs in the USA, and have served as the source of LPAIVs that crossed over to infect small and large commercial flocks [143, 144]:

1. H5N2 LPAIV in Pennsylvania that infected 100 commercial flocks during 1983, and mutated to H5N2 HPAIV [58, 204]
2. H5N2 LPAIV that infected 21 flocks in New York, New Jersey, Massachusetts, and Ohio during 1986 [58]
3. H7N2 LPAIV that infected 24 commercial poultry flocks in Pennsylvania between 1996 and 1998 [41, 159, 181, 211]
4. H7N2 LPAIV that infected seven commercial poultry flocks in Pennsylvania during 2001–2002 [44, 159, 181]
5. H7N2 LPAIV that infected 210 commercial flocks in Virginia, West Virginia, and North Carolina during 2002 [143, 147, 159, 181]
6. H7N2 LPAIV that infected 3.9 million chickens in a large layer company (four farms) in Connecticut during 2003 [143, 181]
7. H7N2 LPAIV that infected 32 000 layers in a single flock in Rhode Island during 2003 [143, 181]
8. H7N2 LPAIV that infected three broiler flocks in Delaware and Maryland during 2004 [146].

No LPM system-linked infections of commercial poultry were identified between 2005 and 2012, but sporadic infections of backyard and commercial poultry were reported from wild bird sources [124, 148, 149].

Other examples of poultry infection (more than 100 flocks) by LPAIVs include the following:

1. H5N2 LPAIV, endemic in non-commercial and commercial chickens in Mexico beginning in 1993 and still continuing in 2016 [124, 127, 140, 200–202]
2. H9N2 LPAIV, endemic in non-commercial and commercial chickens in many developing countries of Asia and the Middle East, beginning in the late 1990s and continuing today [14, 30, 74, 87, 93]
3. H7N1 epidemic of LPAIV in turkeys in Italy during 1999 that mutated to HPAIV
4. H7N3 LPAIV epidemic in Northern Italy during 2002–2003 [87]
5. H1N1, H1N2, and H3N2 swine influenza viruses in meat and breeder turkeys in multiple countries [28, 49, 167, 186, 189]
6. H7N9 LPAIV, widespread in live poultry markets of China during 2013–2014 [77, 203].

Additional outbreaks in poultry (commercial and non-commercial), ratites, pet birds, fighting cocks, and other birds have been described over the past 34 years, and are reported in the proceedings of the first to ninth International Symposia on Avian Influenza [18, 47, 48, 77, 169–171, 176, 179, 182]. A compilation of these LPAI outbreaks is beyond the scope of this chapter.

Features of low-pathogenicity avian influenza

Infections with LPAIV in the field typically produce respiratory disease or drops in egg production, but mortality is usually low unless the LPAIV is accompanied by secondary agents such as bacteria or viruses, which can result in mortality as high as 80% in turkeys and 75% in quail [134, 185]. In some cases, LPAIVs have caused severe and economically important disease in the field when accompanied by secondary infection and other stressors. Reproduction of the field syndrome by experimental inoculation of chickens with LPAIVs alone usually produces no morbidity or mortality, thus pointing to the need for concurrent bacterial or viral infections, or other environmental factors, in the field disease. A detailed discussion of LPAIVs, especially H9N2 in Eurasia and H5N2 in Mexico and Central America, is presented in Chapter 11 of this book.

High-pathogenicity avian influenza (1959–2015)

Over the past 56 years, since the development of consistent diagnostic and control strategies, 37 epidemics or limited outbreaks of HPAI have been documented worldwide, and these are listed in Table 8.1. All of these HPAIVs were of the H5 or H7 HA subtype. There have been no HPAI outbreaks with AIVs of the other 14 HA subtypes (H1–4, H6, H8–16), but laboratory-generated H2, H4, H6, H8, H9, and H14 AIV have been produced by reverse genetic systems with a polybasic HA cleavage site of HPAIV, and they have been highly lethal in chickens [59, 60, 96]. However, several naturally occurring non-H5/H7 AIVs have expressed high lethality in chickens in the intravenous pathogenicity test, including A/mandarin duck/Singapore/805/F-72/7/1993 (H10N5), A/turkey/England/384/79 (H10N4), and derivatives of A/chicken/Alabama/1975 (H4N8), although these viruses were not highly lethal on intranasal inoculation, and lacked the HA cleavage site sequence compatible with HPAIV [32, 209]. In addition, the mechanism responsible for the high lethality was renal failure due to extensive replication in the kidney tubular epithelium, a mechanism previously reported to occur in intravenous inoculated chickens using a variety of different LPAIVs [172–175, 209]. Thus the high lethality results for these three avian influenza isolates were a laboratory phenomenon, and these three viruses are not true HPAIVs.

Clinically, these 37 HPAI epidemics or limited outbreaks initially were recognized with three distinctly different presentations:

1. initial detection as high-mortality disease in chickens and other gallinaceous poultry – that is, detection of HPAIV in the index case with high mortality rates (epidemics 1–3, 5–7, 9–13, 15, 17, 18, 21, 26, 29, 31–35, and 37)
2. detection of HPAIV in domestic waterfowl, ratites, or gallinaceous poultry as the index case, but without high mortality (epidemics 16, 24, 25, and 30)
3. appearance of H5 or H7 LPAIV in the index case with an abrupt change to HPAIV in the index or additional cases after a period of a few weeks to 1 year – that is, initially detected as LPAIV which mutated to HPAIV (epidemics 4, 8, 14, 19–23, 27, 28, and 36) (Table 8.1).

In Chapter 1, the mechanisms for the mutation of H5 and H7 LPAIVs to HPAIVs were discussed. The number of epizootics, the number of cases (i.e. farms), and the number of birds affected by HPAI have increased geometrically since 1959. The number of birds affected in HPAI outbreaks between 1959 and 1998 has been calculated to be 23 million, while between 1999 and early 2004 over 200 million birds were involved [37]. With the implementation of vaccination for control of H5N1 HPAI in Asia and H7N3 HPAI in Mexico, the numbers of affected birds have declined. For the 10-year time period 2005–2014, as reported officially to OIE, 91 million poultry died or were culled in association with HPAI outbreaks [121].

Since 1959, the primary control method has been stamping out, which has been documented with eradication of the virus in 32 of the 37 epidemics (epidemics 1–13, 17–25, 27–31, and 33–37). In five outbreaks (epidemics 14, 15, 16, 26, and 32), vaccination programs with some depopulation have eliminated the clinical HPAI disease, but demonstration of eradication by surveillance programs was not completed (Table 8.1). The H5 Gs/GD-lineage HPAIV which appeared in 1996 as H5N1 (epidemic 16) has become the largest HPAI outbreak of the past 50 years, with more than 400 million

Table 8.1 Details of the 37[a] documented pandemics, epidemics, or limited outbreaks of HPAI since the discovery of AIVs as the cause of fowl plague in 1955.

No.	Date	Prototype AIV	Subtype	Hemagglutinin proteolytic cleavage site deduced amino acid sequence	Number that had high mortality or were depopulated[b]	References
1	1959	A/chicken/Scotland/59	H5N1	PQRKKR/GLF	Scotland, Aberdeen: one site, chickens (*Gallus gallus domesticus*), but total number of birds affected not reported	[15, 82, 129]
2	1961	A/tern/South Africa/61	H5N3	PQRETRRQKR/GLF	South Africa, Western and Eastern Cape (provinces): 1000 miles of coastline between Port Elizabeth and Lambert's Bay, with counts in four small areas of 1300 common terns (*Sterna hirundo*), in one area had 25–30% mortality; no deaths in Arctic terns (*Sterna paradisaea*), swift terns (*Sterna bergii*), white-fronted sandplovers (*Charadrius marginatus*), or the scavenging black-backed gulls (*Larus marinus*) and cape ravens (*Corvus albicollis*)	[15, 26, 82, 139]
3	1963	A/turkey/England/63	H7N3	PETPKRRRR/GLF	England, Norfolk (county): two farms; 29 000 breeder turkeys (*Meleagridis gallopavo*) in outdoor and indoor pens	[15, 205, 208]
4	1966	A/turkey/Ontario/7732/66	H5N9	PQRRRKKR/GLF	Canada, Ontario (province): two indoor farms; 8100 breeder turkeys; mutation of progenitor LPAIV from turkeys to HPAIV	[81, 82, 135, 183]
5	1976	A/chicken/Victoria/76	H7N7	PEIPKKREKR/GLF	Australia, Victoria (province): two farms; 25 000 indoor laying chickens, 17 000 indoor broilers, and 16 000 indoor and outdoor ducks (*Anas platyrhynchos*); minimal biosecurity, wild bird access and untreated surface water used	[16, 22, 155, 191]

(*continued*)

Table 8.1 (*Continued*)

No.	Date	Prototype AIV	Subtype	Hemagglutinin proteolytic cleavage site deduced amino acid sequence	Number that had high mortality or were depopulated[b]	References
6	1979	A/chicken/Leipzig(Germany)/79	H7N7	PEIPKKKKR/GLF PEIPKRKKR/GLF PEIPKKRKKR/GLF PEIPKKKKKKR/GLF	Germany, Saxony (state): two farms: 600 000 chickens, 80 geese (formerly East Germany)	[13, 64, 137]
7	1979	A/turkey/England/199/79	H7N7	PEIPKKRKR/GLF PEIPKRRRR/GLF PEIPKKREKR/GLF	England, Norfolk (county): three commercial farms, 9262 turkeys	[6, 7, 15, 208]
8	1983–1984	A/chicken/Pennsylvania/1/83 (LPAIV) A/chicken/Pennsylvania/1370/83 (HPAIV)	H5N2	PQKKKR/GLF (LPAIV) PQKKKR/GLF (HPAIV) – HPAIV lacked a glycosylation site at amino acid residue 13	USA; Pennsylvania, Maryland, and Virginia (states): 452 flocks, 17 million birds; most were chickens or turkeys, plus a few chukar partridges (*Alectoris chukar*) and guinea fowl (*Numida meleagris*)	[49, 50, 82, 193]
9	1983	A/turkey/Ireland/1378/83	H5N8	PQRKRKKR/GLF	Ireland, Monaghan (county): four farms; 8120 turkeys, 28 020 chickens, and 270 000 ducks died or were depopulated	[10, 15, 82, 90]
10	1985	A/chicken/Victoria/1/85	H7N7	PEIPKKREKR/GLF	Australia, Victoria (province): one farm; 24 000 broiler breeders, 27 000 laying chickens, and 61 000 broilers. Used untreated surface water	[21, 40, 145, 155]
11	1991	A/turkey/England/50-92/91	H5N1	PQRKRKTR/GLF	England, Norfolk (county): one farm; 8000 turkeys	[11, 145, 208]
12	1992	A/chicken/Victoria/1/92	H7N3	PEIPKKKR/GLF	Australia, Victoria (province): two farms, one backyard flock, and one hatchery; 17 000 broiler breeders, 5700 ducks, 105 000 day-old chicks, and 540 000 hatching eggs	[142, 155, 206]
13	1995	A/chicken/Queensland/667/95	H7N3	PEIPRKRKR/GLF	Australia, Queensland (province): one farm; 22 000 laying chickens	[127, 206]

14	1994–1995	A/chicken/Mexico/31381-7/1994 (LPAIV) A/chicken/Puebla/8623-607/94 (HPAIV) A/chicken/Queretaro/14588-19/95 (HPAIV)	H5N2	PQRETR/GLF (LPAIV) PQRKRKTR/GLF (HPAIV) PQRKRKRKTR/GLF (HPAIV)	Mexico, Puebla, and Queretaro (states): chickens – stamping-out policy was not used for control. Concurrent circulation of LPAIV and HPAIV strains, but HPAIV only from late 1994 to mid-1995. Unknown number of birds infected with HPAIV, but 360 commercial chicken flocks were "depopulated" for avian influenza in 1995 through controlled marketing	[49, 57, 126, 157, 200]
15	1994–1995, 2004	A/chicken/Pakistan/447/95 A/chicken/Pakistan/1369-CR2/95 A/chicken/Pakistan/16/95	H7N3	PETPKRKRKR/GLF PETPKRRKR/GLF PETPKRRNR*GLF	Pakistan: "stamping-out" policy was not used for control. Surveillance, quarantine, vaccination, and controlled marketing were used as the control strategy. Two incursions: 3.2 million broilers and broiler breeder chickens (northern part of country, 1994–1995; 2.52 million layers (Karachi, 2004)	[19, 49, 97, 98, 100, 101]
16	1996	A/goose/Guangdong/1/1996 (GS/GD)	H5N1	PQRERRRKKR/GLF ($n = 979$) Clade 1, PQREGRRKKR/GLF Clade 2.1, PQRESRRKK/GLF ($n = 12$) Clade 2.2, PQGERRRKKR/GLF ($n = 436$) Clade 2.3.1, PQRERRRKR/GLF ($n = 204$) Clade 2.3.2, PQRERRRKR/GLF ($n = 361$) Clade 2.3.3, PQRERRRKR/GLF ($n = 304$) Clade 2.3.4, PLRERRRKR/GLF ($n = 73$) Clade 2-like, PQRERRRKKR/GLF ($n = 84$) Clade 7, PQIEGRRRKR/GLF ($n = 5$)	70 countries in Asia, Europe, Africa, and North America: unknown number of commercial and non-commercial flocks (village poultry); over 400 million birds died or were culled between 2003 and early 2012, mostly chickens, but also ducks, geese, Japanese quail, and some wild birds. The H5 and N1 gene lineages have been maintained among the HPAIVs from outbreaks in various Asian, African, and European countries (1996–2007), and the six internal gene segments have undergone reassortment with other AIVs in Asia. Beginning in 2008, the NA gene has undergone reassortment[c]	[53–55, 99, 152, 165, 210]

(*continued*)

Table 8.1 (*Continued*)

No.	Date	Prototype AIV	Subtype	Hemagglutinin proteolytic cleavage site deduced amino acid sequence	Number that had high mortality or were depopulated[b]	References
17	1997	A/chicken/New South Wales/1651/97	H7N4	PEIPRKRKR/GLF	Australia, New South Wales (province): three farms; 160 000 indoor broiler breeders and 261 outdoor emu (*Dromaius novaehollandiae*)	[127, 155]
18	1997	A/chicken/Italy/330/97	H5N2	PQRRRKKR/GLF	Italy; Veneto and Fruili-Venezia-Giulia (regions): eight rural flocks (hobby and backyard only); 2116 chickens, 1501 turkeys, 731 guinea fowl, 2322 ducks, 204 quail (species unknown), 45 pigeons (*Columba livia*), 45 geese (species unknown), and 1 pheasant (species unknown)	[15, 35, 82]
19	1999–2000	A/turkey/Italy/977/99 (LPAIV) A/turkey/Italy/4580/99 (HPAIV)	H7N1	PEIPKGR/GLF (LPAIV) PEIPKGSRVRR/GLF (n = 360) PEIPKGSRMRR/GLF (n = 3) PEIPKRSRVRR/GLF (n = 1)	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, 1737 backyard poultry, and 387 ostriches	[15, 20, 36]
20	2002	A/chicken/Chile/176822/2002 (LPAIV) A/chicken/Chile/4322/2002 (HPAIV) A/chicken/Chile/4325/2002 (HPAIV)	H7N3	PEKPKTR/GLF (LPAIV) PEKPKTCSPLSRCRETR/GLF PEKPKTCSPLSRCRKTR/GLF	Chile, Valparaíso (region): two farms of one company, multiple houses; 617 800 broiler breeders died or destroyed (150 500), 18 500 turkey breeders (two houses) destroyed	[88, 138, 168, 183]
21	2003	A/chicken/Netherlands/621557/2003	H7N7	PEIPKRRRR/GLF	Netherlands, Gelderse Vallei and Limburg (region): 255 infected flocks, and 1381 commercial and 16 521 backyard/smallholder flocks depopulated. 30 million died or depopulated, of which the majority were chickens; backyard/smallholder, 175 035 birds; commercial, 25 million infected or pre-emptive culled birds; 4.5 million birds for welfare reasons Belgium, Limburg and Antwerp (provinces): 8 farms; 2.3 million chickens Germany, Nordrhein Westfalen (state): one farm; 419 000 chickens	[15, 51, 64, 199]

22	2004	A/chicken/Canada/AVFV1/04 (LPAIV) A/chicken/Canada/AVFV2/04 (HPAIV)	H7N3	PENPKTR/GLF (LPAIV) PENPKQAYRKRMTR/GLF ($n = 3$); later HPAI isolates PENPKQAYQKRMTR/GLF ($n = 24$) PENPKQAYKKRMTR/GLF ($n = 4$) PENPKQAYHKRMTR/GLF ($n = 3$) PENPKQAHQKRMTR/GLF ($n = 1$) PENPRQAYRKRMTR/GLF ($n = 1$) PENPKQACQKRMTR/GLF ($n = 1$)	Canada, British Columbia (province): 42 commercial and 11 backyard flocks infected (1.2 million poultry) – approximately 16 million commercial poultry depopulated, most were chickens	[67, 102, 125]
23	2004	A/chicken/Texas/298313/2004	H5N2	PQRKKR/GLF	USA, Texas (state): one non-commercial farm (6608 chickens) and two live poultry markets infected and depopulated; three additional LPMs voluntarily depopulated	[82, 103]
24	2004	A/ostrich/South Africa/N227/2004	H5N2	PQREKRRKKR/GLF	South Africa, East Cape (province): 2004 – eight farms, culled. 23 625 ostriches, 3550 other poultry (chickens, turkeys, geese, ducks, and pigeons), 1594 ostrich eggs, and 1707 other farmed bird eggs	[1, 2, 15, 104]
25	2006	A/ostrich/South Africa/AI1091/2006	H5N2	PQRRKKR/GLF	South Africa, Western Cape (province): 24 farms, 7334 ostriches culled	[1, 106]
26	2005	A/chicken/North Korea/1/2005	H7N7	PEIPKGRHRRPKR*GLF	North Korea: three farms, 218 882 layer chickens culled; number dead not reported	[105] (P. Selleck and H. Heine, personal communication, 2007)
27	2007	A/chicken/Saskatchewan/HR-00011/2007	H7N3	PENPKTTKPRPRR/GLF	Canada, Saskatchewan (province): one farm, 10 barns, 49 500 broiler breeder hens and roosters; 540 deaths, 48 500 culled; LPAIV appeared first, with rapid mutation to HPAIV	[27, 107]
28	2008	A/chicken/England/1158-114061/2008	H7N7	PEIPKRKKR/GLF	England, Oxfordshire (county): one farm; 25 000 free-range layer chickens; 10 000 deaths, 15 000 culled; HPAIV derived from LPAIV on premises	[31, 42, 91, 108]

(*continued*)

Table 8.1 (*Continued*)

No.	Date	Prototype AIV	Subtype	Hemagglutinin proteolytic cleavage site deduced amino acid sequence	Number that had high mortality or were depopulated[b]	References
29	2009	A/chicken/Spain/6279-2/2009	H7N7	PELPKGTKPRPRR/GLF	Spain, Guadalajara (province): one farm, five barns; 308 640 layer chickens; 30 000 deaths, 278 640 culled	[71, 99, 109]
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, 1178 culled, and 39 812 slaughtered on 50 premises; loss of export markets	[99, 112]
31	2012–2013	A/chicken/Taiwan/A1997/12	H5N2	PQRRKR*GLF	Chinese Taipei; Chang-Hua, Pingtung, Yunlin, and Penghu (counties): six premises (native chickens = 4; broiler breeder = 1, layer chicken = 1); 47 151 chickens, 5697 deaths, 41 454 culled (for further details, see Chapter 10 of this volume)	[99, 111, 113]
32	2012	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: June 13, 2012 to September 29, 2012; January 3, 2013 to time of writing (for further details, see Chapter 10 of this volume)	[99, 110, 119]
33	2012	A/chicken/New South Wales/12-3121-1/2012	H7N7	PEIPRKRKR/GLF	Australia, New South Wales (province): one site, free-range layers, 50 000 affected, 5000 deaths, 45 000 culled (for further details, see Chapter 10 of this volume)	[114] (Frank Wong, AAHL, Geelong, Australia, March 18, 2015)
34	2013	A/chicken/Italy/13VIR4527-11/13	H7N7	PETPKRRERR/GLF	Italy, Emilia-Romagna (region): six premises, layers, 952 658 affected, 5676 deaths, 946 982 culled (for further details, see Chapter 10 of this volume)	[99, 115]

35	2013	A/chicken/New South Wales/13-02811-1/2013	H7N2	PEIPRKRKR/GLF	Australia, New South Wales (province): two premises, free-range and caged layers, 490 000 affected, 18 620 deaths, 471 380 culled (for further details, see Chapter 10 of this volume)	[120] (Frank Wong, AAHL, Geelong, Australia, March 18, 2015)
36	2015	A/chicken/England/26352/2015 (H7N7)	H7N7	PEIPRHRKGR/GLF	England, Lancashire (county): one premise, colony and free-range laying chickens, 179 865 affected, 34 604 deaths, 145 261 culled (for further details, see Chapter 10 of this volume)	(www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?page_refer=MapEventSummary&reportid=18116) (Brown, AHPA, Weybridge, UK, November 25, 2015)
37	2015	A/chicken/Germany/AR1385/2015 (H7N7)	H7N7	PEIPKRKRR/GLF	Germany, Lower Saxony (state): one site, laying chickens, 10 104 affected, 50 deaths, 10 054 culled (for further details, see Chapter 10 of this volume)	(www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?page_refer=MapEventSummary&reportid=18234) (Brown, AHPA, Weybridge, UK, November 25, 2015)

Data modified from references [13, 178, 180], and [185].

[a]Since the drafting of this chapters, three additional HPAI outbreaks have occurred: 1) H5(N1, N2 and N9), France, 2015; 2) H7N8, USA, 2016; and 3) H7N7, Italy, 2016.

[b]Most outbreaks were controlled by "stamping out" or depopulation policies for infected and/or exposed populations of birds. Chickens, turkeys, and birds in the order Galliformes had clinical signs and mortality patterns consistent with HPAI, whereas ducks, geese, and other birds lacked or had low mortality rates or infrequent presence of clinical signs.

[c]The initial H5N1 HPAI outbreaks were reported in China (in 1996), with three incursions in Hong Kong (in 1997, 2001, and 2002). This was followed by regional extension with outbreaks in 2003–2005 within South-East Asia (South Korea, Vietnam, Japan, Indonesia, Thailand, Cambodia, Laos, China, and Malaysia). During 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 the autumn of 2005. In 2006, outbreaks were reported in Africa. Initially, chickens were the main species affected by disease and death, but in many of the outbreaks, domestic ducks have emerged as a major species involved in the maintenance and epidemiology of the viruses. Various wild birds have succumbed to infection. In 2008, the first reassortant appeared with a different NA gene (H5N5), with the appearance in subsequent years of 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 clades. In 2014, clade 2.3.4.4 H5N8 spread into Europe and North America as a result of wild bird movements, and reassortants of this virus appeared in North America.

birds affected (by natural deaths or culling), and it has had an impact on more birds than all the other 36 epidemics combined [54, 187]. This epidemic has spread from its initial cases in China during 1996 to affect poultry and wild birds in over 70 countries in Africa, Asia, Europe, and North America [55]. A few of these countries have implemented successful eradication campaigns, but the endemicity of the virus in village poultry and LPM systems in many countries (especially in domestic ducks), the lack of movement controls on village poultry and LPM systems, and the infection of migratory waterfowl have resulted in recurring outbreaks of disease within countries and, in some instances, reintroduction into countries that were declared free of HPAI in 2004 and 2005 (i.e. Japan, South Korea), late 2006 to early 2007, 2010, and 2014 [69, 78, 118, 121].

Wild aquatic birds are the primordial reservoirs for all AIVs, and these AIVs or their genes have appeared in AIVs that have infected domestic poultry and captive birds (see Chapter 7). However, the immediate source of low-pathogenicity and high-pathogenicity epidemic viruses is not always determined as feral wild birds, captive wild birds, village poultry, commercial poultry, etc. Some LPAIVs or HPAIVs, though, have been adapted to poultry and have been maintained in village, backyard, or hobby poultry and LPM systems before introduction into commercial poultry. For example, some began in the LPM system (e.g. the 1983–1984 H5N2 AIV of the north-eastern USA, and the H5N1 HPAI in Hong Kong during 1997), or they began in range-reared layers (e.g. the H7N7 LPAIV outbreak in the Netherlands in 2003) before spreading into commercial poultry sectors [82, 204, 207]. Others were detected in the LPM system and were eliminated before they spread to commercial poultry (e.g. the H5N2 HPAIV in Italy during 1997, and the H5N2 HPAIV in Texas during 2004) [35, 82]. Some HPAIVs appeared to have emerged after the introduction of LPAIV in commercial poultry (e.g. H7N3 viruses in Chile during 2002 and in Canada during 2004) [29, 168]. In other outbreaks, the lack of proper surveillance means that the initial source of infections cannot be determined, but blame is more likely to be attached to the commercial sectors, as they undertake the majority of surveillance, whereas the village or rural sectors conduct the least. However, when AIV infections do occur in commercial industries, they sometimes spread rapidly throughout the integrated system from farm to farm, resulting in epidemics of HPAI or LPAI, depending on how effectively the biosecurity measures are containing the spread.

Detailed information on the 1878–2007 HPAI outbreaks can be found in the first edition of this book [188], in Chapters 7 [75], 8 [183], 9 [15], 10 [155], and 11 [154]. In the current volume, Chapters 9 and 10 provide an update on the epizootics from 2008 to 2015.

Conclusions

AIV is a global virus that knows no geographic boundaries, has no political agenda, and can infect poultry irrespective of their agricultural or other anthropocentric systems. AIVs or evidence of infection with these viruses have been detected in poultry and wild birds on all seven continents. However, the reported frequency of avian influenza is greatly skewed by the availability of diagnostics, the quantity and quality of surveillance undertaken, the type of birds and production sector tested, the time of year, geographic location, climatic conditions, and other undefined factors. The most thorough and intensive surveillance in domestic and wild birds has been undertaken in North America and Europe, because of scientific interest, the availability of virological and serological tests, and the availability of financial resources. Because influenza is an international problem, solutions will require international efforts and cooperation.

Historically, five major scientific advances have changed our definition of avian influenza and thus had an impact on the reported frequency of this disease in the world:

1. early diagnosis of fowl plague in domestic poultry was based primarily on clinical features, lesions, and animal studies
2. recognition of LPAIVs and their infections in domestic poultry was based on serology and virus isolation
3. the discovery of avian influenza in asymptomatic wild bird reservoirs
4. the discovery that HPAIV can arise from mutation of H5/H7 LPAIV
5. the unprecedented global spread of the H5 Gs/GD-lineage HPAIV, including reassortment

of various genes and episodic periods of wild bird transmission.

However, some very specific discoveries have improved our understanding of the pathobiology of avian influenza and how to better control the disease at its source:

1 From 1878 to 1955, fowl plague was described as a high-mortality disease of poultry in many countries throughout Europe, Asia, North and South America, and Africa, and the etiology was demonstrated to be a filterable virus.

2 Between the 1930s and the 1950s, fowl plague disappeared as an endemic disease in most of the world, to be replaced by Newcastle disease.

3 In 1949, the first case of a low virulent disease in chickens caused by AIVs was reported (i.e. the first LPAIV).

4 In 1955, the etiology of fowl plague was determined to be IAV, which was subsequently identified as the H7 subtype.

5 In 1959, the first fowl plague outbreak caused by the H5 subtype of AIV was described in chickens.

6 In 1961, the first wild bird infections and deaths from AIV were reported in common terns in South Africa.

7 In 1966 and 1971, the first H5 and H7 LPAIVs, respectively, were identified. Prior to this, only HPAIVs had H5 and H7 subtypes.

8 In 1970, the AGID serological test was introduced, which allowed rapid and easy identification of AIV-infected poultry flocks.

9 In 1972, the first isolations of LPAIVs from asymptomatic wild birds (ducks in the USA and shorebirds in Australia) were obtained.

10 In 1981, the term "highly pathogenic avian influenza" was accepted as standard nomenclature for fowl plague and related synonyms.

11 In 1983, LPAIV was observed mutating to HPAIV during an LPAI field outbreak, and specific genomic changes in the proteolytic cleavage site of the HA were identified as responsible for this virulence change.

12 In the late 1980s and early 1990s, molecular criteria were added to the definition for classifying an AIV as HPAI.

13 In 2002, the first reports of infections and deaths in a wide variety of wild bird species from AIV (i.e. H5N1 HPAI virus) appeared.

14 The H5 Gs/GD lineage of HPAIV spread via wild birds in 2005, 2010, and 2014, and spread from Eurasia to the Americas in 2014.

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9 Multi-continental panzootic of H5 highly pathogenic avian influenza (1996–2015)

Leslie D. Sims and Ian H. Brown

Introduction

When a high-pathogenicity avian influenza (HPAI) virus (HPAIV) of the H5N1 subtype was isolated from geese in Guangdong Province, China, in 1996 [186] (hereafter referred to as Gs/GD-lineage H5 HPAIV), few realized that this would portend a major panzootic[1] of HPAI, the first of this disease to affect poultry across four continents. This panzootic has been noteworthy not only because of the extent of spread, but also for the breadth of species affected, which include not only chickens, but domestic ducks, other poultry [140], a broad range of non-domesticated avian species [41, 43, 164], and some mammals [161, 165], including members of the Felidae [59, 80, 151], Canidae [152], Viverridae (including Owston's palm civet) [132], and Mustelidae (including mink and stone marten) [128, 165], and humans [7, 122]. Not all strains of Gs/GD-lineage H5 HPAIV appear to have the same capacity to cause severe disease in mammals, including some strains of virus detected in Europe and North America in 2014–2015 (clade 2.3.4.4 viruses of the H5N8, H5N1, and H5N2 subtypes) [76, 184].

Gs/GD-lineage H5 HPAIV and its descendants have circulated as high-pathogenicity viruses since they emerged in 1996, causing a panzootic of unprecedented proportions. Prospects for global eradication remain remote, also setting this apart from other outbreaks of HPAI.

In 1997, international interest in HPAIVs increased dramatically following the occurrence of fatal disease in poultry and humans in Hong Kong associated with the H5N1 strain of HPAIV [31]. These human cases provided the first indications to the international community of the possibility of H5N1 HPAIVs being the precursor to a human pandemic virus. These concerns were amplified because of the high fatality rate among those who became ill, raising the specter of a human influenza pandemic akin to that seen in 1918. These concerns persist, even though there is as yet no direct evidence to indicate that a strain of this virus will ever develop the capacity to transmit readily between people [122]. Some authors have suggested that perhaps these viruses may never produce a severe pandemic [103], and the response to the increase in human cases in Egypt in 2014–2015 has been remarkably muted compared with that observed between 2004 and 2006, when Gs/GD-lineage H5 HPAIVs spread over three continents and fewer human cases occurred in any individual country.

The virus found in Hong Kong SAR in 1997 (H5N1/97-like) [54] was eradicated in late 1997, but other H5N1 HPAIVs within the Gs/GD lineage persisted in the region [142]. Viruses derived from these H5N1 viruses caused intermittent outbreaks of disease in poultry and wild birds in Hong Kong SAR from 2001 to 2003 [41, 142]. In late 2003 and early 2004, outbreaks of HPAI caused by H5N1

[1]The term "panzootic" is used to distinguish disease in animals from a potential influenza pandemic in humans caused by an avian-derived virus.

Animal Influenza, Second Edition. Edited by David E. Swayne.

viruses were reported almost simultaneously in poultry in eight Asian countries (China, Cambodia, Indonesia, Japan, Korea, Lao PDR, Thailand, and Vietnam), followed later in 2004 by an outbreak of the disease in Malaysia [143]. The impact of these viruses was particularly severe in Thailand and Vietnam, where there was widespread disease in poultry and there were multiple fatal human cases. In these two countries alone, around 100 million poultry were culled or died in 2004. From May 2005, cases of disease associated with a novel, but closely related sub-lineage of H5N1 virus (clade 2.2) occurred in migratory birds in North-West China, centered around Qinghai Lake [25, 27]. Strains of this virus were then detected subsequently across southern Russia, in Kazakhstan, and in northern Mongolia, affecting poultry and/or wild birds. In 2006, related viruses were reported in Europe, Africa, the Middle East, Pakistan, and India [153].

Concurrently, the number of human cases began to increase in places where virus was already established, including Indonesia and China, and in newly infected countries, such as Turkey, Egypt, Azerbaijan, and Iran. The number of new human cases fell in Thailand and Vietnam, coinciding with improved control of infection in poultry [182]. Further outbreaks in poultry from 2007 onwards, including cases reported since 2013, resulted from the introduction of virus to countries that had stamped out infection previously (including the Republic of Korea, and Japan, Nigeria, Germany, India, amd Russia), the occurrence of disease in a number of countries that had not reported disease in poultry (including Bangladesh, Nepal, Bhutan, Kuwait, Saudi Arabia, the UK, the Netherlands, Ghana, Togo, the USA, and Canada), cases of disease in parts of infected countries where disease had not been reported previously (e.g. Russia), and additional cases in countries where infection remains endemic or has been recorded regularly in the past (e.g. Indonesia, Vietnam, China, Cambodia, Myanmar, and Egypt). These countries, together with parts of the Gangetic Plain (Bangladesh and India), are regarded as having enzootic infection with these viruses, although incursions and detection of new strains of virus (different clades within the Gs/GD lineage) have also occurred in all of these areas except for Egypt, where all viruses are derived from the original clade that was detected in 2006 (clade 2.2).

From late 2003 to the end of April 2015, infection associated with Gs/GD-lineage H5 HPAIVs was recorded in poultry and/or non-domesticated birds in 66 countries, as well as in Chinese Taipei, Hong Kong SAR, and the Palestinian Autonomous Territories, along with 840 laboratory-confirmed human cases of infection and disease (of which 447 cases were fatal) from 16 countries, including one imported case in a traveler returning to Canada [182]. From 2008 to May 2015, human cases were recorded in Bangladesh, Egypt, Cambodia, Indonesia, China, and Vietnam. The only countries with 20 or more reported human cases per annum were Indonesia (2008 and 2009), Egypt (2009, 2010, 2011, 2014, and 2015), and Cambodia (2013) [182].

Gs/GD-lineage H5 HPAIVs have evolved into a number of HA clades and genotypes. Some of these viruses acquired neuraminidase (NA) proteins of a different subtype through reassortment, especially viruses within clade 2.3.4.4. In addition, for the first time the Gs/GD lineage of H5 HPAIVs spread to North America following the transglobal spread of clade 2.3.4.4. After detection both in wild birds and in poultry, the viruses underwent further genetic reassortment, acquiring genes from North American-lineage avian influenza viruses [68, 120].

This is the most serious HPAI epizootic ever experienced in terms of the number of infected flocks and the geographical extent of the disease. Furthermore, uncontrolled spread has led to the virus "spilling over" to other animals and humans on a scale not detected in the past.

The threat posed by these viruses to public health and to the livelihood of farmers and village communities led to a concerted effort by authorities in infected countries, supported by international agencies, to control and, in many places, to eliminate infection. In Asia, a number of countries succeeded in eradicating the disease, notably Japan, Malaysia, and the Republic of Korea, but experienced re-incursions of Gs/GD-lineage H5 HPAIVs. Disease was quickly eliminated from Western Europe on each of the occasions it occurred there, and from several African countries that reported infections between 2006 and 2008. This occurred relatively quickly in countries with low

poultry density and hot dry climates (e.g. Niger), and over a longer period elsewhere (e.g. 3 years in Nigeria). West African countries reported outbreaks again in 2014–2015, and authorities in some of these countries may find it more difficult to contain the virus, given the increase in size of the poultry sector since the last outbreak [45].

The outbreak in the USA in 2014–2015 proved difficult to contain, especially in the states of Iowa and Minnesota, but the virus has been eliminated from poultry, with the last case reported on June 17, 2015. Virus elimination is possible where veterinary infrastructure is strong and industry has been able to strengthen practices to reduce the risk of virus introduction.

It is now acknowledged that the virus is endemic to some countries and areas, and will be extremely difficult, if not impossible, to eradicate globally. Local elimination is possible in parts of some of these countries, but these zones or compartments are vulnerable to incursions of virus from the remaining infected areas, as experiences from the previous 12 years demonstrate.

This paper provides a chronology of major events and some key features of the H5 HPAI panzootic. It examines the viruses that have emerged and the effects of these on key countries and regions. It reviews information on the pathways of spread and discusses the control measures implemented in selected countries, including changes in rearing and marketing practices (often referred to as restructuring of the poultry industry) that are necessary for long-term control of this disease. It also discusses likely developments in this panzootic.

There are still gaps in our knowledge relating to Gs/GD-lineage H5 HPAI due, in part, to limited epidemiological studies of many outbreaks, especially those that occurred in the early phase of the panzootic. Even in 2015, the source of individual outbreaks in many developing countries cannot be traced. Cases of infection in poultry are going unreported, as demonstrated by the presence of human cases in areas where no cases of infection are reported in poultry [144], although marked improvements in capacity in this area have been achieved. The failure to detect or to report infection also hampers attempts to control this disease, and to understand the evolution of these viruses and the diseases that they cause.

Genetic studies and nomenclature of GS/GD-lineage H5 HPAIVs

Although there are many avian influenza viruses present in Eurasia, the only ones considered in detail in this chapter are HPAIVs belonging to the H5 subtype (i.e. those with a hemagglutinin protein (HA) of the H5 subtype that can be linked phylogenetically to the H5N1 virus that was first identified in Guangdong Province, China, in 1996). Until relatively recently, the vast majority of these viruses had an NA protein of the N1 subtype. However, since 2008 a number of strains of other N subtypes have been detected. In some ways it is remarkable that other N subtypes had not emerged, given the co-circulation of avian influenza viruses in poultry, including H5N1 and H9N2 viruses. The main subtypes found in this lineage, other than H5N1, have been H5N2, H5N3, H5N5, H5N6, and H5N8 viruses [68, 183, 191]. Other reassortant viruses have also been detected recently in North America (H5N2 and H5N1), in which the N1 and N2 genes (and two to three other genes that encode for internal proteins) are derived from North American-lineage influenza viruses [68]. Most of these reassortant viruses fell within clade 2.3.4.4, but some in Asia were within clade 7.2.

The one constant with regard to influenza A viruses is that they will change over time. This has certainly been the case with Gs/GD-lineage H5 HPAIVs isolated over the past 19 years. These viruses exhibit considerable genetic and antigenic heterogeneity as a result of drift in individual genes as well as genotypic variability through genetic reassortment. The common feature is that they have retained an HA gene that can be linked back to that of the original virus in this lineage detected in geese in 1996. Molecular studies have provided unique insights into the evolution of the H5 HPAIVs as they emerged, which has enabled tracking of their movement around the globe. The nomenclature of HA clades has been standardized, but the terminology for genotypes and antigenic variants of these viruses is still not governed by an internationally consistent set of rules. The various systems used to describe these viruses by different research teams led to considerable scope for confusion [24, 54, 105, 137, 149, 168, 173]. In 2005, the first attempt was made to define the various clades of virus, based on genetic relationships of

HA genes [173]. This system was further refined in 2006 and 2007 [176], and new clades have been added as they emerged through a process of continuous review under the auspices of a World Health Organization (WHO)/World Organisation for Animal Health (Office Internationale des Epizooties [OIE])/Food and Agriculture Organization of the United Nations (FAO) working group [37, 177].

According to the system of nomenclature developed by the WHO/OIE/FAO H5N1 evolution working group [177], the viruses were divided into 10 first-order clades numbered from 0 to 9. Clades were distinguished by sharing of a common clade-defining node in the phylogenetic tree with a bootstrap value of > 60 at the clade-defining node, and average pairwise nucleotide distances between and within clades of > 1.5% and < 1.5%, respectively. Viruses that fell within clade 3 in the earlier related classification scheme developed in 2005 were renamed as clade 0 to reflect the fact that these were the precursors of all other clades. Clade 2 was initially divided into five second-order clades (2.1–2.5). Currently some viruses in clade 2.3 have evolved to fifth-order clades, with the fifth order specified by a letter rather than a number (e.g. subclade 2.3.2.1c). Eleven H5 clades actively circulated during 2011 and 2012 [181]. Subsequently, three new clade designations were recommended based on division of clades 2.1.3.2a (Indonesia), 2.2.1 (Egypt), and 2.3.4 (widespread detection in Asia, Europe, and North America) that include newly emergent Gs/GD-lineage HPAIV subtypes H5N2, H5N3, H5N5, H5N6, and H5N8 classified as clade 2.3.4.4 [37]. By March 2015 the only clades that were known to be or had been circulating recently were derivatives of clades 2.1 (Indonesia), 2.2 (Egypt with spillover to neighboring countries), 2.3.2 (several strains widespread in Asia and one in West Africa), and 2.3.4 (China, Republic of Korea, Japan, Europe, and North America). Others, such as derivatives of clades 1 and 7, may still be circulating in the Mekong and China, respectively, but have not been reported.

Genetic studies

Prior to 1996, only two HPAIVs of the H5N1 subtype had been identified – one from poultry in Scotland during 1959 [123], and one from turkeys in Norfolk, England, during 1991 [5]. Although these viruses were the same subtype as the Gs/GD-lineage H5 HPAIV, they were only distantly related, forming part of the broader Eurasian H5 lineage. Similarly, a number of H5N1 low-pathogenicity avian influenza viruses (LPAIVs) have been isolated in Asia, the Americas, and Europe, but none of these are closely related to the Gs/GD-lineage H5 strains. LPAIVs of the H5 subtype have not become established in terrestrial poultry in Asia [38], but are still found in wild birds. In Europe, due to active surveillance in commercial poultry, they have been detected more frequently.

The 1996 H5N1 HPAIV (A/Goose/Guangdong/1/96, hereafter referred to as Go/GD/96) lies at, or near, the root of the Gs/GD-lineage H5 HPAIV, comprising multiple distinct clades and genotypes that, as described above, have emerged and, in many cases, disappeared during subsequent years [24, 54, 55, 91, 108]. All H5N1 viruses isolated in the Gs/GD-lineage H5 HPAIV since 1996, regardless of the species of origin, meet the OIE definition of HPAIV (intravenous pathogenicity index > 1.2) [110], although some variability in the intravenous pathogenicity index has been described, especially in some strains of virus from ducks and geese [24, 189].

Apart from reports from the first half of the twentieth century and Pakistan in the 1990s with H7N3 viruses, this is the first time that HPAIVs are known to have persisted in poultry and/or wild birds for an extended period of time, and the only time that this has occurred across such a wide geographical range.

The precise origins and history of the Go/GD/96 virus are not known [38]. Presumably, like all avian influenza viruses, it came from an unidentified LPAIV precursor virus circulating in wild aquatic birds that then crossed into domestic poultry where mutation to virulence occurred, as is the case with other HPAIV strains [6]. However, surveillance data prior to 1996 for poultry and wild birds in Asia, especially during the period from 1980 to 1996, are limited. The HA gene of Go/GS/96 shares some similarities with other Eurasian viruses from wild birds, including virus from swans in Hokkaido in 1996, but these were not the direct source of the HA gene [38]. Likewise, the origin of the NA gene of Go/GD/96 virus is also unknown. It shares approximately 95% nucleotide similarity with the NA of A/Duck/Hokkaido55/96, an H1N1 LPAIV, which again suggests only a distant relationship

between the NA genes of these viruses. In fact a direct H5N1 precursor virus or viruses have never been detected.

The H5N1 HPAIVs that emerged in 1997 (H5N1/97-like viruses) and caused severe disease in poultry and humans in Hong Kong differed from those in 1996 in that they were reassortants with different NA and internal protein genes to the H5N1 HPAIVs first identified in geese in 1996, but with a closely related HA gene [53]. These H5N1/97-like viruses were considered to form a different genotype within the H5N1 subtype. Subsequently, a wide range of genotypes emerged through reassortment, presumably through multiplication in waterfowl. Different genotypes were assigned alphabetic characters (e.g. "V", "X", "Z"), largely based on the configuration of the genes they possessed encoding internal proteins, although there was inconsistency in the nomenclature and interpretation of the genetic findings between different research groups [24, 54]. Given the extent of reassortment in the past 18 years, a simple system for describing genotypes is no longer possible. The different genotypes all retained the parent HA gene derived from Go/GD/96-like virus but, as expected, this gene has also varied over time due to genetic drift, resulting in the formation of the different clades described above. This genetic drift is evident from phylogenetic trees which demonstrate considerable variation in the composition of nucleotides of the HA genes from the 1996 viruses to recent isolates. The phylogenetic trees produced from these studies also demonstrate epidemiological linkages between isolates (Figure 9.1).

Several useful observations have been made on the genetic characteristics of these viruses. The wide range of genotypes and clades found in China, including Hong Kong SAR, and elsewhere demonstrates that there has been significant replication of viruses over the past 18 years, in both poultry and wild birds [24, 55, 148]. Genetic information suggests initial single introductions of different strains of Gs/GD-lineage H5 HPAIVs to Indonesia (clade 2.1) and Thailand (clade 1) in 2003, and to Egypt in 2006 (clade 2.2), with subsequent evolution of these viruses in these countries. In Indonesia these viruses became endemic and evolved into fourth-order clades. Of these, clade 2.1.3.2 became the dominant strain and was subsequently split into fifth-order clades 2.1.3.2a and 2.1.3.2b [37]. A second strain (clade 2.3.2.1c) was introduced to Indonesia in 2012 [36]. Additional strains were also introduced to Thailand but did not become established [156]. Multiple strains of virus have been introduced to the Republic of Korea and Japan (clade 2.5 in 2003–2004, clade 2.2 in 2006, clade 2.3.2.1 in 2008, clade 2.3.2.1c in 2010–2011, and clade 2.3.4.4 in 2014–2015) [71]. Based on the timing of introductions, the epidemiology of the outbreaks and, in some cases, the detection of virus in wild birds before it was found in poultry, it is highly likely that migratory birds were the means of primary introduction of each of these strains of virus to Japan and the Republic of Korea. Vietnam has also experienced multiple introductions of virus, but the more likely mode of introduction is via illegal trade in poultry [84].

From 2005 to 2009 virtually all of the viruses that were isolated north and west of Myanmar fell within clade 2.2 and its derivatives. This included the H5N1 viruses detected in wild birds or poultry in Europe, the Middle East, South Asia, and Africa. These clade 2.2 viruses could be clearly distinguished from other H5N1 HPAIV clades that, at the time, were restricted geographically to East and South-East Asia. Among the exceptions were viruses detected in Europe in intercepted smuggled birds from Asia [166].

Soon after the introduction of clade 2.2 H5N1 HPAIVs into Europe, West Asia, the Middle East, and Africa, a number of distinguishable sub-lineages were apparent, indicating a dynamic situation in which the viruses continued to evolve.

Analysis of selected viruses, predominantly from Africa, but also including strains from Europe and the Middle East, identified at least three sub-lineages [137] within clade 2.2 (i.e. viruses linked to those identified in wild birds in Qinghai Province of China). These viruses were at the time not designated as separate clades, but were sufficiently different to be referred to as sub-lineages EMA1, EMA2, and EMA3 [137]. The subtle differences between these viruses provided support for epidemiological studies. For example, the presence of EMA1 and EMA2 in Nigeria demonstrated that more than one virus had been introduced to Nigeria in 2005–2006. Viruses detected in India (EMA3) were more closely related to those in Mongolia, Azerbaijan, Italy, and Afghanistan, and in the following year these were recorded in Russia,

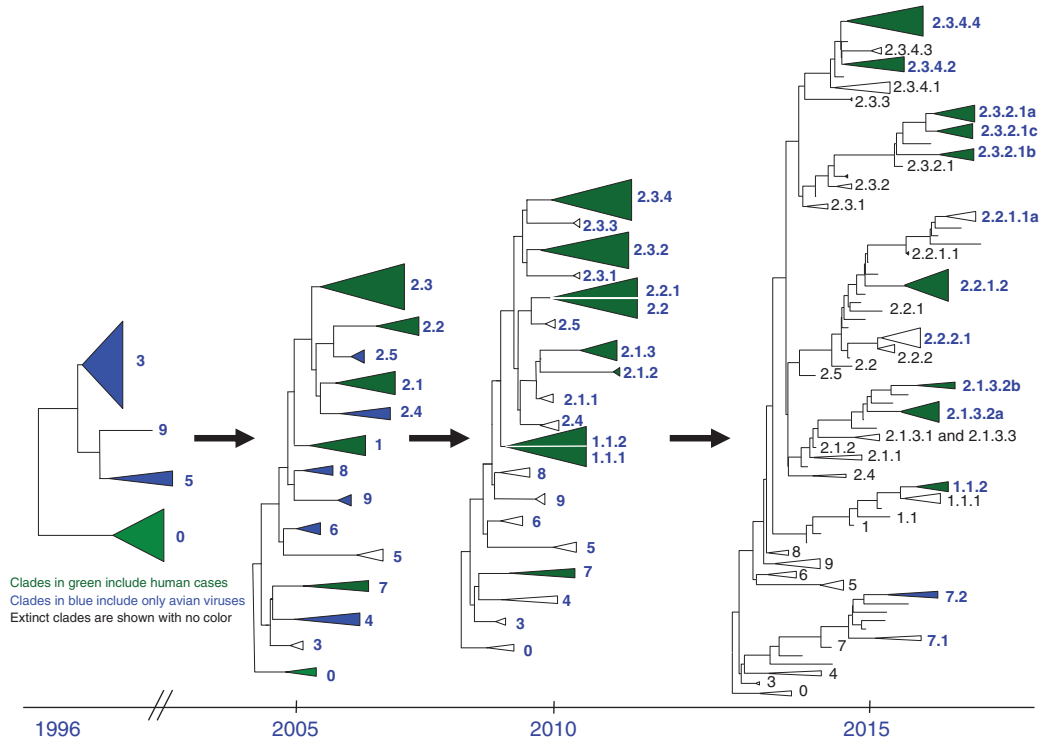


Figure 9.1 Phylogenetic relationships of the HA gene of Gs/GD-lineage H5N1 HPAIVs over time. The WHO/OFFLU H5 Evolution Working Group has kept under continuous review the nomenclature for Gs/GD-lineage H5 HPAIVs as they have evolved since their first emergence and detection in 1996. Discrete monophyletic groups appear within a specific clade, and when those groups meet the nucleotide divergence criteria (as well as having bootstrap values greater than 60, and within-clade average pairwise distances of less than 1.5%) they are split into second-order clades (but still considered part of the first-order clade). As a second-order clade continues to evolve it may reach a similar level of genetic diversity, at which point it may be split into third-order clades, and so on. The same clade designation criteria apply to first-, second-, and any higher-order clade designations. Extinct clades that are believed to be no longer circulating are shown without color, clades that have only been reported in avian species are shown in blue, and clades that include viruses which have been detected both in humans and in avian species are shown in green. Courtesy of Todd Davis, CDC Atlanta. *See Plate section for color representation of this figure.*

Pakistan, Kuwait, Turkey, and the Czech Republic. The EMA3 sub-lineage was not detected in Africa. The emergence of new clusters of H5N1 HPAIVs in Egypt has been noted on several occasions, presumably as a result of endemic cycling of virus. By 2007, clade 2.2 viruses in Egypt had evolved to the extent that they were (later) designated as clade 2.2.1 In late 2007, a subclade of antigenic drift variants, later designated 2.2.1.1, emerged from clade 2.2.1 and expanded (clade 2.2.1.1a) in commercial poultry in Egypt. By late 2010 it had disappeared [11]. Viruses of clade 2.2.1.1 that emerged in 2007 have now been replaced by clade 2.2.1.2 [37], which appears to have become predominant across all poultry production sectors [12], and furthermore has been associated with an increased number of human infections that might be related to increased viral burden in the poultry sector. This example demonstrates the rapid rate of evolution that is occurring in these viruses and the replacement of one clade by another, which often leads to the replaced clade disappearing altogether.

Some interesting changes have been observed when mammals have been infected. Despite the fact that most cases of infection in mammals are derived directly from poultry, differences in the genes of these viruses in the mammalian and avian hosts have occurred, apparently following a single or limited passage in a mammalian host. The mutation in the PB2 gene at position 627 (E627K) has

been reported as significant, but a number of other changes have also been associated with passage through mammals [57]. These changes are probably due to the fact that any population of influenza viruses in a single host is a quasispecies and that, in an aberrant host, a different "dominant" strain of virus is selected from this quasispecies, due to differences in the host environment.

Resistance to the antiviral chemicals amantadines has been identified in some H5N1 (and H9N2) viruses, suggesting selection pressure from use of this drug in Asia, and this correlates with reports of its use in commercial poultry [33].

Experimental studies in which H5N1 viruses were modified either by reverse genetics or by serial passage in ferrets have demonstrated that changes in these viruses in both the HA gene and other genes allow virus to be transmitted aerogenously between ferrets (with reduced pathogenicity). So far this combination of mutations has not been detected in nature [62, 67].

Cleavage site

For all influenza A viruses, the HA glycoprotein is produced as a precursor, HA0, which requires post-translational cleavage by host proteases before it is fully functional and virus particles are infectious. The genetic motif at the HA0 cleavage site in HPAIVs typically contains multiple basic amino acids and can be found in all of the H5 Gs/GD-lineage viruses. The original Go/GD/96 virus had the configuration PQRERRRKKR*GLF, but since then there has been considerable variation at this site, with variations also apparent between viruses from different clades (Table 9.1). All of these viruses remain highly pathogenic for gallinaceous poultry, with many isolates having an IVPI in chickens of 3.0.

Antigenic change

Marked antigenic change in the HA protein of Gs/GD-lineage H5 HPAIVs has occurred since they first emerged. Studies using both polyclonal and monoclonal antibodies demonstrate significant differences between strains from different locations. This is probably being driven by a combination of systemic circulation of virus in ducks, which develop an immune response to these viruses, interspecies transmission leading to strong immune selective pressure and, possibly, some impact from vaccination. Ongoing studies are being conducted to ensure that vaccine antigens remain appropriate for protection against the prevailing field strains, and to evaluate the impacts, if any, of antigenic drift in field viruses. China has been a leader in this field, and has introduced a number of new vaccine antigens once antigenic variant strains were detected [89]. Antigenic cartography has also been used as a means of demonstrating antigenic differences [1, 65, 159], but a global system for utilizing this information is lacking.

Table 9.1 Variability in HA0 cleavage site motifs of selected H5 HPAIVs.

Subtype	Clade	Cleavage site consensus$_2$	HP
H5	LP	PQRETR/GLF	–
H5N1	GS/GD lineage	PQRERRRKKR/GLF	+
	Clade 1	PQREGRRKKR/GLF	+
	Clade 2.1	PQRESRRKK/GLF	+
	Clade 2.2	PQGERRRKKR/GLF	+
	Clade 2.2.1	PQGEKRRKKR/GLF	+
	Clade 2.3.1	PQRERRRKR/GLF	+
	Clade 2.3.2	PQRERRRKR/GLF	+
	Clade 2.3.3	PQRERRRKR/GLF	+
	Clade 2.3.4	PLRERRRKR/GLF	+
		PLREKRRKR/GLF	
	Clade 2-like	PQRERRRKKR/GLF	+
	Clade 7	PQIEGRRRKR/GLF	+

Examples of variability of amino acids at the cleavage site of the HA0 gene of selected H5 viruses, derived from published sequences and unpublished data from viruses submitted to the European Union International Reference Laboratory for Avian Influenza, Animal and Plant Health Agency, Weybridge, UK.
HP = highly pathogenic, H5N* = various low-pathogenicity viruses of the H5 subtype but with variable N subtype, D = aspartic acid, E = glutamic acid, F = phenylalanine, G = glycine, K = lysine, P = proline, Q = glutamine, R = arginine, T = threonine.
Some data are derived from www.offlu.net/fileadmin/home/en/resource-centre/pdf/Influenza_A_Cleavage_Sites.pdf

Deletions in virus proteins

Several protein deletions have developed as the Gs/GD-lineage H5 HPAIVs have evolved. The original goose viruses (Go/GD/96) did not have a deletion in the NA stalk, but all of the 1997 strains did. This change is generally regarded as an adaptation to gallinaceous poultry. A similar but

not identical 19-amino-acid deletion occurred in the NA protein of most isolates from 2001 onwards (with the notable exception of some wild bird viruses from Hong Kong SAR in 2002, and those from humans in 2003) [55]. Recent H5N2 HPAIVs from North America do not have a deletion in the NA stalk, probably reflecting the wild bird origin of the NA gene [120]. A 5-amino-acid deletion has also been identified in the NS protein of many isolates [93]. The significance of this is unclear, but the maintenance of this change across a heterogeneous population of viruses implies higher virus fitness in key host species. It may also be a contributory factor in determining host range. It has been associated with higher virulence in ducks [93].

The history of the GS/GD-lineage H5 panzootic

The following section describes the development of the panzootic and is derived from a range of published material, including gene databases, and therefore does not only reflect official notifications of confirmed outbreaks to the World Organisation for Animal Health (OIE). Some official data appear biologically implausible, such as the lack of formal reports of disease in poultry in places other than Hong Kong SAR from 2001 to early 2003, despite the presence of H5N1 HPAIVs in an arc extending from Hanoi to Jilin [143, 178].

The absence of data from countries with poorly developed veterinary infrastructure and surveillance systems has sometimes been erroneously interpreted as lack of infection [144]. Over the past 10 years, most countries have improved their diagnostic and surveillance capabilities and also their transparency as a result of investments by national and subnational governments supported by international donors and financial institutes. The effect has been a clearer understanding of the extent of infection, although not all surveillance studies are reported, especially those with negative results. Political considerations still have the potential to lead to withholding of timely information on some disease outbreaks. Farmers do not always recognize or report all cases of disease [144], and in some countries they have few incentives to do so.

1996–1997: emergence of H5N1 viruses in China and disease in Hong Kong SAR

The first formally reported cases of serious disease associated with Gs/GD-lineage H5 HPAIV in Asia involved fatal disease in poultry and humans in 1997 in Hong Kong SAR [31, 142]. However, these cases had been preceded by infections with H5N1 HPAIVs in geese in Guangdong Province in 1996. The first avian cases in Hong Kong SAR were diagnosed on a farm in March 1997. The first human case (a 3-year-old child) was detected in May of that year, although the identity of the virus in the human case was not confirmed until 3 months later. No direct links between infected farms and the child were established, and testing of live poultry markets was not undertaken at that time. No further cases were reported until November 1997, when additional human cases were identified, followed by detection of avian cases in live poultry markets and on one farm. By the end of December 1997, a total of 18 human cases had been detected, six of which were fatal. Case–control studies suggested an association with visits to poultry-selling markets in the week prior to onset of illness [104].

The causal H5N1 viruses were reassortants with an HA gene derived from a Go/GD/96-like virus, and the other seven genes were derived from different (non-H5) avian influenza viruses [171]. This particular H5N1/97-like genotype has not been detected in the field since it was eliminated in late 1997 following the culling of all poultry in markets and virtually all chickens on farms [142]. A virus of the same genotype was apparently detected on duck and goose eggs imported from Vietnam to China [92], but phylogenetic analysis of over 100 H5N1 viruses from poultry in different locations in Vietnam from 2001 to 2006, and of thousands of viruses subsequently, has not confirmed the circulation of this particular clade and genotype.

It is still not known whether these H5N1/97 viruses arose in Hong Kong SAR or elsewhere. However, they had ample opportunity to multiply, largely unchecked, especially in live bird markets that sold a wide range of poultry, including terrestrial and aquatic species and some wild birds. Uncontrolled replication and persistence of these viruses in these markets would have provided considerable opportunity for them to undergo genetic changes. This could have occurred through point

mutations arising from the poor fidelity of influenza A virus polymerases during virus replication, or through reassortment following co-infection of poultry with other avian influenza viruses known to be present in these markets. However, these conditions were not unique to live poultry markets in Hong Kong SAR, as similar conditions existed in markets in mainland China and elsewhere in Asia at that time.

It has been reported that around 20% of chickens in markets in Hong Kong SAR were affected with H5N1 viruses just prior to the culling of birds in 1997 [141]. This widely quoted figure is a single point estimate of prevalence taken under unusual conditions, and may not reflect the true prevalence in markets in the months leading up to the outbreak (when similar surveillance studies were not conducted). The samples were collected when the demand for poultry had collapsed, and therefore poultry were kept in infected markets for longer periods, providing ample opportunity for viral transmission within market stalls.

Changes in the HA genes of the H5N1 HPAIVs isolated in 1997 suggested evolution in the 9-month period from March to December during which avian and human cases were detected. The presence of two distinct sub-lineages suggested that two strains of virus had been introduced to Hong Kong [193]. Other H5N1 viruses in the Gs/GD-lineage H5 HPAIVs, but different from those isolated in Hong Kong SAR, were also isolated from chickens in Hubei Province, China, in 1997, demonstrating that cases at this time were not confined to Hong Kong SAR (see, for example, A/chicken/Hubei/wh/1997) [178].

1998–2000: circulation of virus in China

No formal reports of H5N1 HPAI were made to the OIE between 1998 and 2000 by any country. However, H5N1 HPAIVs closely related to the original 1996 genotype (Go/GD/96-like) continued to circulate in geese in southern China in the late 1990s [21]. By 2000, multiple genotypes had also been detected in domestic ducks [24, 54]. Molecular studies suggest that these reassortant viruses acquired new genes coding for internal proteins in various combinations from unidentified avian influenza viruses, presumably circulating in aquatic birds [54].

This expansion of the host range of H5N1 HPAIVs from geese to ducks is considered to be a key event in the genesis of the subsequent panzootic. Experimental studies suggest that the HK/97-like viruses were poorly adapted to ducks [125]. It is now recognized that domestic ducks play a vital role in the maintenance of these viruses and their spread to terrestrial poultry [51, 154, 155, 162]. This expansion of host range probably played a role in the spread of these viruses back to wild birds (and in their transmission from wild birds to ducks), given the close phylogenetic relationship between domestic ducks and species of wild Anatidae, and their shared environment in many parts of Asia, including China [20].

2001–2002: outbreaks of disease in Hong Kong SAR, and continuing circulation and evolution of virus in mainland China

Detection of infection in ducks and geese continued into 2001, with an upsurge in the number of subclinically infected domestic waterfowl detected in consignments transported from mainland China to a dedicated duck and goose market/slaughterhouse in Hong Kong SAR [142]. During this period, viruses within clades 0, 1, 2.1.1, 2.4, 3, 4, 5, 6, 8, and 9 were detected in Hong Kong SAR and mainland China, demonstrating their rapid evolution [178]. A small study of poultry in markets in northern Vietnam in 2001 resulted in the isolation of two clade 3 H5N1 viruses from 33 samples from geese [109]. Given the small number of samples, it is extremely unlikely that these represented the only infected geese (or other poultry) in the country at the time. It was not until 2 years later that outbreaks of disease were reported from Vietnam (Figure 9.2).

New cases of infection with H5N1 HPAIVs in terrestrial poultry were reported in Hong Kong SAR and mainland China in 2001, the first since 1997 [142]. Based on the combination of genes coding for internal proteins, seven distinct H5N1 genotypes (one Go/GD/96-like and the rest reassortant viruses) were identified in terrestrial poultry in Hong Kong SAR and Guangdong Province of China in that year, and at least another five genotypes were identified in terrestrial poultry in Hong Kong SAR in 2002 [54, 142]. These viruses caused

Figure 9.2 Distribution of Gs/GD-lineage H5 HPAIV HA clades during the period 1996–2002. Source: Adapted from World Map centered at the Pacific Ocean, $200inaire, 4 July 2012. https://commons.wikimedia.org/wiki/Category:Blank_maps_of_the_world_without_Antarctica#/media/File:White_World_Map(Pacfic-centered)_Blank.png Used under CC BY-SA 3.0. http://creativecommons.org/licenses/by-sa/3.0/.

outbreaks of disease in Hong Kong SAR for the first time since 1997. Disease occurred in live poultry markets in May 2001 (mainly clade 3), and on farms and in markets in early 2002 (mainly Clades 0, 4, and 8).

There were no official reports of disease from mainland China in 2001 and 2002. However, H5N1 HPAIVs were isolated from a range of species, including chicken, duck, quail, and partridge, with cases extending from Guangxi to Jilin Province [24, 178].

Among the viruses isolated from terrestrial poultry in Hong Kong SAR in 2002 was one referred to as the "Z" genotype. This subsequently became the dominant (but not the only) genotype associated with the panzootic for much of the first decade of the twenty-first century. The first known representative of the "Z"-genotype virus was isolated from a healthy duck in Guangxi Province, China, in 2001 [24].[2] These viruses have continued to evolve (through mutation in individual genes), such that "Z"-genotype viruses isolated from the same location at different times, and even those isolated from different countries at the same time, have significant genetic and antigenic differences based on gene sequencing and antibody profiling [55, 91]. The combination of internal protein genes that make up the "Z" genotype was associated with multiple HA clades.

The year 2001 also saw the first reported cases of H5N1 HPAIVs infecting mammals other than humans. Pigs were found to be infected in Fujian Province, China, in 2001. It is not clear whether clinical disease reported in some of these pigs was due to infection with H5N1 virus [90]. In addition, at least one tiger was found to be infected in Harbin in 2002 [23]. Based on experiences from Thailand in 2004, the most likely source of infection would have been infected poultry.

Infected duck meat was imported from China into the Republic of Korea in 2001 [163], although no outbreaks of disease associated with this particular virus or related strains of viruses were detected in the importing country. This finding demonstrated the potential for spread of infection via contaminated or infected poultry products.

An additional novel finding emerged in late 2002, when wild birds in two zoological collections in Hong Kong SAR developed fatal disease and were found to be infected with Gs/GD-lineage H5N1 HPAIV. The first outbreak involved a zoological collection in Penfold Park (Shatin, New Territories) [41], and the virus belonged to clade 1. The park, in the center of a racecourse, contained a small lake that was home to a collection of waterfowl. Little egrets (*Egretta garzetta*) had access to this site, and at least one of these birds (found dead near the park) was infected with this virus [41, 55]. The second outbreak in birds in a zoological collection occurred at Kowloon Park in late 2002 and early 2003, around 13 km from the first outbreak. This outbreak was halted using a combination of isolation, limited depopulation, and vaccination, and involved a wide range of captive species. The source of the virus was not determined [41].

2003: emergence of H5N1 virus into other parts of Asia

More cases of infection were detected in live poultry markets in Hong Kong SAR, and outbreaks of

[2]Referred to here as the "G" genotype.

disease also occurred on several chicken farms, where it was controlled using a combination of limited depopulation and vaccination [42]. The last H5N1 HPAIV isolate from live poultry markets in Hong Kong SAR, until June 2008, was detected in November 2003, just prior to the introduction of vaccination for all poultry from mainland China that were destined for Hong Kong SAR [42]. In early 2003 the next two human cases of disease associated with H5N1 viruses were identified. These were detected in Hong Kong SAR, but the patients developed clinical disease in Fujian Province before returning to Hong Kong SAR [55, 121]. These viruses were closely related to viruses detected in wild birds in late 2002 (i.e. they had a similar gene constellation and no amino acid deletion in the NA stalk). The HA gene of these viruses grouped within clade 1. A similar virus was detected in a condor in Guangdong Province, China, in 2003 [70]. This virus was a reassortant with a PB2 gene apparently derived from North American influenza viruses and a different NA gene to the human isolates. Among the clades detected for the first time in poultry in mainland China during 2003 were viruses from clades 2.3.2, 2.3.1, and 2.5, again demonstrating the extent of viral circulation and evolution [178].

The emergence of severe acute respiratory syndrome (SARS) in 2003 may have led to the misdiagnosis of some cases of severe H5N1-related disease in humans. This possibility was demonstrated by the detection of such a case in Beijing in late 2003. This case was caused by a clade 7 virus and was not identified as being due to a Gs/GD-lineage H5N1 HPAIV until several years after the event [194].

The first official report of disease associated with H5N1 HPAI outside of Hong Kong SAR in 2003 was from the Republic of Korea. Results of investigations suggest that subclinical infection in ducks preceded detection in domestic chickens [172]. Other countries where disease had already emerged in poultry, captive animals, or humans by the end of 2003 included Indonesia [34], Vietnam [84], Japan [99], Thailand [72], and Cambodia [35], although disease was not reported until 2004.

In 2003, a Gs/GD-lineage H5N1 HPAIV in a new sub-lineage was detected in the Xinjiang autonomous region of China in geese [32], the first report of infection in the north-west of the country. Market studies conducted in a range of southern provinces identified infection in China in both terrestrial and aquatic poultry [91]. Serological evidence of infection with H5 virus in pigs was also reported in Fujian and Guangdong Provinces. In addition, one H5N1 virus was isolated from a pig in an area in Fujian Province where infection in pigs had been detected previously [90].

2004: responses to outbreaks in Asia and viral evolution

In the first 2 months of 2004, outbreaks of H5N1 HPAI were reported officially in quick succession from a number of countries in Asia, including those listed above, as well as Lao PDR and China. In August 2004, Malaysia also reported infection. The disease and the control measures implemented in the region (based largely on stamping out in a wide ring around known infected premises, with concurrent movement controls) resulted in massive losses of poultry. Some countries (e.g. Japan, Republic of Korea, and Malaysia) eliminated the virus, but the measures used did not result in elimination of infection from the region. Success was achieved in countries where infection was detected relatively early, the virus had not disseminated widely, and veterinary infrastructure was well developed. Lack of success in eradication in other places was due to a range of factors, including limited veterinary capacity, failure of farmers to report or recognize disease, widespread infection in dispersed non-biosecure farms with complex market chains often involving sale of birds through poorly regulated live poultry markets, maintenance of virus in silently infected domestic waterfowl, and difficulties in implementing appropriate movement controls [144].

In 2004, no infection was reported in commercial poultry in Hong Kong SAR, but positive samples were obtained from free-flying birds, including a peregrine falcon (*Falco peregrinus*) in early 2004 (clade 9), and gray herons (*Ardea cinerea*) (clade 2.3.2) [178]. H5N1 viruses were also isolated from free-flying tree sparrows (*Passer montanus*) in Henan Province of China. These were reassortants forming new genotypes within clade 7 [79]. These findings demonstrated the potential importance of a range of wild birds in the local transmission of Gs/GD-lineage H5 HPAIVs (Figure 9.3).

During 2004, there was a marked increase in the number of human cases, especially in Thailand and

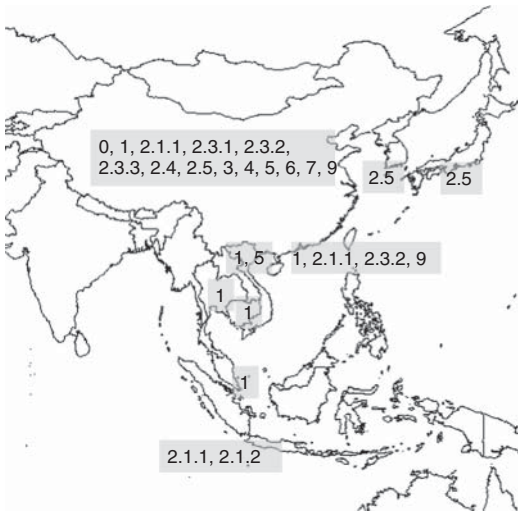


Figure 9.3 Distribution of Gs/GD-lineage H5 HPAIV HA clades during the period 2003–2004. Source: Adapted from World Map centered at the Pacific Ocean, $200inaire, 4 July 2012. https://commons.wikimedia.org/wiki/Category:Blank_maps_of_the_world_without_Antarctica#/media/File:White_World_Map(Pacfic-centered)_Blank.png Used under CC BY-SA 3.0. http://creativecommons.org/licenses/by-sa/3.0/.

Vietnam, as well as additional mammalian cases [182]. One notable outbreak in tigers in a sanctuary in Thailand led to the death of over 45 tigers, and may have resulted in limited tiger-to-tiger transmission [160]. Most of the cases were probably the result of feeding infected chicken carcasses, although it is now recognized that close contact between experimentally infected domestic felids can lead to transmission of infection [130]. This conclusion is also supported by subclinical cases in domestic cats in Austria in 2006 that occurred in an animal shelter as a result of exposure to infected wild birds kept in nearby pens. Spread of infection in this Austrian case was considered to be through close contact, rather than via ingestion of infected birds [88].

Viruses isolated from the newly reported outbreaks in 2003–2004 demonstrated considerable genetic diversity of isolates from different countries, yet showed remarkable genetic homogeneity between isolates within infected countries or groups of countries, suggesting either single introductions or multiple introductions of virtually identical viruses. Viruses in Indonesia (clade 2.1) differed from those in Thailand, Vietnam, Lao PDR, Cambodia, and Malaysia (clade 1), and from those isolated in Japan and South Korea (clade 2.5). The only exception was China, where a range of virus genotypes and sub-lineages had already been identified and several new clades were identified, including clade 2.3.3. Even within single provinces there was variation in the viruses isolated in 2004 [189].

2005: Qinghai Lake and the westward movement of virus

Events in 2005 were dominated by the detection and emergence of a new clade of H5N1 virus that was identified in Qinghai Province in north-western China in migratory birds (clade 2.2). The detection of this virus was preceded by the discovery of H5N1 HPAIVs with similar genes to those found in Qinghai in wild ducks at Poyang Lake in Jiangxi Province of China earlier in 2005 [26], although migratory links between the two sites have still not been established. The origin of this clade remains unclear [25, 27]. Viruses in this clade differed from those isolated previously from wild birds or poultry in that virtually all of the viruses in clade 2.2 isolated post detection at Qinghai had an E627K mutation in the PB2 protein, a signature normally associated with viruses of mammalian origin. It is not clear how or where this mutation first appeared, as neither the initial viruses isolated from Qinghai nor those from Poyang Lake possessed this mutation [25, 27], in contrast to virtually all other viruses in this clade isolated subsequently. These events raised concerns that H5N1 viruses with a signature for potential mammalian infection and increased mammalian pathogenicity would be spread by wild birds during their movement or migration. Strains of this clade were subsequently detected in wild birds and poultry in southern Russia and Kazakhstan, and in wild birds in Mongolia [27]. The outbreaks in southern Russia and Kazakhstan predominantly involved small poultry flocks in isolated locations where poultry and wild birds shared a common environment. The cases in wild birds in Mongolia occurred in areas where there were no poultry farms.

In early October 2005, a closely related virus belonging to clade 2.2 was associated with outbreaks of disease in poultry in Turkey and Romania.

The outbreak in Turkey continued into 2006, and extended from the east of the country to the west, with cases detected in poultry and wild birds. It was brought under control following "stamping out", movement restrictions, and quarantine. Infection spread in Romania where the virus became temporarily established in village poultry in the Danube Delta. Reported cases of disease in Romanian poultry were confined mainly to small village flocks and several large farms between October 2005 and June 2006 [170]. In addition, virus was reported in poultry and wild birds in the Ukraine. Perhaps most significantly, in October 2005, virus was detected in Croatia in a dead mute swan (*Cygnus olor*) in the absence of any infection in poultry in the wider region, followed by another 16 cases in mute swans, mallards, and black-headed gulls that appeared to be healthy [138]. Furthermore, an H5N1 virus in clade 2.2 was detected in a live wild teal in Egypt in late 2005 [135], the first report of an H5N1 HPAIV from Africa.

Asian countries including China, Thailand, Vietnam, Cambodia, and Indonesia continued to detect and report cases of infection and disease in poultry and humans caused by strains of virus other than those in clade 2.2 (although some cases in Liaoning Province in northern China were associated with viruses from clade 2.2 [178], as was one case in a tiger in Shanghai [106]). By 2005 at least three different sub-lineages of virus were present in Vietnam [84] (clades 1, 2.3.2, and 8), indicating additional introductions of virus from elsewhere [84, 149]. The first viruses of clade 2.3.4 were detected in China in 2005 [174]. This particular clade has been an important cause of disease for a number of years. Viruses derived from this clade spread to North America and Europe in 2014–2015.

One infected Chinese pond heron (*Ardeola bacchus*) was found in Hong Kong SAR through a dead bird surveillance program (clade 2.3.2), but no cases of infection were detected in poultry. Additional mammalian cases were detected in 2005 in captive Owston's banded palm civets (*Chrotogale owstoni*) in northern Vietnam [133].

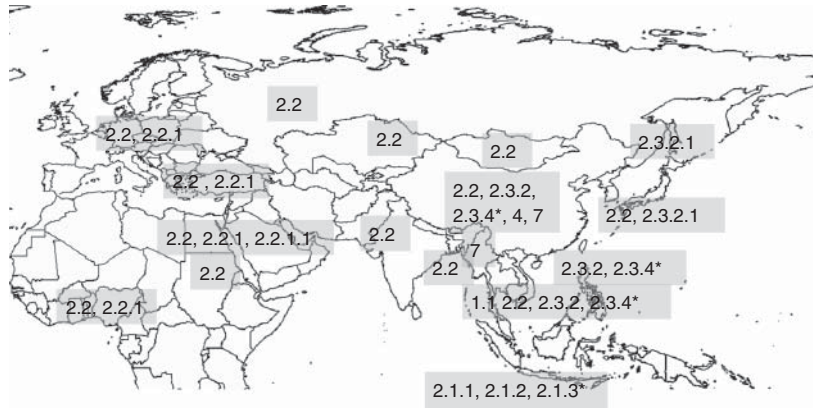
2006–2007: outbreaks in three continents

In 2006, infection with H5N1 viruses of clade 2.2 extended their range through West Africa, Western Europe, West Asia, and the Middle East, affecting wild birds, poultry, humans, and other mammals. Additional cases of infection and disease continued to be found in Asia, including cases in countries where disease had not been reported for 3 years, and in those that had never reported disease. Many of these cases were caused by viruses belonging to clades 2.3.2 and 2.3.4.

In Africa, infection and disease associated with H5N1 viruses of clade 2.2 were reported in poultry, initially in Nigeria [39], and later in other countries in West Africa, including Niger, Burkina Faso, Cameroon, Côte d'Ivoire, Ghana, and Togo. Detection in Nigeria was soon followed by detection of infection in Egypt, Sudan, and Djibouti. The disease in Egypt and Nigeria was present in both commercial and backyard poultry.

Disease, mainly in poultry, was also reported in Myanmar, Pakistan, India, Afghanistan, Iraq, Iran, Israel, Jordan, and Palestine in the first quarter of 2006. The high number of cases detected across Western Asia and the Middle East within a relatively short period of time indicated that the viruses were already widespread throughout this region. The source of infection and route of introduction for most of these countries was not determined, but all of the viruses detected between 2006 and 2008 from these countries, with the exception of one from Myanmar [176], belonged to clade 2.2 or its derivatives (Figure 9.4). The limited occurrence of this virus in poultry in East Asia, and the fact that it was detected in wild birds without being detected in poultry (as well as repeated incursions in later years) provide strong circumstantial evidence for a role for wild birds in the introduction of the virus to new regions. This applies in particular to those places with no direct trade in poultry or poultry products from countries where these strains of virus were present. However, the possibility of illegal trade in live birds and/or poultry commodities in some cases cannot be excluded.

In European Union (EU) member states between January and May 2006, H5N1 viruses were detected in or isolated from 748 individual dead wild birds from over 60 species [127]. The peak incidence occurred in mid-March, coinciding with adverse weather conditions. Clusters of H5N1-positive birds were detected in some areas, such as the Baltic Sea and Danube Delta, but incursions into poultry were limited, with only five



*Vietnam includes clades 2.3.4.2 and 2.3.4.3
China includes clades 2.3.4.2, 2.3.4.3, 2.3.4.4 and 2.3.2.1
Hong Kong SAR includes clades 2.3.4.3 and 2.3.2.1
Indonesia includes clades 2.1.3.1, 2.1.3.2 and 2.1.3.3

Figure 9.4 Distribution of Gs/GD-lineage H5 HPAIV HA clades during the period 2005–2008. Source: Adapted from World Map centered at the Pacific Ocean, $200inaire, 4 July 2012. https://commons.wikimedia.org/wiki/Category:Blank_maps_of_the_world_without_Antarctica#/media/File:White_World_Map(Pacfic-centered)_Blank.png Used under CC BY-SA 3.0. http://creativecommons.org/licenses/by-sa/3.0/.

member states (Denmark, France, Germany, Hungary, and Sweden) reporting outbreaks. These were mainly in outdoor production systems involving various types of poultry [127]. By early July, infection had been reported in 26 European countries, of which 25 reported affected wild bird populations and 11 reported outbreaks in poultry. Of the latter 11 countries, four (Denmark, France, Germany, and Sweden) successfully contained single outbreaks. However, extensive spread in poultry was reported in Hungary, Romania, the Russian Federation, Turkey, and Ukraine, predominantly in outdoor production systems. The presence of virus in wild birds in many countries in the absence of reports of disease in poultry provided further evidence for probable introduction of virus to these countries via wild birds. In 2007 (in both early and late winter) there were a number of limited outbreaks associated with clade 2.2 viruses in domestic poultry in the Czech Republic, Germany, Hungary, Poland, Romania, and the UK, while consistent with the epidemiology in 2006 there were also wild bird cases in the Czech Republic, France, Germany, and Poland.

Further to these episodes in poultry and wild birds, five countries in the Middle East and Africa reported human infections/fatalities in 2006–2007, including Azerbaijan (8/5), Djibouti (1/0), Egypt (43/19), Iraq (3/2), and Turkey (12/4) [182]. All of these cases apparently occurred in places where there was close association between poultry and humans. Some cases in Azerbaijan were possibly associated with defeathering of dead wild swans [175], suspected of being infected with H5N1 HPAIV. Cases in Egypt were mainly associated with household poultry.

Infection in poultry in Indonesia remained endemic. Genetic analyses of virus isolates revealed that they all grouped together within clade 2.1 and its derivatives, but were relatively heterogeneous, reflecting evolution as they spread across the country [149]. By the end of 2006, infection had spread as far east as West Papua, and had involved 29 of 33 provinces. Consistent with widespread infection in Indonesian poultry, an increasing number of human cases were identified (97 cases with 82 fatalities in 2006–2007). By August 2006, Indonesia had recorded more human fatalities from H5N1 HPAIVs than any other country. This included at least one large cluster of cases in Sumatra in which limited human-to-human transmission probably occurred, although it was difficult to prove that there were no other sources of exposure in this and other clusters [122]. Further human cases in Asia in 2006 and 2007 were reported from China (18 clinical cases/11 fatal cases), Thailand (3/3),

Lao PDR (2/2), Pakistan (3/1), Vietnam (8/5), and Cambodia (3/3) [182]. No human cases were reported in Vietnam during 2006, which probably related to the many measures introduced to control the disease in poultry, including large-scale poultry vaccination. No further human cases were detected in Thailand after 2006 or in Lao PDR after 2007.

New cases of disease in poultry were reported in Thailand (after a period of more than 6 months without a reported case), Lao PDR, and Cambodia. Some of the cases in Thailand were caused by viruses from clade 1, indicating that these viruses were still circulating in the region. However, other human and poultry infections in Thailand and Lao PDR were due to viruses from clade 2.3.4, indicating the introduction of a different lineage from the ones that were isolated in 2004 [30] (Figure 9.4).

Vietnam detected virus by targeted surveillance of unvaccinated ducks, indicating that the risk of infection of terrestrial poultry remained high. No outbreaks of disease were reported in poultry in Vietnam in 2006 until December, when unvaccinated ducks in the south of Vietnam in the Mekong Delta developed clinical disease. Sporadic cases of disease were detected in 2007, mainly in unvaccinated Pekin and Muscovy ducks. Viruses in the south of the country belonged to clade 1.1, whereas those in the north were grouped within clades 2.3.2 and 2.3.4 and their derivatives [84].

In the first half of 2006, infection in wild birds was reported again in north-west China, largely in Xinjiang, Tibet, and Qinghai [179]. Virus was also detected in wild birds in Mongolia at the same time [52]. This clade 2.2 virus was not detected again in these areas in subsequent years. New outbreaks of disease occurred in poultry in several northern provinces, mainly in layer farms. One of these outbreaks, in Shanxi Province in June 2006, resulted in the culling of more than 1.7 million poultry and was caused by a novel virus, antigenically and phylogenetically distinct from earlier strains, belonging instead to clade 7 [37]. The precise origin of this strain remains unknown, although it still falls within the GS\s/GD-lineage H5 HPAIV. A new vaccine based on this viral antigen was produced after this outbreak, for use in China. Clade 7 viruses continued to evolve in China to form clade 7.2, which was also detected in Vietnam.

Market samples from southern China were also found to be positive for H5N1 viruses in 2006 and 2007 – predominantly, but not only, clade 2.3.2 and 2.3.4 viruses [148]. The rate of recovery of virus from swabs collected in markets conformed largely to patterns seen in previous years, in which the rate of isolation increased in the winter.

Active surveillance in Hong Kong SAR led to the isolation of viruses from dead free-flying birds from 15 locations in the first quarter of 2006, and 14 locations in the first quarter of 2007, using similar surveillance strategies [43]. Two smuggled chickens were also positive for H5N1 virus in the first quarter of 2006. Viruses examined in 2006 belonged to clade 2.3.4, whereas those examined in 2007 were largely from clade 2.3.2, demonstrating incursions of different viruses. Clade 2.3.4.3 viruses were first detected in wild birds in Hong Kong SAR in 2007.

Virus was isolated from a range of passerine species and also from birds of prey. In 2007, no virus-positive dead water birds were found, in contrast to previous years, in which dead water birds such as herons, gulls, and egrets were found to be infected. A seasonal pattern was apparent, with all cases in 2006 and the majority of cases in 2007 detected between January and March, despite intensive surveillance of dead birds being conducted throughout the year.

Further cases of infection and disease in mammals were reported, including cats (in Germany, Austria, and Indonesia), stone marten (in Germany), and mink (in Sweden) [128]. All of these incidents were believed to result from close exposure to infected poultry or wild birds, or from feeding on dead birds. There were also some reports of infection and disease in Plateau pikas in western China [192].

In February 2007, disease occurred on a turkey farm in Suffolk, England [119]. Analysis of the genes of these viruses demonstrated almost 100% similarity with viruses from concurrent outbreaks in geese in Hungary. Further investigations revealed that unprocessed meat from Hungary was being transported to a related processing plant adjacent to the turkey farm. However, links between this meat and infected farms in Hungary were not established. Biosecurity breaches were detected on the turkey farm [8]. Another outbreak occurred in November 2007 in a flock of free-range turkeys, again in Suffolk. It was not directly related to the original outbreak, and the virus was almost certainly introduced through wild birds. Elsewhere in

Europe, virus was detected in poultry in Germany, Poland, and Romania. All of these cases were due to clade 2.2 viruses.

In 2007, outbreaks of disease were also reported widely in Bangladesh, a country that had not reported infection previously. This was followed by reports of disease in West Bengal in India. These outbreaks involved clade 2.2 viruses. Disease also affected a number of layer farms in Kuwait, where it was managed by stamping out of most of the country's layer flock. The disease was first detected in February 2007 in backyard poultry, but then affected commercial poultry. Saudi Arabia reported its first outbreak in April 2007. A clade 2.3.4 virus was detected in Malaysia in 2007.

By 2007, clade 1.1 viruses were circulating in southern Vietnam and Cambodia. Viruses in clades 2.3.4.1 and 2.3.4.3 had also been identified in Vietnam (Figure 9.4).

2008–2010: spread of clade 2.3.2.1 and evolution of clade 2.3.4

The period 2008–2010 saw a transition with regard to the predominant viruses. Clade 2.2 viruses that had been detected in Europe since 2005 were not detected there after January 2009, and were followed by the next westward wave of H5N1 viruses in 2009 and 2010, this time involving viruses belonging to clade 2.3.2.1c. This strain of virus reached as far as Bulgaria and Romania. It would appear likely that introduction was via wild birds. This clade had spread to and presumably been maintained (at least transiently) in wild birds, as surveillance further to the east had detected these strains predominantly in water birds but also in raptors. The last wild bird case in Europe associated with a clade 2.2 virus was detected in a mallard in Germany in January 2009. Clade 2.2 viruses persisted throughout 2010 in South Asia, and were responsible for new outbreaks in Nepal and Bhutan. These viruses were similar to those in India and Bangladesh. Co-circulation of clade 2.3.2.1 and 2.2 viruses occurred in this sub-region in 2011. Clade 2.3.4 viruses continued their evolution to form a number of fourth-order clades, some of which have disappeared while others have persisted.

It was also a period when it was acknowledged that H5N1 viruses were entrenched in a number of countries, and that virus elimination was unlikely to be achieved in these places at least in the next 10 years [44]. Nevertheless, some major successes had been achieved. By 2009, both Thailand [156] and Nigeria [113] were no longer detecting cases of H5N1 HPAI, although Nigeria re-experienced outbreaks due to a new incursion of virus in 2015. During this period, new cases of H5N1 HPAI were no longer being reported from Pakistan.

In 2008, Hong Kong SAR reported its first H5N1 HPAI cases in commercial poultry since 2003, when a novel clade 2.3.4 virus was detected in several retail markets in June and then in a commercial farm in December [146]. This particular strain of virus was an antigenic variant against which the vaccine in use provided suboptimal protection. The outbreak in December only involved one farm, but resulted in considerable disruption to trade in poultry. The route of introduction was not determined, but wild birds were considered the most likely source, given that they were present on the farm, roosting around chicken houses.

A similar virus was also isolated from a wild peregrine falcon at about the same time. In addition, clade 2.3.2.1 viruses were detected in wild birds in Hong Kong SAR in 2008 and 2009 [150].

In December 2010, Hong Kong SAR detected the first of a number of dead chickens and ducks infected with H5N1 HPAIV that were washed ashore, mainly on the outer islands [145]. The origin of the birds was not determined, but they were found on beaches at the head of the Pearl River Delta, which suggests that they may have floated downstream or been discarded overboard from boats carrying poultry.

An imported human case was also detected in Hong Kong SAR in 2010 [179]. The virus belonged to clade 2.3.2.1c. The case was a Hong Kong resident who had a history of travel to several mainland cities in and around Jiangsu and Shanghai.

Throughout this period the Chinese Ministry of Agriculture continued to report cases of infection in poultry detected during routine surveillance. A higher proportion of samples from ducks were positive than from chickens [101]. Human cases in China during this period were mainly caused by clade 2.3.4 viruses and their derivatives, with only three cases caused by clade 2.3.2.1 viruses (including the imported case in Hong Kong SAR) [169].

In 2008, Japan and the Republic of Korea both reported outbreaks of disease associated with a clade 2.3.2.1 virus. However, unlike the previous two outbreaks, these occurred in April. Only wild bird cases were detected in Japan. A very similar virus was subsequently found in far eastern Russia [150], but was not detected further west, and there was no apparent invasion of the flyways in North America, as occurred in late 2014 and early 2015. One of the closest relatives to these viruses in Japan was detected in Hunan Province in China in 2007.

Both Japan and the Republic of Korea also experienced outbreaks of disease in poultry in 2010–2011 as a result of entry of a clade 2.3.2.1c virus found in both countries in December 2010. Detection of virus in wild birds preceded cases in poultry in Japan, and involved a captive swan in a zoological collection and other wild birds elsewhere. These cases were followed in 2011 by outbreaks on a small number of poultry farms. The outbreak in the Republic of Korea was much larger, involving 53 farms, but resulted in depopulation of 286 farms and destruction of around 6.4 million head of poultry [77].

H5N1 HPAIVs continued to evolve in China. By 2010, clade 2.3.2.1 viruses that had first emerged in 2007 had evolved into three distinct lineages. These were A/Hubei/1/2010-like viruses (clade 2.3.2.1a), A/HK/Barn swallow 1161/10-like viruses (2.3.2.1b), and A/HK/6841/2010-like viruses (clade 2.3.2.1c). All three clades were detected in China, including Hong Kong SAR, as well as in Vietnam. However, a rapid expansion in range occurred with differences evident between the fifth-order clades that also resulted in some antigenic differences. Clade 2.3.2.1a viruses have predominated in Bangladesh and India primarily in poultry since 2011, whereas clade 2.3.2.1.b viruses have spread from China to Vietnam and have been found primarily in poultry. It is noteworthy that despite being an antigenic variant that would be expected to give this virus a selective advantage in vaccinated poultry, viruses in Clade 2.3.2.1b have not been reported in Vietnam since 2012, and were last reported from China in 2014. Clade 2.3.2.1c showed an apparently broader host range, including a wide range of wild bird hosts and poultry. Presumably as a result of dispersal via wild bird populations, these viruses have been detected across a broad geographical region since 2009 [180], including parts of Asia other than China and Vietnam (i.e. Nepal, Mongolia, Japan, Republic of Korea, and Iran), and Europe (Bulgaria and Romania). The geospatial and temporal patterns indicate that this clade can be maintained in wild birds perhaps, at times, independent of poultry, although spillback of fitter strains from poultry probably occurs intermittently. Related but not identical strains of clade 2.3.2.1c virus were found in Russia, Bulgaria, Romania, and Nigeria in 2014–2015 (Figure 9.5).

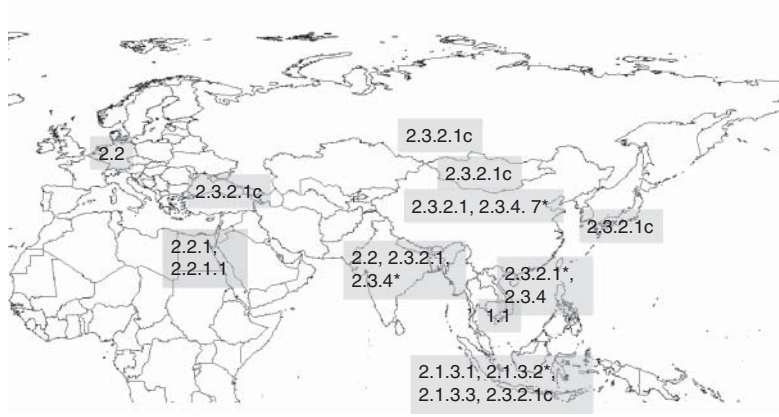
Clade 2.3.4 viruses also continued their evolution into fourth-order clades, including the first detections of clade 2.3.4.4 viruses (initially referred to as clade 2.3.4.6) [95, 191] that were to eventually be transmitted to Europe and North America in 2014–2015. Clade 7.2 viruses continued to evolve. These were last reported in outbreaks in vaccinated poultry in 2014 [185].

Clade 2.3.4.1 viruses were identified in human cases in Hunan Province in 2009 and also in poultry in Guizhou Province and in Vietnam and Lao PDR in 2009–2010. Clade 2.3.4.2 viruses were detected in Guizhou Province and Vietnam, and subsequently in Lao PDR and Myanmar. Clade 2.3.4.3 continued to circulate in Vietnam until 2009.

Clade 2.3.4.4 viruses, including a number of reassortants with N2, N5, or N8 genes, were detected from 2008 onwards. These were the precursor viruses for the strains of virus that infected poultry and wild birds in Korea in 2014–2015 and spread to Europe and North America in the winter of 2014–2015.

Viruses related to a clade 2.3.4 virus isolated from a peregrine falcon in Hong Kong in 2009 were detected in multiple Chinese provinces. They included an H5N2-subtype virus detected in a chicken in Tibet in 2010 (a reassortant virus carrying genes derived from H9N2 viruses) [96]. Somewhat surprisingly, the cases in poultry in Tibet for which gene sequences are available (other than one isolate from 2011) were caused by strains of virus that were not found in migratory birds in Qinghai Province or in poultry in South Asia, despite being in between the two populations on migratory bird pathways.

In May 2009, deaths in wild birds caused by H5N1 HPAI were reported in Qinghai Province. This was



*South Asia includes clades 2.3.2.1a and c, 2.2.2 and 2.3.4.2
Indonesia includes clades 2.1.3.2a and 2.1.3.2b
China includes clades 2.3.2.1a, b, and c, 2.3.4.1, 2.3.4.2, and 2.3.4.4.
Vietnam includes clades 2.3.2.1a, b, and c, 2.3.4.1, 2.3.4.2.

Figure 9.5 Distribution of Gs/GD-lineage H5 HPAIV HA clades during the period 2009–2012. Source: Adapted from World Map centered at the Pacific Ocean, $200inaire, 4 July 2012. https://commons.wikimedia.org/wiki/Category:Blank_maps_of_the_world_without_Antarctica#/media/File:White_World_Map(Pacfic-centered)_Blank.png Used under CC BY-SA 3.0. http://creativecommons.org/licenses/by-sa/3.0/.

followed by reports of infection in a wild swan in Mongolia, and in June by cases in Tyva Republic. By November an outbreak was reported in western Russia around Moscow. These were all caused by clade 2.3.2.1c viruses.

Myanmar reported cases of H5N1 HPAI caused by viruses in clade 2.3.4.2 around Yangon in February 2010. Similar viruses were also detected elsewhere in Myanmar and in Bangladesh, but this particular strain did not become established there [102].

Thailand reported several outbreaks of disease in poultry in January 2008, associated with clade 1 viruses and their derivatives. This contrasted with outbreaks in 2007 that were caused by introduced viruses, and it suggested that clade 1 virus was still persisting in the country. Some differences were detected between viruses from different parts of Thailand, including some reassortment between the clade 1 strains [9].

In South Asia, outbreaks continued to occur in Bangladesh and India. Nepal experienced its first outbreak in January 2009. Disease was detected in a number of new provinces in India, including Sikkim in January 2009. The first outbreaks in Bhutan occurred in free-range village chickens near the Indian border in 2010. The virus was closely related to those elsewhere in South Asia within clade 2.2 which subsequently evolved into clade 2.2.2. The first introduction of clade 2.3.2.1 viruses was recognized in Nepal in February 2010 [107].

Viruses were continuing to evolve in Indonesia, with some antigenic variant strains identified that were causing problems for parts of the poultry sector using vaccination [34].

Ukraine and Turkey reported additional outbreaks in January 2008 in backyard flocks. Cases in the Ukraine were in Crimea, whereas those in Turkey were located along the Black Sea coast in backyard poultry and were associated with clade 2.2.1 viruses. Wild bird cases were detected in swans over an extended period in and around a swan nursery in southern England [8]. Wild bird cases were also detected in Germany and France.

Cases of H5N1 HPAI were still occurring in early 2008 in a number of West African countries, including Benin. Nigeria had a new incursion of clade 2.2 virus that was most closely related to wild bird isolates from Europe in 2008. Egypt continued to experience outbreaks of disease. By 2008 the viruses in Egypt had evolved to form two clades, 2.2.1 and 2.2.1.1, with the latter predominantly found in the commercial sector. This particular strain was an antigenic variant, with the antigenic changes possibly driven in part by the vaccination program [65].

Israel detected a clade 2.2.1 virus in a petting zoo in Haifa in January 2008. This was the first time that virus had been detected in December, a pattern that was repeated for all outbreaks in the area around Haifa, including another outbreak in 2014–2015.

Iran also reported an outbreak in 2008 associated with a clade 2.2 virus that was closely related to a virus detected there in 2006, suggesting that the virus may have persisted in the area for several years.

2011–2013: virus largely restricted to countries with entrenched infection

The period from 2011 to 2013 was marked by additional cases in Egypt, Vietnam, Cambodia, China, Indonesia, and South Asia – places where infection was well entrenched. Outbreaks that commenced in Japan and the Republic of Korea in 2010 extended into 2011. Several outbreaks in Israel and the Palestinian Autonomous Territories were associated with spillover of viruses circulating in Egypt.

Cambodia experienced a marked increase in the number of human cases in 2011 (8 cases, all fatal) and again in 2013 (26 cases, 14 fatal), accounting for two-thirds of global cases in that year. The viruses all belonged to clade 1.1.2, with some strains being reassortants carrying one gene from a clade 2.3.2.1c virus. The reason for the increase was not determined, although some of the increase related to enhanced testing capacity and active surveillance programs. Changes in receptor-binding characteristics were found in human but not avian isolates, suggesting these changes occurred after human infection [131].

The period from 2011 to 2013 was noteworthy for the absence of new outbreaks in Europe despite continued levels of passive surveillance, especially in the poultry sector. The absence of incursions probably also relates to the relative risk and level of infection in wild bird populations. By this time, clade 2.2 and its derivatives had most probably disappeared from wild bird populations. There is some evidence that these viruses became highly adapted to gallinaceous poultry and had reduced infectivity for domestic waterfowl (B. Z. Londt, personal communication). Clade 2.3.2.1c viruses continued to circulate in Asia and were responsible for outbreaks in Iran in 2011 (previous outbreaks had been caused by clade 2.2 viruses) [78].

Virus evolution continued in China. Human cases in Guizhou Province were associated with viruses in clade 2.3.4.2 in 2012 and 2013. Other human cases were caused by viruses in clade 2.3.2.1. Clade 2.3.4.4 viruses continued to reassort and acquire different neuraminidase genes. A human case that was detected in Canada in December 2013 in a traveler returning from China was found to be infected with a clade 2.3.2.1c virus. It was not possible to determine how the exposure occurred, but it was deemed to be an imported case [115]. A case in a tiger in a zoo in Jiangsu Province was reported in 2013. This virus was a reassortant with a novel PB2 gene [60].

Vietnam continued to experience occasional outbreaks of disease in poultry and infection in ducks. Clade 2.1.3.2c viruses became the dominant strain, and spread to southern Vietnam, complicating the use of vaccination as two distinct viruses were circulating there. Only occasional human cases were reported.

It was not until 2011 that clade 2.3.2.1a viruses arrived in India and Bangladesh. During 2011, three different clades of virus were circulating in Bangladesh – clades 2.2, 2.3.4, and 2.3.2.1a. Human cases in 2011 were caused by clade 2.2 viruses. From 2012 onwards, apart from several clade 2.3.2.1c viruses in Bangladesh in 2012, only clade 2.3.2.1a viruses have been detected. In addition, considerable reassortment of H5N1 viruses was detected in Bangladesh, both between H5 isolates and with H9N2 viruses [49]. An outbreak of disease in crows marked the introduction of these viruses to Bangladesh, although it is likely that poultry were also affected at that time, given that traces of viral RNA were detected in live poultry markets [73]. By 2013, antigenic variation had been detected in these viruses in Bangladesh.

Similar clade 2.3.2.1a viruses were also detected in Nepal, India, and Bhutan in 2011, and continued to evolve [180]. Intermittent reports of disease in India continued in 2011 in Tripura, Assam, and West Bengal. In 2012, seven outbreaks were reported, of which five were on government farms (Orissa, Tripura, and Meghalaya states). All of these cases occurred during the first 4 months of the year. In 2013, outbreaks were reported in Bihar and Chhattisgarh,

with two of the three outbreaks occurring in university- or government-managed flocks. This over-representation of cases in government institutions suggests considerable under-reporting by the private sector.

Bhutan and Nepal reported additional outbreaks in 2012 and 2013 associated with clade 2.3.2.1a virus that had been circulating and evolving in the wider region.

Indonesia experienced an incursion of clade 2.3.2.1c virus in 2012 that was detected following investigations of high mortality in ducks [36]. The presence of this virus complicated control of this disease, especially for those farms that were using vaccination. The original clade 2.1 viruses in Indonesia have now evolved into fifth-order clades – clades 2.1.3.2a and 2.1.3.2b [37]. Clade 2.3.2.1c virus was detected in Austria in smuggled songbirds that originated from Indonesia [17].

Gs/GD-lineage H5 HPAIVs have also been evolving in Egypt. The antigenic variant clade 2.2.1.1 that had emerged in the commercial poultry sector had disappeared by 2011 [11] (Figure 9.6).

2014–2015: the third wave of intercontinental spread

The period from 2014 to 2015 was remarkable because, for the first time in this panzootic, virus found its way to North America, almost certainly being transferred by wild birds [86]. This period was also noteworthy because two separate viral clades (clades 2.3.2.1c and 2.3.4.4) were transported long distances from Asia to the Middle East and beyond.

In early 2014, the Republic of Korea reported outbreaks of HPAI in poultry, especially in ducks, that extended to virtually all parts of the country. These were caused by clade 2.3.4.4 viruses of the H5N8 subtype. Wild bird cases were also detected. Wild waterfowl migration and domestic duck density were important in the emergence and persistence of H5N8 in the Republic of Korea. Specifically, H5N8 entered the country via the western province of Jeonbuk and spread rapidly among the other western provinces, where densities of overwintering waterfowl and domestic ducks were generally higher, but rarely persisted in the east. The most recent common ancestor of H5N8 was estimated to have arrived during the peak migration of overwintering birds into the country. Recent outbreaks in 2014–2015 in the Republic of Korea are more likely to represent re-introductions via winter bird migration [63].

Similar viruses were also present in China at this time, a similar virus was isolated from a wild bird in eastern Russia in September 2014, and related viruses returned to Korea the following winter.

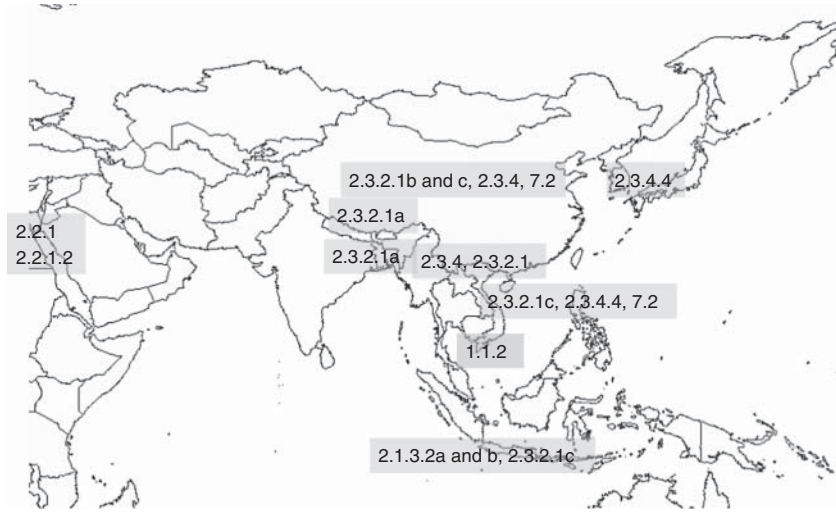


Figure 9.6 Distribution of Gs/GD-lineage H5 HPAIV clades during the period 2013–September 2014. Source: Adapted from World Map centered at the Pacific Ocean, $200inaire, 4 July 2012. https://commons.wikimedia.org/wiki/Category:Blank_maps_of_the_world_without_Antarctica#/media/File:White_World_Map(Pacfic-centered)_Blank.png Used under CC BY-SA 3.0. http://creativecommons.org/licenses/by-sa/3.0/.

This virus was also introduced to Japan in early 2014, and caused disease on one farm. In addition, virus was detected in wild birds. More cases in wild birds were detected in late 2014, with a strain isolated from a crane in Kagoshima showing signs of evolving.

Viruses similar to those detected in Korea and Russia were also found in Europe, starting with an outbreak on a turkey farm in northern Germany [58], and followed in rapid succession by multiple outbreaks in the Netherlands [15], additional cases in Germany, and a single outbreak in northern England [57]. The two unusual aspects of these cases were that the affected farms reared birds predominantly indoors, and no similar viruses had been detected between eastern Russia and Europe. Cases were also detected subsequently in turkeys in Italy and domestic ducks in Hungary in February 2015 (Figure 9.7).

Further to these incursions into commercial poultry there was an increased awareness and enhancement of surveillance in wild birds, and a number of cases were detected, principally in healthy wild waterfowl in several countries, including Germany and the Netherlands. These viruses were very closely related to the strains detected in poultry. Furthermore, all of the viruses detected in Europe were closely related and formed a distinct monophyletic cluster, but contained some strains from late 2014 in the Republic of Korea and in Japan, and separated from the emergent clade 2.3.4.4 viruses in North America.

Similar viruses to the one that was isolated from a crane in Kagoshima were found in North America, starting in western Canada, but included reassorted viruses that had acquired genes from American-lineage influenza viruses from wild birds. Viruses detected included the original H5N8 strain plus reassortant viruses of the H5N2 and H5N1 subtypes [68, 120]. All HA genes fell within clade 2.3.4.4. These were the first recorded incursions of Gs/GD-lineage H5 viruses into North America despite intensive surveillance in previous years, and these cases demonstrated that viruses could move from Eurasia to North America. The spread of virus to North America has resulted in the largest epizootic on that continent in poultry, with multiple sectors and introductions involved, especially turkey and layer farms, with around 49 million poultry killed or destroyed.

At the same time a new clade 2.3.2.1c virus was found in Russia, Bulgaria, Romania, Nigeria, Burkina Faso, Côte d'Ivoire, Niger, Ghana, and India. These viruses could be clearly differentiated from those that had previously been detected in Europe, and they showed a high degree of similarity, potentially indicating a common progenitor strain. The reappearance of these viruses, including detections in wild water birds such as Dalmatian pelicans, as well as the pattern of spread strongly suggest initial dispersal via wild birds. The detections in Africa represent the first recorded for clade 2.3.2.1c viruses, and raise questions about the mode of spread.

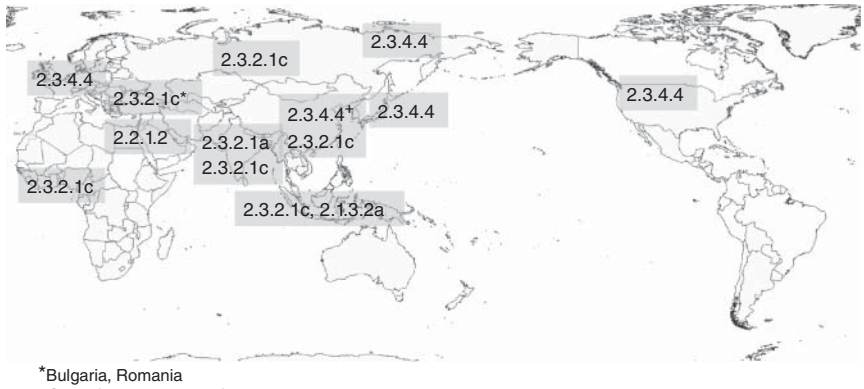


Figure 9.7 Distribution of Gs/GD-lineage H5 HPAIV clades during the period October 2014–April 2015. Source: Adapted from World Map centered at the Pacific Ocean, $200inaire, 4 July 2012. https://commons.wikimedia.org/wiki/Category:Blank_maps_of_the_world_without_Antarctica#/media/File:White_World_Map(Pacfic-centered)_Blank.png Used under CC BY-SA 3.0. http://creativecommons.org/licenses/by-sa/3.0/.

Egypt experienced a marked increase in poultry and human cases in 2014–2015, with the increase in human cases likely to be due to greater exposure to virus resulting from the increased number of cases in poultry, rather than to mutations in the virus. The virus is referred to as clade 2.2.1.2, and has replaced existing strains over the past few years. Similar viruses were detected in Israel and the Palestinian Autonomous Territories.

Human and avian cases associated with clade 2.3.4.4 viruses (H5N6) were reported in China and were widely distributed across the country. Cases involving wild birds and a cat were also detected [190]. Cases of infection with H5N6 virus were also detected in poultry in Laos and Vietnam. Taiwan experienced its first recorded major outbreak of Gs/GD-lineage H5 HPAI, with a range of clade 2.3.4.4 viruses (H5N2, H5N3, and H5N8) causing disease predominantly in goose and duck flocks. By May 2015, over 800 premises were affected [112].

Sources of infection and reasons for spread

It has proved difficult to determine the precise origin of many outbreaks of Gs/GD-lineage H5 HPAI. This section reviews some of the factors that are likely to have been involved in the spread and persistence of these viruses.

The single largest risk factor for spread of Gs/GD-lineage H5 HPAIVs is mechanical transfer of infective feces and/or oral secretions that may contain high concentrations of virus. Therefore any infected bird and any item or commodity that is contaminated with infective feces or respiratory secretions can be a source of virus for a susceptible population. As a result, poorly regulated trade in live poultry, as occurs in many countries, represents the highest risk, especially if this occurs between infected and uninfected areas and/or populations.

Trade in poultry and other birds: anthropogenic factors

Historically and conventionally, the main route of spread of HPAI has been through trade in live poultry or items related to the poultry industry. This has almost certainly been a major factor in many outbreaks of Gs/GD-lineage H5 HPAI, especially in places where virus is already endemic. For example, in Hong Kong SAR the high-level trade in live poultry was regarded as a major contributory factor in the recurring outbreaks in 1997 and between 2001 and 2003. On several occasions, virus introductions were detected via testing of live, healthy ducks and geese on arrival at a wholesale market or slaughterhouse [142].

International trade in day-old chicks has been suggested as a potential means of spread of infection. Day-old chicks hatched in properly managed mechanical incubators are unlikely to be exposed to virus when they hatch, unless virus from infected hens survives on the surface of eggs during incubation. However, naturally hatched chicks (often incubated using surrogate birds, such as Muscovy ducks, in parts of Asia) could conceivably become infected via exposure to infected feces contaminating the surface of the egg or the environment in which the chicks are hatched. If transport containers for day-old chicks are reused or are contaminated once they leave the hatchery, exposure and infection of chicks after hatching could occur. In many parts of Asia, trade in day-old chicks is poorly controlled, with considerable mixing of poultry from different sources. Most of the global trade in live poultry involves movement of day-old chicks. Trade in these birds, when conducted and regulated in accordance with international animal health regulations [111], is unlikely to pose a significant threat.

In one study, the extent of international trade in live poultry was used as a possible indicator of risk of introduction of virus from infected to uninfected countries [75]. However, as this mostly involves trade in day-old chicks, it is not a particularly reliable indicator.

Considerable illegal trade in poultry occurs, especially across land borders between countries where there are significant differences in the market price of poultry. For example, illegally imported live poultry are known to be moved across the border between China and Vietnam, and this trade is driven by the higher price available for poultry in Vietnam. This trade is notoriously difficult to eliminate, although since the emergence of H7N9 LPAIVs in China attempts have been made to curtail it in Vietnam [19]. The volume of illegal trade

is hard to estimate, but it remains a significant risk factor for introduction of avian influenza viruses.

It has been strongly suggested by some that the spread of H5N1 HPAIVs from Asia to Europe during 2006 was largely anthropogenic, with routes such as the Trans-Siberian Railway (and others) proposed as possible modes of dissemination [48]. However, the repeated movement of Gs/GD-lineage H5 HPAIVs over long distances, including introduction to North America, provides very strong circumstantial evidence for the role of wild birds in initial introductions. It is also noteworthy that many of the cases in Siberia occurred in isolated settlements located between lakes [94] distant from rail links and to which no poultry had been introduced recently.

H5N1 HPAIVs were detected in legally imported duck meat in South Korea [163] and Japan [98] before outbreaks were reported in these two countries. Trade in infected meat could contribute to the spread of infection if uncooked meat scraps are fed to poultry. However, neither of the strains of virus found to date in imported duck meat have been associated with cases of disease in these two countries. One 2001 isolate of H5N1 HPAIV from duck meat imported into the Republic of Korea is very similar genetically to viruses found in ducks in and around Shanghai, the place of origin of the meat [24].

Trade in songbirds and birds for religious release in Asia has also been proposed as a potential route of spread of infection [100]. Experimental studies of the infection dynamics of H5N1 HPAIVs in passerine species have shown that these viruses can spread in sparrows, and that infected sparrows can infect poultry [56, 188]. Dead birds representing species likely to be used for religious release in Hong Kong SAR have been shown to be infected, but it is unclear how they acquired this infection. Extensive testing of healthy, legally imported songbirds in markets did not detect virus until a single case was identified in June 2007. The possibility of illegal imports cannot be ruled out. The role of passerines beyond very local spread remains highly uncertain.

The threat of introduction of Gs/GD-lineage H5 virus via trade in captive birds was demonstrated by three incidents in Europe prior to the detection of virus in free-living birds or poultry on this continent. The first incident occurred in October 2004, when H5N1 HPAIV was detected in two crested hawk-eagles (*Spizaetus nipalensis*) that were confiscated at Brussels Airport after being smuggled from Thailand. The recovered virus was genetically closely related to those isolated in Thailand (clade 1) [167]. A second incursion occurred in October 2005 when routine investigation of deaths in quarantine of captive cage birds imported from Taiwan to the UK showed them to have been caused by infection with Gs/GD-lineage H5N1 HPAIV. The viruses recovered were genetically most closely related to viruses from southern China (clade 2.3.) [8]. The third case occurred through a seized consignment of captive birds illegally smuggled into Austria at Vienna Airport during 2013. There was a high mortality across a wide spectrum of birds, and these viruses, belonging to clade 2.3.2.1c, showed similarity to strains reported at the time in Indonesia [17]. In these cases, incursion beyond the point of detection was prevented, but due to the risk demonstrated by these cases, legal importation of captive birds from outside the European Union (EU) was banned. The possible role of illegally imported falcons, or birds used to feed falcons, in the spread of infection in the Middle East warrants further study [97].

Live bird markets

Live bird markets are well recognized as important sites for the maintenance and exchange of avian influenza viruses [146], including Gs/GD-lineage H5 HPAIVs, which are found regularly in large markets. Detection of Gs/GD-lineage H5 HPAIVs in markets has been reported frequently, but has only occasionally been associated with detectable signs of disease or reported increases in mortality [91, 142], making them appear to be silent reservoirs of virus. This finding is largely considered to be due to under-reporting of disease and rapid turnover of poultry [81, 144]. The possibility that some cross-protective cell-mediated immunity is afforded by previous infection with avian influenza viruses of a different subtype, allowing multiplication of virus without disease, cannot be ruled out [66, 139]. Vaccinal immunity may also play a role in permitting some shedding if suboptimally protected birds are infected. Multiple species of birds, both wild and domestic, are held in live poultry markets in many parts of the world, especially (but

not only) in Asia, and unless markets are very well managed this practice can facilitate the exchange and spread of viruses. Contaminated equipment and infected birds that are moved between markets and farms represent a significant threat. By the very nature of these markets, different species of bird are brought together that might otherwise have less opportunity for direct contact, thus aiding interspecies transmission of virus.

It is possible to operate live bird markets in a manner that prevents infection with these viruses, as has been demonstrated in Hong Kong SAR since 2003. However, even with stringent restrictions on sources of poultry for these markets, and improved market hygiene, infection with H5N1 HPAIVs in Hong Kong SAR was only prevented by ensuring that all poultry entering these markets came from vaccinated flocks and all consignments were tested for evidence of protective antibodies against H5 AIV. The risks associated with live poultry markets are exacerbated in locations where large numbers of poultry are reared in facilities with poor biosecurity, as is the case in many Asian countries where there is a mismatch between the threat of virus incursion to poultry flocks and the level of biosecurity implemented on farms.

Domestic ducks

In 2000, changes in the genetics of H5N1 HPAIVs corresponded with an expansion of the host range into ducks. Prior to this, H5N1 virus had been found in ducks in Hong Kong SAR in 1997, but only at a time when the prevalence of infection was very high [141]. Experimental studies of the Hong Kong/97 H5N1 virus demonstrated that this virus was not well adapted to ducks, but grew and was shed in low titers from the respiratory and alimentary tracts [125].

Since 2002, Gs/GD-lineage H5 HPAIVs have continued to be found in domestic waterfowl in Asia and beyond [91], with some, but not all, of these viruses being associated with disease [4, 118, 154]. The reasons for the variation in pathogenicity and the apparent preferential tropism for the respiratory tract of avian species are still not fully understood. The differences are strain related and the mortality is age related (i.e. there is higher lethality in younger ducks) [118]. Surveillance in live bird markets in southern China in early 2004 revealed high infection rates in clinically normal domestic ducks, with around 25% of samples yielding H5N1 HPAIV [91]. Mallard ducks experimentally infected by intratracheal challenge with H5N1 viruses shed virus asymptomatically for up to 17 days [64], demonstrating their capacity to contaminate the environment with these viruses. They are a potential source of infection for chickens and other birds, including wild species. Furthermore, it would appear that as these viruses become highly adapted to galliform species, their ability to infect and transmit between domestic waterfowl, and thus potentially affect wild waterfowl, is attenuated, with these species being almost resistant to infection (B. Z. Londt, personal communication).

The existence of high levels of infection in clinically normal domestic ducks is regarded as an important factor that contributed to the epidemic, especially in countries such as Thailand and Vietnam, and in southern China, where ducks are commonly present in households, and ducks are unprotected from wild birds and range freely on ponds and rice paddies. China and Vietnam alone rear approximately 65% of the world's ducks.

Wild birds

Wild birds are now recognized as having played a role in the long-distance spread of Gs/GD-lineage H5 HPAIVs, although the relative contributions of migratory birds and anthropogenic factors associated with the poultry industry still remain unclear in some places. This uncertainty is compounded by limited knowledge of wild bird host factors, including the range of susceptible species, infection dynamics in these birds, and precise details of their migratory and other movement patterns.

The role of wild birds in medium- to long-distance spread was initially demonstrated by the introduction of virus to parts of Western Europe and Mongolia where there were few poultry, or no accompanying cases of infection in poultry. The introduction of virus to North America in 2014 provided additional strong circumstantial evidence for transmission by migratory species. It is still not clear which species are involved in spreading the virus, but Anatidae remain high on the list of suspicion, given that Gs/GD-lineage H5 HPAIVs have been found in some apparently normal wild ducks [20, 28, 68], and mallards (*Anas platyrhynchos*) have

the capacity to be silently infected, at least for short periods of time [71]. Preliminary results indicate that emergent clade 2.3.4.4 viruses are more attenuated in mallards which do not demonstrate overt clinical signs but still shed large quantities of virus [71, 87].

The spread of H5N1 HPAI from Russia and Kazakhstan to the Black Sea basin in 2005 is consistent in space and time with migratory movement of ducks [50]. Furthermore, as surveillance programs in poultry in Europe are generally well developed, the presence of H5N1 HPAIV in dead wild birds in the absence of infection in poultry appears to be significant. Given that it may only take a single infected bird to introduce virus to a susceptible population or local environment, even a low prevalence of infection in wild birds, which is difficult to detect using active surveillance, is potentially significant.

The timing of bird migrations does not always match the timing of initial disease reports in poultry [100]. However, this does not rule out the possibility that these birds are a potential source of infection, as the timing of disease reports does not always reflect the date of the initial viral incursion [143]. Virus could spread from wild birds to domestic waterfowl (or resident wild duck populations), with subsequent silent or undetected amplification, before infection of terrestrial poultry, resulting in a lag between movements of infected wild birds and detection of disease. "Bridge" species between migratory birds and poultry populations could also play a role in virus dissemination. Recent clade 2.3.4.4 viruses in Europe and North America from wild birds and poultry have shown a close relationship, and epidemiological investigations indicate fomite transmission to poultry flocks, most likely influenced by the persistence of the wild bird-derived virus in the environment.

Although it has not yet been demonstrated to occur, relay transmission of virus may be significant. In this scenario, infected free-flying birds move over relatively short distances to stopover sites where they mix with and infect other birds, some of which then transport the virus to another location [46]. This could help to explain the pattern of spread of viruses from Asia to Europe, given that no wild birds are known to migrate directly over this particular route, and that there is a relatively slow speed of spread, which is not consistent with spread via migration [48]. There are still gaps in our understanding, such as how clade 2.3.4.4 Gs/GD-lineage H5 HPAIVs have traveled from eastern Russia and east Asia to multiple sites in western Europe in late 2014 without any cases of infection being detected in between these points. This contrasts markedly with the transmission of clade 2.2 viruses during 2005–2006 from western China to Europe and beyond, in which virus was detected in multiple locations in between.

All introductions of Gs/GD-lineage H5 HPAIVs into the Republic of Korea and Japan are considered likely to be due to introduction by wild birds, given the location of the affected farms, the timing of the outbreaks, and the molecular characteristics of the isolates. Phylogeographic studies have revealed that wild waterfowl migration and domestic duck density were important in the emergence and persistence of H5N8 in the Republic of Korea [63]. In Japan, virus was also detected in wild birds before it was found in poultry, demonstrating that well-targeted surveillance resulting in early detections in wild birds can provide information to farmers to strengthen biosecurity [136].

In a number of cases, including crows in Japan [99] and magpies in South Korea [82], infected wild birds detected during targeted surveillance were believed to have been infected by exposure to diseased poultry (or material contaminated by infected poultry), rather than having been the source of virus for poultry. Spread to vulture populations provided the first evidence of spillover from poultry to local wild bird populations in Africa [40]. In other cases, free-flying birds were thought to have been infected by captive birds in zoological collections [41]. Nevertheless, the interface between wild birds and domestic waterfowl in shared environments is sufficiently extensive in many parts of Asia and also in Europe to provide significant opportunity for exchange of viruses (in both directions) between wild birds and domestic poultry, especially where ducks are free ranging or raised on open ponds. In some areas, such as Eastern Europe in 2006, transmission in both directions is implicated by genetic data.

It is also noteworthy that not all clades of virus have been detected in wild bird populations. However, in particular clades 2.2, 2.3.2.1c, and more recently 2.3.4.4 have been detected in numerous species and locations over time that might indicate

they have a greater propensity to establish infection and spread within such populations. This might lead to independent maintenance without the need for continued reinfection from exposure to infected poultry in order to act as a reservoir for the virus. The dynamics have changed over time, with the apparent disappearance of clade 2.2 viruses since 2009 coinciding with the emergence first of clade 2.3.2.1c viruses and then subsequently of clade 2.3.4.4 viruses.

There is still insufficient evidence to determine the exact means of introduction of virus to Nigeria, the first African country to report infection and disease. Wild birds were initially assumed to be the source of infection for the outbreak detected in 2006 [39]. Multiple incursions of similar viruses have occurred, and until 2009 the viruses from Europe, the Middle East, and Africa showed a close genetic relationship [137]. All of them belonged to clade 2.2, despite the fact that they were collected from a broad geographic region covering three continents. In addition, viruses from this clade were not widely found in commercial poultry in Asia at that time.

Others suggested that the virus was introduced via illegal or poorly regulated trade in day-old chicks [166]. However, if trade in poultry from Asia was the source of infection, it is difficult to explain why introduction did not occur earlier than the winter of 2005–2006, and why only viruses from clade 2.2 emerged there (and in Europe and the Middle East), rather than other clades found widely in poultry across Asia for a number of years.

Genetic mapping of virus strains in Nigeria suggests that there were at least two independent introductions of virus in 2006, with a third introduction several years later [39, 47, 137]. Agro-ecological studies in Nigeria also suggested a role for wild birds in the introduction of virus [22].

The introduction of virus to Nigeria in 2014 follows a similar pattern to that in 2005–2006, in that similar, but not identical, viruses within clade 2.3.2.1c were detected in wild birds at stopover points on migratory pathways in Asia and Europe before being detected in Nigeria.

In many infected countries, including some in Africa, epidemiological investigations have been complicated by late detection of infection in poultry (i.e. the first reported case was not necessarily the first case of infection) and the multitude of potential pathways for introduction of virus, many of which are illegal and difficult to trace [144, 166].

Seasonal effects

Temperature and festivals

Disease patterns have varied between countries. The peak of reports during the Asian H5N1 epidemic occurred in winter 2003–2004. Earlier surveillance studies of markets in China, including Hong Kong SAR, showed that H5N1 HPAIVs were more frequently isolated during the winter months [91]. This could be attributed to better survival of virus at low temperatures, combined with the increased movement of and trade in poultry associated with winter festivals. However, outbreaks of disease have occurred in Asia in all seasons. The first outbreaks of H5N1 HPAI on chicken farms in Hong Kong were reported in March, April, and May 1997 [142]. The first post-1997 outbreak of HPAI in terrestrial poultry in live bird markets in Hong Kong SAR occurred in May 2001, although H5N1 HPAIV had first been detected in a market in February of that year [142]. Low temperatures do not appear to have been a major factor in the emergence of disease in Thailand, where cases have been detected throughout the year, although there appears to be greater susceptibility in late summer and early autumn (perhaps associated with storm activity).

Seasonal bird migrations

Cases in wild birds in north-western China have corresponded to the migratory movements of these birds, with most cases occurring in birds as or just after they return to and congregate on breeding grounds after winter (e.g. those in Qinghai in 2005 and 2009). The first reported incursions of Gs/GD-lineage H5 HPAIVs into the Russian Federation in summer 2006, followed by spread to Europe in autumn of the same year, coincided with seasonal patterns of migration and dispersal of some wild bird species. This alone does not provide definitive evidence of spread by wild birds, although movements of ducks to the Black Sea basin in autumn were consistent with H5N1 HPAI spread in the region at that time [50]. These movements of birds will also be influenced by weather patterns. For example, movement of Anatidae usually coincides with or precedes the first autumn

frosts in the Western Palearctic [50]. The spread of virus to western, northern, and southern Europe in 2006 was associated with unusually severe weather conditions at the time, which resulted in exceptional movements of aquatic birds from the Caspian and Black Sea regions (a wintering area) in a westerly and southerly direction [46, 114, 129]. This large displacement of birds brought many species into close contact on an atypical scale, which may have increased the extent of transmission within these populations. In addition, some populations of birds were displaced to areas outside of their regular wintering range. Outbreaks in North America, Japan, and the Republic of Korea also fitted with the timing of migrations.

Pathology of H5 HPAI

The pathology of HPAI caused by H5 viruses in avian species and selected mammals has been well documented [41, 85, 120, 124, 125, 130, 157]. Experimental studies of Gs/GD-lineage H5 HPAIVs in chickens have demonstrated that infection is systemic and causes severe damage to endothelial cells and parenchymal organs [117].

However, not all naturally infected chickens that die of Gs/GD-lineage H5 HPAI develop classical signs, such as swelling of the head, edema of the subcutis, and severe hemorrhages. These lesions were found in some dead chickens, but in many cases the only changes found were congestion and cyanosis of the wattles and combs, dehydration, some subcutaneous and subserosal hemorrhages, serous fluid exuding from the nares, congestion and edema of the lungs, and congestion and enlargement of the spleen. Pancreatic mottling suggesting focal necrosis was not a consistent feature in chickens, but was reported in other species, such as magpies in South Korea [83]. Cecal tonsils were usually slightly enlarged and hyperemic. Histologically, multifocal necrosis and inflammation were evident in the spleen, brain, pancreas, and heart. Viral antigen was detectable in most tissues, with high concentrations in the vascular endothelium and cardiac myocytes [85].

Waterfowl and other wild birds that died from H5N1 HPAI had non-specific gross lesions, including dirty ruffled feathers (typical of changes seen in birds with neurological disease that cannot groom properly), dehydration, and congestion of visceral organs. Hemorrhages were found in cranial bones. Some mild edema of the lungs and increased fluid in the pharynx and trachea have been seen in naturally infected waterfowl and other wild birds in Hong Kong SAR. Corneal opacity has been reported in a number of cases in ducks in Asia [187]. Experimental infection of Pekin ducks resulted in a range of lesions, including dehydration, splenomegaly, and thymic atrophy. The intestines of inoculated birds were empty [116]. A yellowish nasal discharge was detected in some ducks. Histological lesions identified included multifocal non-suppurative encephalitis and malacia, multifocal myocardial degeneration and necrosis accompanied by minimal inflammation, ulcerative rhinitis and degeneration, and necrosis of pancreatic and adrenocortical epithelial cells. Congestion and interstitial inflammation were detected in the lungs, and lymphoid depletion and necrosis in the spleen, in experimentally infected ducks [116].

None of these gross or histological lesions are pathognomonic of Gs/GD-lineage H5 HPAIV infection, and detection of virus is required to confirm the diagnosis.

Diagnostic aspects

The OIE *Terrestrial Manual of Diagnostic Tests and Vaccines* [110] provides recommendations for appropriate tests for diagnosis. Methods should be well standardized, appropriately validated, and shown to be fit for purpose. Diagnosis can be made by the detection of infectious virus, viral antigen, or viral nucleic acid. The use of serology for Gs/GD-lineage H5 HPAI in highly susceptible birds such as chickens is not recommended, as the course of infection is usually very short, leading to death before a detectable immune response is induced. Detection of antibody is an appropriate method for detecting previous exposure in domestic waterfowl, because many cases of infection are subclinical.

Appropriate samples

Cloacal and tracheal swabs have been used as samples of choice for field samples, as the carcass of affected birds does not have to be opened, reducing

the risk of infection for those handling sick or dead animals. Strains of contemporary Gs/GD-lineage H5 HPAIV appear to be shed at higher levels via the respiratory route than via the cloaca, and therefore it is advisable to collect samples from both sites. In places where tissue samples can be collected safely, a range of organs (including the brain) should be collected into specified viral transport medium and kept cool (at 4°C). In remote locations, collection of samples into lysis buffer or onto Flinders Technology Associates (FTA) filter paper [2] will preserve viral RNA without a cold chain, allowing detection of virus by polymerase chain reaction (PCR), but precludes the use of culture. Tissue samples should be collected into 10% neutral buffered formalin solution for histological and immunohistochemical examination.

Feathers from infected birds are also a valuable specimen for detection of Gs/GD-lineage H5 HPAIV [119]. Conjunctival swabs have also been used in wild birds, and in one study were found to have higher concentrations of virus than tracheal swabs [16].

Culture in 9- to 11-day-old embryonating chicken eggs is the gold standard technique for isolation of Gs/GD-lineage H5 HPAIVs, followed by HA and NA subtyping. Provided that suitable eggs are available from either specific pathogen-free or influenza-antibody-negative flocks, virus isolation can be a rapid diagnostic tool because most embryos inoculated with positive clinical samples die within 24–48 hours, which reduces the lag time normally associated with virus isolation, at least on positive cases. It also provides a source of virus for subsequent molecular studies and pathogenicity testing.

To speed up diagnosis, a number of different techniques for detection of specific AIV antigen or nucleic acids are routinely utilized. Rapid antigen detection tests have proved useful as a "rule in" test for clinical cases in gallinaceous birds, particularly when applied to multiple birds from a suspected flock [29, 134]. Few false-positive results have been reported, and although not as sensitive as culture or PCR, this method is nevertheless capable of detecting infection if three or more dead or sick chickens from an infected farm or market are tested (using cloacal and tracheal swabs). However, negative samples should still be submitted for laboratory testing. Furthermore, these tests are not suitable for screening of clinically normal poultry, such as fecal samples from markets, and are of limited use for testing cloacal or tracheal swabs from infected waterfowl, due to the low sensitivity. This relates to the low concentration of virus in these samples.

Various molecular techniques are also used to detect infection. Standard reverse transcriptase–polymerase chain reaction (RT-PCR) was used originally in many countries, but has now largely been replaced by real-time RT-PCR (rRT-PCR) targeting matrix gene conserved among all influenza A viruses, or specific tests for Gs/GD-lineage H5 HPAIVs. The former allows detection of any avian influenza virus, and subtype-specific assays for detection of H5 and N1 (and other N-subtype) viruses are also in routine use [3, 147]. Given the changes to the HA gene of H5 HPAIVs, it has been necessary to update primers and probes to ensure that all isolates are detected. These tests are highly sensitive, several orders more so than virus isolation, but with the advantage that they can deliver results within around 4–6 hours. Clade-specific tests for Gs/GD-lineage H5 HPAIVs have also been developed [61].

All cases in which type A influenza is detected should ideally be cultured to determine whether infectious virus is present, and these cultures can then be used to perform standard characterization tests as described in the OIE *Terrestrial Manual of Diagnostic Tests and Vaccines* [110].

Disease control

Control strategies used for H5N1 HPAIVs have varied considerably, demonstrating that there is no single approach which is appropriate to all situations. These measures are discussed in Chapter 14. Individual control plans must match the local disease situation, the likely risk of reinfection, and the available resources. No control measure used alone is likely to lead to elimination of virus.

Control and elimination of Gs/GD-lineage H5 HPAIVs from poultry are also dependent on a fully functional surveillance system that allows early detection of infection and disease. This usually requires incentives for disease reporting, such as appropriate levels of compensation, and active surveillance systems in place to detect subclinical infection, as occurs in domestic ducks and live poultry markets. This type of surveillance depends

on a well-resourced and trained veterinary service. Most veterinary services in locations where Gs/GD-lineage H5 HPAIVs remain entrenched are still developing. Control programs should also be supported by public education and behavioral change campaigns. It is important that these programs fully engage and involve all stakeholders in order to ensure success. It is also essential to examine the factors that allow infection to occur and persist, and to make appropriate changes to production and marketing practices to reduce the risk of infection. Greater consideration of socio-economic factors will be required in the future to increase the likelihood of a positive outcome.

This section briefly reviews the control and preventive measures used in selected countries, with a particular focus on Asia, where the mix of measures used has varied and the disease (or threat of infection) has been present for up to 18 years. In reviewing the effectiveness of control and preventive measures, the biggest challenge has been to assess the precise benefits of individual measures, given that these are not applied individually and that there is no directly comparable control population.

In addition, when comparing the apparent effectiveness of control measures, the nature of the poultry sector in the affected location needs to be considered. For example, comparisons between Thailand and Vietnam need to take into account the large export sector in Thailand, and the relative lack of live poultry sales (among other factors), both of which assisted Thailand in its disease control efforts.

Hong Kong SAR

In 1997, Hong Kong SAR had approximately 1000 separate retail market stalls selling live poultry, scattered across the territory in wet markets and individual shops. There were also 200 poultry farms, largely located in the northern and north-western part of the New Territories, which supplied about 20% of the live poultry sent for sale in markets. The remaining 80% came from mainland China. There were two large wholesale markets through which most poultry were sold. There were few controls on the movement of poultry between farms and markets, or even from markets to farms. Hygiene standards in markets were generally poor, especially in the older markets. A range of poultry were sold at individual stalls, including live chickens, ducks, geese, quail, chukar, pheasants, and in some cases wild birds, such as owls and wild ducks [142].

The outbreaks of disease in Hong Kong SAR resulted in the introduction of a range of control and preventive measures, and these differed from outbreak to outbreak. The 1997 outbreak resulted in the depopulation of virtually all commercial chickens on Hong Kong farms and in live poultry markets, but several flocks containing valuable genetic stocks were spared. This measure was backed by the closure of live poultry markets for 7 weeks, and a ban on importation of live poultry during this period. All depopulated farms and markets were thoroughly cleaned and disinfected, and were not reopened until they had passed a stringent veterinary inspection. Backyard poultry were largely excluded from control measures. Once the trade in poultry had resumed in 1998, only certain farms in mainland China were allowed to supply poultry to live poultry markets. Poultry from these were subjected to inspection and serological testing at border entry points. Rapid influenza A detection tests were performed on cloacal swabs from dead or sick birds if present in the shipment and, over time, these tests were replaced by more sensitive tests based on nucleic acid amplification. A segregation policy was introduced which separated domestic waterfowl from terrestrial poultry during rearing, transport, and slaughter. A dedicated waterfowl wholesale market and slaughterhouse was established, with the bagged carcasses being sold at retail markets.

Market stalls that were previously licensed to sell wild birds had their permits to do so revoked. Wooden cages used for transport were replaced by plastic ones that could be more easily cleaned and disinfected. Special cage-washing equipment was installed in the main wholesale market. Affected farmers and traders were paid generous compensation and *ex gratia* allowances to cover the loss of poultry and lost business during the 7-week shutdown.

These measures apparently prevented virus from re-establishing in retail markets until 2001, when several Gs/GD-lineage H5N1 HPAIVs were detected. Markets were again depopulated and closed for 1 month. Imports of live poultry were banned. As there were no outlets for live poultry

reared on local farms (there was no central poultry slaughterhouse in Hong Kong SAR for terrestrial poultry), market-weight poultry on farms had to be destroyed. Again compensation and allowances were paid to affected traders and farmers. New measures were introduced to improve market hygiene, including monthly rest days in retail markets that required a total depopulation of market stalls for 24 hours on one day every month (synchronized to prevent movement of poultry from stall to stall). A segregation policy was also introduced for quail.

Further outbreaks of disease in 2002 and 2003 on farms and in markets resulted in the introduction of vaccination as an additional control and preventive measure, along with improvements to farm biosecurity. These outbreaks were controlled by limited culling of poultry in the affected area, and the use of ring vaccination. Following these outbreaks, additional measures were implemented to enhance market hygiene, including an additional rest day and strict limits on the number of poultry allowed in market stalls. Eventually this resulted in bans on keeping poultry in markets overnight, and a ban on keeping backyard poultry. Between December 2003, when vaccination of all poultry destined for live bird markets was made mandatory, and February 2015, there have been only two occasions when virus has managed to penetrate the system (retail markets were found to contain infected poultry in June 2008, and one commercial farm was found to be positive in December 2008), despite outbreaks occurring in poultry in Guangdong Province (the source of imported poultry) throughout this period. Wild birds infected with Gs/GD-lineage H5 HPAIVs were detected regularly throughout this period, yet no additional farm cases were reported. These cases were found as a result of the intensive surveillance program in place covering poultry in markets and on farms. This includes the keeping of unvaccinated sentinel chickens in all batches of vaccinated chickens on local farms, a measure that is not popular with farmers. Strict limits have also been placed on the numbers of chickens that can be raised and sold.

The successful experiences in Hong Kong SAR provide some valuable lessons for other countries, although they cannot all be directly transferred, given the unique features of the small Hong Kong poultry industry and the financial resources available. In particular, these experiences demonstrate the need to use multiple measures, to adopt an iterative approach to control, to modify production and marketing systems, and to tightly regulate the sources and movement of poultry. These experiences also demonstrated that in areas at high risk of exposure to virus, such as live poultry trade, improved farm biosecurity alone was not sufficient to prevent recurrence of infection. This resulted in the decision to use preventive vaccination [146]. One of the major challenges faced by the program is maintaining appropriate protective vaccine antigens, given the rate of evolution of viruses in mainland China.

The unexpected economic consequences of certain control measures were also demonstrated following the implementation of the duck and goose segregation policy. Chilled carcasses produced at the dedicated duck and goose market and slaughterhouse established in 1998 in Hong Kong SAR could not compete with cheaper chilled carcasses from mainland China once trade in the latter was permitted. This dedicated market and slaughterhouse has since closed along with all of the local duck farms. The ban on keeping poultry in retail markets overnight also resulted in a high percentage of market traders surrendering their trading licenses and receiving *ex gratia* payments from the government [146].

Thailand

Among the countries in South-East Asia, Thailand had the most to lose from outbreaks of H5N1 HPAI because of the dependence of its poultry industry and economy on poultry exports. It has also been among the success stories.

Prior to the outbreaks in 2004, the poultry industry in Thailand had grown at a remarkable rate. Much of the industry was vertically integrated, with the majority of poultry grown in the industrial sector. Nevertheless, there were substantial smallholder and non-industrial sectors, including a significant number of grazing ducks that fed in recently harvested paddy fields and were moved between fields by vehicles over considerable distances.

Disease in poultry due to HPAI in Thailand was first reported in 2004, but this was not the first case of infection, given that the first human and feline

cases occurred earlier [72, 162]. Several waves of disease were reported, with the second wave, in October 2004, having the most cases (but also more intense surveillance than during the first wave). The Thai authorities used intensive door-to-door surveillance (so called "X-ray" surveillance) to detect cases, and this played a key role in reducing the levels of infection by enhancing early detection [18]. The "X-ray" survey detected more than 750 separate infected flocks in 51 provinces between 1 October and 9 December 2004. This large number probably reflects the introduction of intensive surveillance as well as an increase in the number of disease outbreaks. Several movement restrictions were used. Movement restrictions prevented long-distance transport of grazing ducks, and controls on fighting cocks were introduced, including the development of a fighting cock passport, although this measure probably had limited benefits in terms of disease prevention [10]. The use of vaccination against HPAI was banned in Thailand, although some consignments of smuggled vaccine were intercepted, and in the past there have been reports of the use of vaccine, especially in layer farms [10].

Stamping out was the main method used, and initially involved culling in a wide zone around infected premises, but this zone was subsequently reduced to the affected farm or village. Around 62 million poultry were destroyed or died in Thailand [18]. Compartmentalization is being used by large integrated operations. The Thai export industry is now based mainly on cooked produce, which is less affected by the presence of HPAI in the country than fresh or chilled meat sales. Biosecurity on farms has been improved, but there are still significant problems with the standard of these measures, especially on smaller farms.

The success of the measures used has been demonstrated by the absence of reports of new cases of infection since 2010, when serological evidence of infection in ducks was last reported [13], and gene sequences of several isolates of Gs/GD-lineage H5N1 HPAIV from chickens and the environment in 2010 were uploaded to GenBank.

Vietnam

By late 2003, H5N1 HPAIV infection was already established in Vietnam with clade 1 and other viruses. By 2004, the disease had affected 24% of communes and 60% of towns. By March 2004, 17% of the poultry population either had been culled or had died from disease, equivalent to around 45 million birds. This initial "wave" of infection and disease was followed by other less severe waves of outbreaks in 2004 and 2005. Stamping out reduced the levels of infection, but did not eliminate the virus. After the first wave, the stamping-out policy was changed from a wide ring around infected premises to affected farms only. Ho Chi Minh City banned the rearing and sale of live poultry, and closed a large number of small slaughterhouses. This led to a shift towards the sale of chilled carcasses in retail outlets within markets from a small number of central slaughtering plants on the outskirts of the city. Hanoi closed a large urban wholesale market and upgraded one other large wholesale market that supplied much of the poultry to the city. Over the next 9 years a number of other smaller markets across the country were upgraded. Special emphasis was placed on ensuring that market workers and staff understood the need for change in behaviors.

In 2005, a national poultry vaccination program was initiated for poultry in high-risk areas. The first round was conducted from October 2005 to January 2006, and resulted in the delivery of 166.3 million doses of vaccine to chickens and 78.1 million doses of vaccine to ducks. This was followed by further rounds of vaccination in high-risk areas in 2006–2007. No outbreaks of HPAI in poultry were reported between December 2005 and late 2006, when disease was detected in unvaccinated ducks in the south of the country. Additional cases were detected in May 2007, predominantly in unvaccinated Pekin and Muscovy ducks. Stamping out was used on the affected farms. The precise contribution of vaccination to control of the disease is not known, as it was implemented with other measures. However, since its introduction there has been a marked reduction in the number of avian and human cases. The vaccination program has been modified over time, with reduced central support for the program, especially following the emergence of clade 2.3.2.1b virus, against which existing vaccines were largely ineffective. When outbreaks of disease are detected, stamping out is used in combination with ring vaccination.

Other measures that have been introduced in Vietnam include increased enforcement activities on poultry smuggling, and some improvements in farm biosecurity. Veterinary services have been strengthened, with a special emphasis on veterinary laboratories, epidemiology services, and community-based animal health workers. Considerable effort has been expended on market surveillance and on post-vaccination monitoring of antibody levels.

Gs/GD-lineage H5 HPAIVs remain endemic to Vietnam, but progress has been made in reducing their impact. Virus elimination remains a long-term objective, and has been recognized as such in national strategies, but it is likely that some infection-free compartments will be developed. The program in Vietnam has been noteworthy for its strong central direction based around inter-ministerial steering committees.

Mainland China

China has used a multifaceted approach to the control of HPAI. Vaccination has been widely used through regular compulsory blanket vaccination campaigns, and is able to achieve high-level immunity in industrial layer and breeder flocks. It is less efficient in ducks and short-lived meat broilers [89].Vaccines are produced locally from registered vaccine plants, with over 100 billion doses of vaccine delivered between 2006 and 2012 to a standing population of around 5 billion chickens and just under 1 billion domestic waterfowl, including geese. The major concern with regard to vaccination is that new antigenic variants are emerging rapidly, and although systems are in place to detect antigenic variants and update vaccines, there is an inevitable lag between the emergence of new strains and the incorporation of new antigens. China has been a leader in vaccine development, and some new products offer some hope for limiting infection in domestic ducks, in particular a duck enteritis virus-vectored vaccine that may help to improve levels of vaccination coverage and flock immunity in ducks [89].

In the event of an outbreak of disease in poultry, a combination of stamping out and ring vaccination is used. Progress has been made in enhancing surveillance capacity, in building veterinary laboratories, and in increasing epidemiology capacity. Nevertheless, the massive size of the Chinese poultry sector, with around 50% of poultry reared by smallholders, presents enormous challenges to veterinary authorities.

Considerable post-vaccination surveillance is conducted by national veterinary authorities. However, as in many other Asian countries, passive surveillance systems need to be strengthened, as demonstrated by the detection of some cases of zoonotic disease in humans before detection of the source of infection in poultry.

The emergence of influenza A(H7N9) virus as a zoonotic agent in 2013 has resulted in many changes to live poultry marketing, especially in locations that experienced multiple human cases. Bans (both short-term and permanent) on live poultry sales have been implemented in a number of large cities in affected provinces. The strong cultural preference for live poultry in southern China has limited the changes in these areas, and it is noteworthy that in 2015 the majority of human H7N9 cases were in the southern provinces. Virus elimination (both Gs/GD-lineage H5 HPAIVs and H7N9 LPAIV) remains a long-term objective.

Cambodia

Cambodia has experienced intermittent cases of disease both in poultry and in humans since 2004. Generally, the country has a low poultry density, with most poultry being reared in village or smallholder flocks. The main control measure used is stamping out, but no formal compensation is offered to support this process. Vaccination is not permitted, and considerable resources have been expended on enhancing public awareness and improving disease surveillance. Work has been undertaken, especially at the village level, to strengthen veterinary services and to introduce measures to control this and other zoonotic diseases. Some changes have been made to poultry markets, but there are few controls on movement of poultry either to major urban centers or across the border with Vietnam.

Republic of Korea

Following the introduction of Gs/GD-lineage H5N1 HPAIV in 2003–2004, the virus was eliminated by a combination of stamping out, movement controls, and rigorous tracing studies.

Subsequent disease outbreaks have been handled using essentially the same approach as in 2003–2004, but have resulted in the destruction of very large numbers of poultry. Active surveillance was required to detect infection in duck flocks with clade 2.3.4.4 H5N8 virus, because most ducks displayed either no clinical signs or only mild signs of disease (e.g. a fall in egg production). Improvements to farm biosecurity are gradually being implemented. Vaccination is not permitted.

Japan

Japan has been very effective in controlling outbreaks of disease caused by Gs/GD-lineage H5 HPAIVs, using stamping out and strict movement controls. After each virus incursion, improvements have been made to farm biosecurity, and early warning systems have been developed through monitoring of migratory birds and their habitats. Improvements in farm biosecurity probably played a role in limiting the occurrence of outbreaks in recent years despite the presence of Gs/GD-lineage H5 HPAIVs in wild bird populations. Vaccination is not permitted.

Indonesia

Infection in Indonesia was present in 2003, persisted through 2004, and at the time of writing is still endemic in many parts of the country, especially Java and parts of Sumatra. A range of control measures has been used, including vaccination, stamping out, a participatory approach to disease detection and control, and measures to improve market hygiene. Vaccines are widely used in the commercial sector, and have reduced the impact of the disease on these farms. Long-term control will require significant restructuring of poultry production and marketing systems, especially given the scale of poultry production in this country.

Russia

The first reported incursions of H5N1 HPAIV into Russia occurred in July 2005, initially in western Siberia, and almost exclusively affected poultry at the village level. Many of these communities were remote, in locations where wild birds and poultry shared environments. Stamping out was deployed together with movement restrictions, quarantine measures, and disinfection. During late 2005 and early 2006 there was spread to southern and central European regions of the Russian Federation. By the beginning of March 2006, more than 1 million poultry had died or been slaughtered [69, 94]. At this time, targeted vaccination was introduced for free-range poultry and other captive birds in areas deemed to be at high risk of infection based on the location of previous outbreaks, proximity to flyways of migratory waterfowl, and the nature of the enterprise (backyard versus commercial). A total of 425 million doses of local vaccine were administered to non-commercial farms and backyard production birds between 2006 and 2010 [158]. The number of cases decreased, and infection apparently only occurred in non-vaccinated or inadequately vaccinated populations. Where infection was reported, stamping out was used. The number of vaccine doses administered has gradually declined, with 16.7 million doses being administered in 2014, as the perceived risk has decreased. Subsequent outbreaks, including those in 2014–2015, have been managed by stamping out.

Egypt

Egypt has used a combination of measures, including stamping out, restrictions on poultry movement, closure of some live bird markets, and vaccination, although the emergence of antigenic variant viruses has reduced the effectiveness of the latter measure. Vaccination for small-scale producers was undertaken in a manner that was unlikely to be effective [126], using a variety of licensed vaccines. A vectored vaccine based on herpesvirus of turkeys has been deployed [74]. The upsurge in the number of human cases in Egypt in 2015 prompted a review of the existing control measures. The disease remains endemic and will not be controlled unless there is considerable restructuring of the poultry industry, implementation of more effective measures to break the chain of infection in backyard birds, and strengthening of veterinary services.

Nigeria

The main method used for disease control in Nigeria was culling of affected populations. Some have suggested that the payment of high rates of compensation may have assisted the control of the disease following increases in these rates in 2007

[113], whereas others have suggested that the virus was unlikely to remain endemic in Nigeria, given the low rate of transmission between villages [14]. Whether the same successes can be achieved following the viral incursion in 2014–2015 has yet to be determined.

Countries of the European Union (EU)

Community control measures have included stamping out, zoning, movement restrictions, enhanced surveillance, cleansing and disinfection, and controlled repopulation. Pilot preventive targeted vaccination programs under the control of the veterinary authority were approved for use in three member states. These programs were conducted under strict requirements for ongoing surveillance in vaccinated populations, but have not been adopted subsequently, due to the reduced risk of virus incursion and reluctance from industry. All outbreaks in 2014–2015 were successfully managed by stamping out.

North America

North America experienced its first incursions of Gs/GD-lineage H5 HPAIVs in 2014–2015. Outbreaks of disease were managed using stamping out and movement controls around affected premises with cases occurring over a period of 6 months. Biosecurity measures on farms have been improved. If these viruses persist in or re-infect migratory waterfowl populations, the challenge will be to prevent the viruses from gaining entry to farms. Biosecurity measures that until 2014 had been sufficient to keep out LPAIVs were not able to prevent infections with Gs/GD-lineage H5 HPAIVs on 209 commercial farms and 21 backyard premises. Vaccines based on clade 2.3.4.4 H5 virus from North America have been developed. It is not yet clear whether these will be deployed in future outbreaks, especially given the potential for trade restrictions that might be placed on products destined for export even from areas where vaccines are not being used.

Conclusions and the future

Gs/GD-lineage HPAIVs of the H5 subtype have been circulating continuously for over 18 years since they were first detected in China in 1996. They have spread to four continents and remain well entrenched in a number of countries and sub-regions. These viruses have caused considerable losses to the poultry industry, as well as loss of human life, and continue to raise concerns about the potential for emergence of a human influenza pandemic strain of virus.

Although local elimination of H5N1 HPAIVs has been achieved in a number of countries, in some of these more than once, the prospects of global eradication remain remote unless major changes occur in these viruses or significant modifications are made to the methods used for rearing and marketing poultry in endemically infected countries and those at high risk of infection. As the poultry industry in many of these locations still utilizes high-risk practices, such as sales of broiler chickens through poorly regulated live poultry markets, and also supports large numbers of poor smallholders and associated traders, rearing poultry under conditions of minimal biosecurity, this will take many years to achieve. These problems are compounded by the relatively weak veterinary services, administration, and governance in most of these countries. As a result, appropriate preventive veterinary practices are not undertaken, including pre-market checks and movement controls on the millions of poultry in infected locations.

This panzootic has gradually resulted in a shift in attitudes towards measures for control of this disease. The classical emergency response used to control infection, involving stamping out and movement restrictions, while still of value in reducing the effects of the disease and still the first-line approach in newly infected countries, is less appropriate in endemically infected countries. Control in these countries requires longer-term measures to change high-risk industry practices, including improvements in farm biosecurity, market hygiene, and movement management for poultry. Where this is not possible, other measures to mitigate these risks are required, such as vaccination, but in these places vaccination is only able, at best, to contain the problem.

The world has had to learn to live with these viruses, and it is expected that it will need to do so for at least another 10–20 years, despite ambitious and unrealistic plans to eradicate these viruses. This will require implementation of measures that

reduce the risk posed by these viruses to poultry and public health, while at the same time balancing the social, economic, and environmental effects of the control programs used.

The outbreaks in 2014–2015 demonstrated that as long as countries remain endemically infected, Gs/GD-lineage H5 HPAIVs will continue to circulate and, in doing so, to mutate, leading to the emergence of new H5 strains or genotypes that potentially have new biological properties. However, calls by some for these countries to return to stamping-out measures do not recognize where these countries are along the path to disease eradication. Unless the necessary changes to the poultry sector are made before embarking on a final push towards eradication, and unless all cases of infection, including subclinical cases, can be identified early, countries will not succeed. Veterinary services in locations where these viruses remain entrenched are not yet strong enough to achieve this goal.

This panzootic has been unprecedented, and there are now few parts of the northern hemisphere that have not been exposed to this viral lineage. Over the past 18 years, Gs/GD-lineage H5 HPAIVs have demonstrated a remarkable capacity to survive and evolve. This viral lineage emerged at a time when the poultry industry was highly vulnerable to a pathogen of this type. The rapid expansion of poultry production to meet consumer demand over the past 40 years resulted in a shortfall between the methods used for rearing, transport, and sale of poultry and the biosecurity measures required to prevent viral incursions and spread. Even in some rich countries, poultry production systems have been found to be insufficiently biosecure to stop introduction and spread of the virus when challenged. Experiences in the Republic of Korea and Japan during the winter of 2006–2007 and again in 2013–2015 suggest that biosecurity measures in industrialized poultry farms in these two countries, especially in the Republic of Korea, were not able to prevent virus incursions. It is noteworthy that most of the outbreaks in the USA in 2014–2015 have involved farms rearing turkeys and layers, not broilers, which suggest that biosecurity measures on broiler farms might prevent virus incursion. Much remains to be learned about the mode of spread of the H5N2 virus in the USA, and this information will be vital for preventing large-scale outbreaks in the future. This outbreak in the USA will probably result in a reassessment of production methods, especially for turkeys and layers.

For countries such as Vietnam, China, Indonesia, and Egypt, in some cases the factors that have allowed these viruses to persist are changing, albeit slowly. The emergence of H7N9 LPAIV in China will be a catalyst for a shift away from live poultry sales towards centralized slaughter or more efficiently managed markets (as used in the USA and Hong Kong SAR). These changes will take time, and will occur more slowly in other countries that have not yet been exposed to the H7N9 virus. These countries face many obstacles in controlling this disease, including limited resources for disease control, political instability, and provincial autonomy that can limit the capacity to implement national control programs.

We now have ample evidence for concluding that both illegal trade and wild bird movements contribute to the spread of Gs/GD-lineage H5 HPAIVs. Both pathways must be considered when developing and implementing national and regional plans for control of this disease. Debate about the relative roles of wild birds and trade will always be contentious. For long-distance transmission of virus, wild birds should always be considered, especially in cases where patterns follow those seen in the past, where the genetics of viruses suggests entry of very closely related strains across wide geographical areas, and where no other plausible explanations that relate to contact with poultry or poultry products from infected locations can be found.

In the previous version of this volume it was suggested that cycles of infection largely independent of poultry may be established in wild birds, and that repeated incursions of virus along migratory or wild bird movement routes in Asia and elsewhere may occur. This has happened, although it seems that cycles of infection occur with different viral clades every few years. Yet the world does not yet have systems in place to prevent this from recurring. Infection will persist in the poultry sector in countries with large populations of free-ranging domestic ducks, poorly regulated movement of poultry, high concentrations of farms with poor biosecurity, and locations with extensive trade in

live poultry through poorly regulated live poultry markets, providing opportunities for spillover.

Vaccination has been used as a supplementary control and preventive measure in some countries. It will continue to be used in the medium to long term for control of Gs/GD-lineage H5 HPAIVs in places where it is needed. New vaccines using different technologies are expected to help with control of the disease, but will not result in virus elimination in places where the virus is entrenched. At best, vaccination will reduce levels of virus shedding, which is an important objective. However, experiences from the past 10 years also show that antigenic variant viruses do emerge (whether due to vaccination or to other factors), and the inevitable lag time between virus emergence and updating of vaccines creates vulnerabilities. Vaccination must be backed by measures that allow rapid identification of antigenic variants and changes to vaccine antigens when required. Where there is a mismatch between virus and vaccine, spillover of virus from poorly protected vaccinated domestic ducks to wild birds that share the same habitats could well occur. This may have been a factor in the emergence of clade 2.3.4.4 viruses. However, it needs to be recognized that without vaccination this spillover is also expected to occur because of silent infection in domestic ducks. Changes to production and marketing systems will be needed to reduce reliance on vaccination. In implementing these changes, national authorities need to consider the social, economic, and environmental implications and the technical feasibility of any changes they propose, given the adverse impacts that these can have, especially on poor farmers and other vulnerable households in urban and periurban areas. Measures designed to concentrate poultry farms should be approached with caution, as control in these settings can be challenging even in developed countries that are more reliant on structured commercial production.

The possibility of using emergency vaccination in the face of major outbreaks should be investigated further. Trade restrictions placed on exporting countries that use emergency vaccination must be scientifically based and consistent with international regulations on trade in live animals and animal products.

Prior to 2014 it was expected that control measures already implemented globally would gradually reduce the levels of infection, although seasonal peaks and annual variations were still expected to occur. Global eradication has been set back by the extensive global spread since 2014. Even though in most recently infected locations the virus has been eliminated, these places remain vulnerable to re-incursion, given the uncertainties about virus maintenance both in wild birds and in the environment, together with the future evolutionary trajectory of the virus. Experiences from the first 2005–2008 outbreaks in West Africa demonstrate that the virus can be eliminated in this region, and the lessons learned from the earlier outbreak should be applied. However, the poultry sector in West Africa has grown since the last outbreak, and this may complicate virus elimination programs. In addition, the same level of resources that was provided by donors between 2005 and 2011 may not be available in the future.

It seems that clade 2.3.4.4 viruses have not persisted in wild bird populations in North America, following the pattern seen with viruses in wild birds in Asia which have extinguished after one or several years, as was the case with clade 2.2 viruses. There are already signs of divergent evolution of these viruses in different migratory pathways. Much remains to be learned about the ecology of these viruses in wild bird populations.

One lesson that has been learned from this panzootic is that prediction of behavior of H5 HPAIVs is difficult. Nevertheless, certain patterns have been identified. Prior to 2005, when H5N1 HPAIVs were first detected in wild birds in north-western China, few predicted the movement of the virus across Russia into Europe and Africa. Now it is expected, with virus having moved to Europe at least three times since 2005, even if the exact pathways have not been determined.

Much of the effort to control Gs/GD-lineage H5 HPAIVs has been based on the concern that these viruses could become a severe human pandemic strain. This was the driver of massive investments in 2005, but appears to be less so today. Egypt experienced the highest ever monthly total of human cases in early 2015, yet there was no sign of a response similar to that mounted in 2005, when transcontinental spread first occurred and fewer human cases were being reported in individual countries. One important difference between the earlier strains of H5N1 virus and the current strains

in North America is the apparently reduced capacity of North American strains to produce disease in mammals, including humans. If in the future these viruses no longer pose a significant hazard to human health, fewer resources may be available to control them, given that the driving force for donor funds has been prevention of a possible influenza pandemic. Despite the uncertainty as to whether a pandemic strain could emerge, the high impact of such an event provides sufficient justification to prepare for such an eventuality. Reduction of the risk of human infection requires control of infection at source in the animal reservoirs. This action is also appropriate regardless of the human health implications, because this disease remains a serious animal health problem that has marked consequences for poultry producers, including many poorer farmers, and for national and international trade in poultry and poultry products. The potential long-term negative impact on global food security is a real threat, especially in poorer settings, as the human population increases.

Based on their remarkable persistence and evolution for over 18 years, natural extinction of Gs/GD-lineage H5 HPAIVs appears to be a very remote possibility, even though this has occurred with other influenza viruses. Their capacity to infect both domestic and wild ducks without necessarily causing disease has provided a niche in which an otherwise highly pathogenic virus can survive. A number of variant strains of H5 HPAIV have already emerged and disappeared in the past 17 years, presumably having been replaced by fitter versions, and this process will continue.

The past few decades have seen the emergence of a number of animal diseases that have spread globally. The emergence of Gs/GD-lineage H5 HPAIVs has provided information about some of the driving factors behind these outbreaks. Many of the drivers of avian influenza relate to the way that poultry are reared and sold, and to the sheer size of the poultry industry. This has been driven by demand, and the process of development has been left largely to market forces, with insufficient recognition of the problems that the systems that developed could and did produce. Redesigning and reshaping these systems is an extremely difficult task, but one that will have to be undertaken if this viral lineage is to be contained and eventually eliminated.

Acknowledgments

The information in this chapter was drawn from many sources. The assistance provided by colleagues in Vietnam, China, Cambodia, Hong Kong SAR, and the Food and Agriculture Organization of the United Nations (FAO) is greatly appreciated. We are also grateful to Todd Davis (CDC Atlanta) for providing Figure 9.1. Many of the virus sequences were made available through the submission of samples to the Weybridge Reference Laboratory. The countries that provided these samples are gratefully acknowledged.

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10 High-pathogenicity avian influenza outbreaks since 2008, excluding multi-continental panzootic of H5 Goose/Guangdong-lineage viruses

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Introduction

Highly pathogenic avian influenza (HPAI), originally termed "fowl plague", was first recognized as a distinct disease in 1878, and reports of outbreaks were common in many countries in Europe and North Africa during a period covering the late nineteenth century. During the twentieth century there were further more intermittent reports until the last decade, when, principally due to H5N1, the disease and infection by the virus became established in many parts of the world.

Influenza A viruses capable of causing a severe disease in poultry, namely HPAI, derive from low-pathogenic precursors of the H5 and H7 subtypes. It is generally accepted that HPAI viruses (HPAIVs) emerge following the transmission of low-pathogenicity avian influenza (LPAI) viruses (LPAIVs) of the H5 and H7 subtypes to gallinaceous poultry, and through the host adaptation process acquire changes in the hemagglutinin gene that confer high pathogenicity. In some instances the mutation from LPAIV to HPAIV appears to have occurred very quickly after introduction into poultry, but on other occasions, viruses of low virulence of H5 and H7 subtype have circulated for some time before the mutation has taken place, or have circulated as LPAIVs without mutation to HPAIVs. Because of the marked differences in the disease in susceptible poultry caused by influenza A viruses, depending on the virulence of the strain, it is necessary to provide clear definitions of the different viruses for trade and control purposes.

The international approach to the control of avian influenza (AI) in relation to international trade in live poultry and associated products has been to prevent the spread of HPAI, but there should also be measures aimed at LPAIVs of H5 and H7 subtypes. The World Organisation for Animal Health (OIE) has addressed this in the *Terrestrial Animal Health Code* [39] with clear definitions of HPAI and LPAI. The occurrence of HPAI in a compartment, region, or country will have an impact on the ability to trade freely in live poultry or poultry products, in order to reduce the risk of spread of infection. Furthermore, with the increasing global spread of HPAI, there are implications for long-term food security as well as a socio-economic impact, whereby the enzootic nature of HPAI in some regions and countries has a significant effect on local communities.

In this chapter, the reported outbreaks of HPAI during the period 2008–2015, excluding the panzootic of H5N1 (and related H5 HPAIV) of A/goose/Guangdong (Gs/GD)-lineage viruses are described. These consist of a total of 13 outbreaks

Animal Influenza, Second Edition. Edited by David E. Swayne.

from seven countries. The multi-continental panzootic of Gs/GD-lineage H5 HPAIV is described in Chapter 9.

Ostrich HPAIV infections in South Africa during 2004–2011

A history of ostrich farming in the South African Cape Provinces dates back to the late 1800s, when the birds were farmed for feathers. Currently, ostrich meat and leather are the primary value from commercial production, and the region accounts for 75% of global ostrich production. The geophysical area of the Eastern and Western Cape Provinces, which is favorable for ostrich production, is known as the Little or "Klein" Karoo – a semi-arid desert with a climate unique to sub-Saharan Africa, having wet winters and very hot dry summers. Ostrich farms tend to be clustered alongside rivers and irrigation schemes providing water for alfalfa and other small grain crops planted for fodder. Ponds and seasonally filled dams are scattered throughout the region and, together with the irrigated pastures of the Klein Karoo, attract large numbers of grazing wild ducks in winter, at a time when fresh vegetation is scarce in other summer-rainfall regions.

Except for the first 6–8 weeks of their lives, ostriches are farmed outdoors, either in feedlots or on pasture. The ostrich production system is fragmented, with each operation specializing in a specific stage of ostrich growth. Breeder farms focus on the 72-hour process of eggs and hatching. From there the chicks are moved to a chick-rearing farm until the age of about 2–3 months. Chick-rearing farms tend to be located in more arid regions that are conducive to lower mortality rates. After chick rearing, the young ostriches are moved to adult-rearing farms, often of a feedlot type, until the age of about 9–12 months, when they are quarantined and slaughtered [21].

The European Union (EU) is the major trade partner for ostrich meat, leather, and feathers, but this lucrative export market has been devastated by three consecutive yet unrelated outbreaks of H5N2 HPAI over a period of less than 7 years (Table 10.1). It is assumed that the mutation from H5 LPAIV to HPAIV occurred in ostriches, as no notifiable avian influenza virus (AIV) has ever been detected in the South African chicken flocks during the mandatory bi-annual screening of all poultry in that country.

Table 10.1 Summary of ostrich HPAI infections in South Africa during the period 2004–2011.

Location (country; states/provinces)	South Africa; **1**, Eastern Cape, 2004; **2**, Western Cape, 2006; **3**, Western Cape, 2011
HPAIV subtype	H5N2
Outbreak duration and dates[a]	**1** No formal report **2** 38 days: 19 May 2006 – 26 July 2006 **3** 308 days: 1 February 2011 – 5 December 2011
Date resolved[a]	**1** No formal report **2** 27 October 2006 **3** 5 December 2011
Number of positive premises (by production species)	**1** 38 (ostriches) **2** 24 (ostriches) **3** 44 (ostriches)
Birds affected (number and species)	**1** 26 740 ostriches **2** 7334 ostriches **3** 45 411 ostriches
Human infections	None reported
Direct costs	**3** US$6.6 million
Control measures[a]	Stamping out, quarantine, movement control at regional level, screening, zoning, vaccination prohibited, no treatment of affected animals
Source of virus	Initial introduction via wild birds as LPAIV before mutation to virulence and lateral spread

[a]Information obtained from the OIE (World Organisation for Animal Health).

2004 Outbreak in Eastern Cape Province: clinical signs, diagnosis, outbreak development, and control measures

At the end of July 2004 (winter in that region), unexplained deaths in ostriches on two feedlot farms near the towns of Somerset East and Bedford in the Eastern Cape Province of South Africa were reported to provincial veterinary authorities. Typical clinical signs observed on these farms included respiratory signs, swelling of and exudates from the eyes, fluorescent green diarrhea, depression, emaciation, collapse, and death. On post-mortem examination, some cases were found to have prominent liver damage comprising multifocal to diffuse

necrosis and degeneration, often accompanied by hemorrhagic lesions of the heart, lungs, kidney, pancreas, and intestine. Miliary granulomas in the lungs and fungal growth on the air sac membranes due to infection with *Aspergillus fumigatus* were observed in some cases [2]. Various management and environmental factors contributed to the severity of clinical disease. These included very high population density, coalescence of various age groups from several grower farms, adaptation stress associated with new rations and environment, and very cold weather. The mortality rate was around 18%, and was limited to a few camps.

Samples collected on the farm by veterinary officials were inoculated into embryonating specific pathogen-free (SPF) eggs, and an H5N2 HPAIV was isolated which had a hemagglutinin (HA0) proteolytic cleavage site of PQREKRRKKR*GLF. The index ostrich case farm was quarantined, and sero-surveillance was started immediately on all neighboring farms within a 10-km radius. Subsequently, three other farms within the same locality were found to be H5N2 positive, and control measures were extended to a radius of 30 km from the index case. On 6 August 2004 the OIE and all trading partners were informed of the outbreak and the action to be taken in order to control the disease. This was followed by a county-wide export ban on all live ostriches and other poultry, as well as untreated ostrich and other poultry products. The National Directorate of Animal Health opted to cull all infected farms, with compensation paid to farmers whose animals were destroyed as a result of these disease control measures. Culling commenced on 10 August 2004, and on 13 August 2004 an official instruction was issued to all provinces to conduct a national serological survey for avian influenza.

Forward and backward tracing of all ostrich movements associated with the index farm, as well as extensive serological testing, revealed two ostrich farms about 160 km away that tested positive for H5 AI without any birds on the farms showing any clinical signs of infection. A third farm in the area was later found to be positive on serological testing, supported by clinical and post-mortem diagnoses. One set of swabs collected from 50 wild ostriches on a game farm adjacent to a farm in this second cluster was found to be positive on real-time RT-PCR (rRT-PCR). The National Directorate of Animal Health opted to capture and slaughter all of these wild ostriches.

As the serological and molecular surveillance exercise expanded, ostriches on a farm approximately 180 km to the north-west of the index case tested positive for AIV exposure and were culled in early December 2004. Ostriches on two more farms within a 3-km radius were also culled. Based on forward and backward tracing, it was speculated that a truck returning from an abattoir where suspected infected birds from the index farm were also present could have introduced the virus, as the vehicle was not disinfected before leaving the abattoir. A further farm near the town of Jansenville, approximately 150 km west of the index-case farm, had ostriches that tested positive for H5N2 AIV during August 2004. Additional samples taken in early November were also positive, and ostriches on the farm were culled in early December 2004 [22].

A total of 26 740 ostriches from 38 farms were slaughtered in the affected areas of the Eastern Cape Province [47]. Clinical disease was present on five of the affected farms, with deaths on four of these farms. Surveillance testing revealed evidence of exposure to H5 AIV in 10% of Eastern Cape Province flocks.

2004 Western Cape Province

Following the directive of 13 August 2004 to conduct a countrywide survey, a number of suspicious serological results were found in ostriches from various farms in the adjacent Western Cape Province. Farmers, State officials, and private veterinarians did not report any deaths or clinical signs (Animal Health Emergency Follow-Up report). A total of 150 flocks from 50 farms out of a total of 463 farms were found to be seropositive, with an average of 17% of birds in those flocks being seropositive, but molecular detection assays were all negative [53] (M. Sinclair, personal communication). Whereas H5 seropositive flocks in the Eastern Cape had been culled, seropositive flocks in the Western Cape were only held in quarantine. It is generally accepted that the infection spread from the Western to the Eastern Cape Province.

Throughout the rest of the Republic of South Africa a total of 813 ostrich farms (21 596 sera by hemagglutination inhibition, HI) were tested,

and 849 commercial and non-commercial chicken farms (24 550 sera by ELISA). All of the results were negative. The export ban was eventually lifted by the EU in October 2005.

Molecular characterization causally linked the 2004 H5N2 HPAIV with other wild duck viruses isolated during the same period, and with a possible progenitor H5N2 LPAIV detected in an Egyptian goose (*Alopochen aegyptiacus*) that was hunted in the Oudtshoorn region just 2 weeks prior to the report of the index case in the Eastern Cape Province. Transmission from wild birds to ostriches and the subsequent mutation to HPAIV was hypothesized, despite the fact that this was the first reported instance of such mutation in ratites.

The intravenous pathogenicity index (IVPI) for the index farm HPAI H5N2 isolate was only 0.63, but after a further passage in embryonating eggs, a second IVPI test was performed and an elevated value of 1.19 was obtained. Cloacal swabs taken from the initial IVPI birds were inoculated into embryonating chicken eggs, and a third IVPI test was then performed on the resulting allantoic fluid, resulting in an IVPI of 2.73. In each of the first two IVPI tests, the chickens that survived had exhibited marked cyanosis of the wattles, combs, and legs, and became depressed, but by the end of the 10-day test period the birds had returned to an apparently normal clinical state [2].

2006 H5N2 outbreak: clinical signs, diagnosis, outbreak development, and control measures

In June 2006 a localized outbreak of avian influenza was detected on an ostrich farm in the Riversdale area near the town of Mossel Bay on the Western Cape Province coast. Eight moribund birds in a flock of 58 ostrich chicks aged 3–4 months subsequently died, and an H5N2 HPAIV was isolated from necropsy samples. Although approximately 85% of the remaining ostriches were seropositive, no further virus could be detected. The infected farm was immediately placed under quarantine, and a 20-km zone was established around the index farm. All ostriches as well as other poultry on the index farm were culled by 3 July 2006 [1, 23].

One second infected farm with two epidemiological units was identified by serology, and although no clinical signs were observed, the ostriches on this farm were also culled. The rest of the young ostriches and poultry were killed and examined. Positive rRT-PCR results were obtained from tracheal swabs from ostriches on a neighboring farm, but cloacal swabs were rRT-PCR negative. All three locations were clustered along the same river bend, and a total of 7334 ostriches from these two properties were culled [23].

Intensive surveillance covering all 559 ostrich farms in the Western Cape revealed 93 seropositive ostrich flocks, but virus was not detected in any of them, nor had any significant clinical signs or deaths been reported. In August 2006, a second partially related H5N2 LPAIV was isolated from an ostrich farm in the Oudtshoorn area. International trade in ostrich meat from the whole of South Africa was banned from July 2006, and the EU lifted trade restrictions on 1 November 2006 [1].

Full genome sequencing and genetic comparison indicated that the LPAIV and HPAIV 2006 strains were related, but not identical, based on internal gene reassortment [1]. The 2006 virus was also not derived from the 2004 outbreak strain, implying that the control measures instituted in 2004 had been successful and that no chronic carrier situation of H5N2 HPAI had existed in ostriches during this period (Figure 10.1). The IVPI of the 2006 strain was determined to be 0.58 [3], but since the HA0 cleavage site sequence PQRRKKR*GLF was characteristic of HPAIV, the virus was classified as HPAIV.

2011 H5N2 outbreak: clinical signs, diagnosis, outbreak development, and control measures

Serological evidence of H5 infection in the ostrich-farming areas of the Western Cape Province was detected by HI as early as May 2010, and persisted until early 2011. During this period, epidemiologic investigations could not identify viral activity through seroconversion, nor could viral RNA be detected by rRT-PCR. In early March 2011, the first rRT-PCR-positive case was detected in the Oudtshoorn area, and confirmed by sequence analysis as H5N2 with an HPAIV multibasic HA0 cleavage site.

After HPAI had been confirmed, a control area was established around the town of Oudtshoorn, exports were voluntarily suspended, and extensive

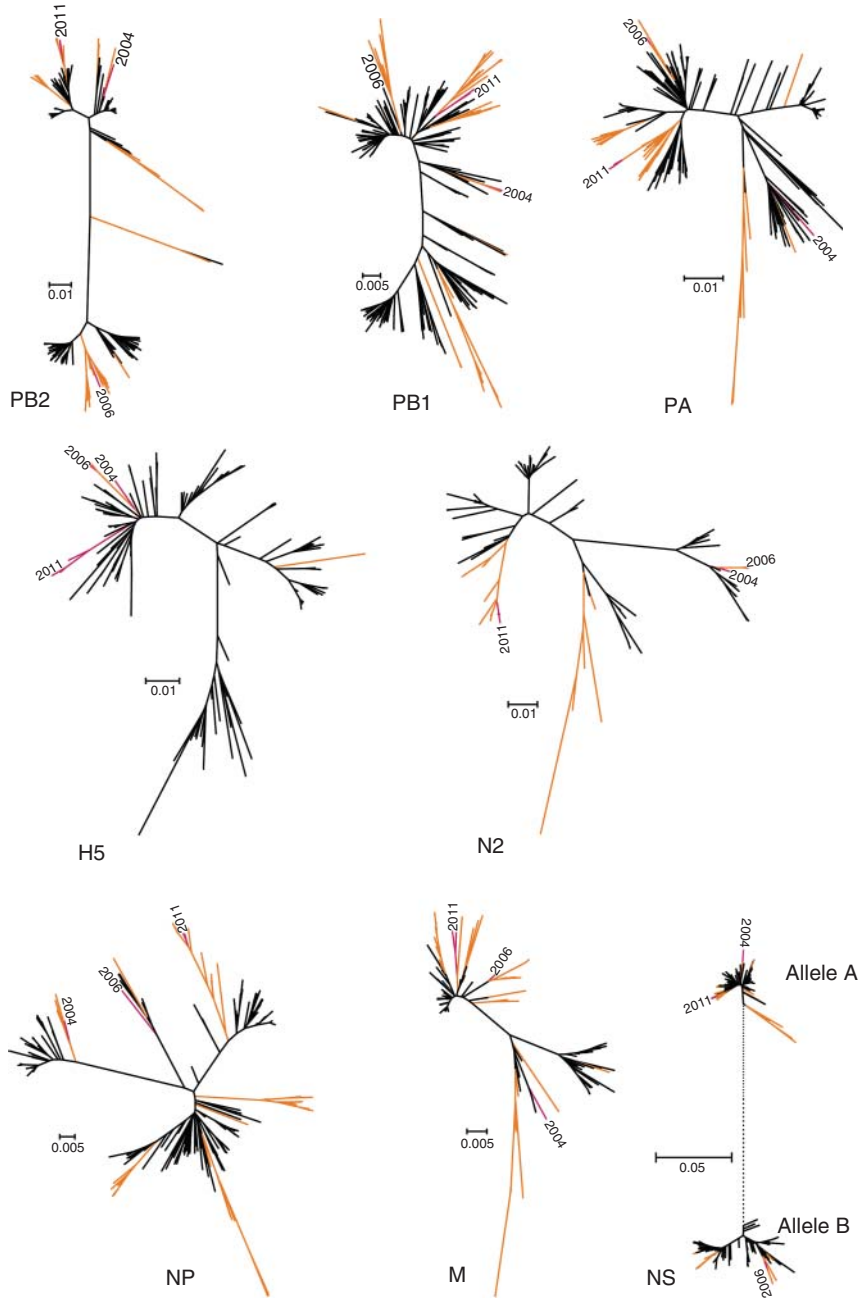

Figure 10.1 Maximum likelihood trees of full gene sequences derived from South African HPAI H5N2 isolates (shown in red), other southern African wild duck and ostrich isolates (shown in orange), and Eurasian strains (shown in black). *See Plate section for color representation of this figure.*

forward and backward tracing from the purported index farm commenced. An unprecedented HPAI outbreak in ostriches emerged from the epidemiological analyses. The formal surveillance for avian influenza on all farms in the area included 211 farms initially, and eight formal rounds of surveillance were completed between April 2011 and February 2012 in the control area. Samples were collected by state officials, and initially sera and cloacal swabs were sampled from each epidemiological group on the farm. By the start of the second round of surveillance, in May 2011, tracheal swabs were collected instead of cloacal swabs based on rRT-PCR results, in agreement with previous findings of more effective replication of AIV in the ostrich trachea compared with that of the cloaca. Tissues from birds that had died were collected during on-farm necropsies.

The index-case farm kept about 400 ostriches of ages 4–14 months that had been moved from a feedlot to alfalfa pasture after a decrease in feed intake was observed. Some birds presented with high fever and concentrated green urine. Initially, 4–5 deaths per day were reported, and these were associated with protein energy deficiency. Mortality suddenly peaked at about 23 deaths per day for a few days, and thereafter decreased to 4–7 deaths per day. In total, around 130 deaths were recorded (33% mortality). At necropsy, macroscopic lesions were observed that included multifocal to coalescent hepatic necrosis, severe diffuse necrotic pseudomembranous tracheitis, and severe diffuse airsacculitis [17]. As in previous outbreaks, the apparent clinical infection was exacerbated by inclement weather conditions and secondary bacterial infections. One of three viral isolates (AI2114) was obtained from this index farm, and an IVPI score of 1.37 was obtained for this isolate.

The second farm reported 150 deaths among a total of 550 birds of ages 7 weeks to 4 months. Two groups or camps of chicks were affected. Group 1 had good body condition but went off their feed, and clostridial enterotoxemia was suspected. High mortalities of more than 20 deaths per day for at least 2 days were recorded initially, but the mortality dropped to 2–4 deaths per day thereafter. There were no further deaths after the chicks were moved to new alfalfa pasture with supplemented feed pellets. A low incidence of green urine, depression, and loss of appetite was observed. The chicks in group 2 were not eating well following severely cold and wet weather. Gross necropsy findings in 37 birds from both groups included hepatomegaly, splenomegaly, and generalized congestion. Cachexia, serous atrophy of fat, airsacculitis, and secondary infections were also observed. Necrotizing hepatitis, splenitis, and airsacculitis were prominent histopathological findings [17]. Abundant virus was detected by immunohistochemistry in the liver, spleen, air sac, and gastrointestinal tract. Infected cells included epithelium, endothelium, macrophages, circulating lymphocytes, and smooth muscle of a variety of organs and vessel walls. No brain lesions were present, and virus was not detected in any of the seven brains that were necropsied [17].

Although clinical cases were rare on this farm, strain AI2214 was isolated from juvenile birds that died rapidly after showing signs of depression and weakness. An IVPI score of 0.8 was determined for strain AI2214. In a similar manner to isolate AI2114 from the index farm, chickens displayed clinical signs of depression, pale combs with cyanotic tips, and discoloration of the feet. However, by day 6 of the experiment, all of them appeared to have recovered with regard to mood and appetite, and cyanosis of the combs was the only clinical sign for the remainder of the experiment.

Over the course of the H5N2 HPAI outbreak, 44 farms tested positive for the virus by rRT-PCR, and the movement of infected ostriches was determined to be the primary source of spread. At least 13 farms thought to have been infected with H5N2 HPAIV were linked through an auction of birds that were subsequently dispersed from one source flock. Other potential transmission pathways were proposed to be via drinking water, wild birds, surfaces such as transport trucks, and the workers who accompanied the birds between locations [21]. The last strain linked to this outbreak was detected in November 2011, at which time a new lineage of H5N2 LPAIV was identified. An H5N2 HPAIV reported in June 2012 was found to be a laboratory-derived contamination. The H5N2 HPAI outbreak in 2011 resulted in the complete eradication of stock on all H5N2-positive farms, including the loss of valuable breeding stock, involving a total of 45 411 ostriches. This led to substantial economic losses within the region, and

government compensation in excess of US$6.6 million [21].

Phylogenetic analysis of the isolates obtained (AI2114, AI2214, and AI2512) indicated recent common ancestors with contemporary wild duck viruses isolated in southern Africa and Eurasia, once again identifying the local wild duck population as the source of the infection (Figure 10.1). The lack of reassortment and phylogenetic topology observed within around 16 genomic sequences obtained during the outbreak pointed to a single infected flock as the source. An interesting aspect was the detection of four variations of the HA0 proteolytic cleavage site sequence: PQRRKKR*GLF, PQRRRKKR*GLF, PQRKRKKR*GLF, and PQRRRKR*GLF.

Prior to September 2011, HI was the standard serological screening test for H5 and H7 AI in ostriches, but a commercial group-specific competitive ELISA (cELISA) was locally validated for use in ostriches, and has been in use since that time. However, positive reactors must still be tested by HI for H5 and H7. HI on ostrich sera is likely to fail to detect up to 35% of positive samples [4], which could partially explain how outbreaks have been able to spread undetected in 2004, 2006, and 2011. These were shown to be separate outbreaks, as demonstrated by phylogenetic analyses of full gene sequences. The 2004, 2006, and 2011 H5N2 HPAI outbreak strain genes are interspersed with those of regional and Eurasian wild duck LPAIVs as opposed to a monophyletic grouping of H5N2 HPAI strains (Figure 10.1).

The extent of the 2011 outbreak was also attributed to the expansion of the ostrich movement network after the 2008 global recession, as farmers attempted to reduce costs and maximize yields by, for example, increasing the survival rate of ostrich chicks by sending them to specialized rearing farms [21].

Specific biosecurity measures and the registration of all ostrich farms in accordance with South Africa's Veterinary Procedural Notice VPN04 are compulsory. These guidelines stipulate requirements for the keeping and registration of ostriches in approved compartments, the movement of ostriches, and specific disease prevention and control measures, among other regulations [10]. Despite these measures, the extensive nature of ostrich farming means that contact with wild birds cannot be prevented, and point introductions of H5 and H7 AI to ostriches are likely to continue. With more sensitive detection methods, such as cELISA, it is hoped that H5 and H7 AI will be detected and controlled before further epidemics occur in ostriches.

H7N7 high-pathogenicity avian influenza in the UK in 2008

On 4 June 2008, an outbreak of AI caused by H7N7 HPAI was confirmed in laying hens on a premises in Oxfordshire, central England (Table 10.2). The infection was confined to a single premises and did not spread. The virus had been introduced as an LPAIV several weeks before a marked clinical presentation characterized by high mortality. Furthermore, clinical evidence from the farm records and virological data confirmed that the H7 HPAIV was derived from a pre-existing LPAIV precursor that transmitted between epidemiological groups on the infected premises, during which time it mutated to high pathogenicity, having presumably been introduced from a wild waterfowl source. No human cases were reported, and the financial cost was in excess of £1 million (US$1.5 million).

Table 10.2 Summary of H7N7 HPAI infections in the UK in 2008.

Location (country; states/provinces)	UK; Oxfordshire, England
HPAI subtype	H7N7
Outbreak duration and dates[a]	48 days: 22 May 2008 – 8 July 2008
Date resolved[a]	28 August 2008
Number of positive premises (by production species)	1 (chicken layers)
Birds affected (number and species)	25 000 chickens
Human infections	None reported
Direct costs	c. £1 million (US$1.5 million)
Control measures[a]	Stamping out, movement control inside country, screening, zoning, disinfection of infected premises and establishments
Source of virus	Introduction of virus as LPAIV progenitor from wild birds

[a]Information obtained from the OIE (World Organisation for Animal Health).

Table 10.3 Summary of laboratory results of serological, PCR, and sequencing analyses on samples from the infected premises.

Shed	Percentage of birds positive at report[a]		Percentage of birds positive at culling		HA CS[b]
	H7 HIT	H7 rRT-PCR	H7 HIT	H7 rRT-PCR	
1	83	Not sampled	95	17	a, b
2	50	Not sampled	42	28	a, b, c, z
3	38[c]	90	97	44	a, c
4	0	100	1	92	a, c

[a]At the Initial Report, 50% of the 20 birds selected for sampling in each shed were clinically ill.

[b]HA CS = hemagglutinin gene cleavage site nucleotide sequence. One LPAIV (z, from Shed 2 feces) and three HPAIV (a, b, and c, from poultry samples) sequences were identified. z = PEIPKKR/GLF, a = PEIPKKKKR/GLF, b = PEIPKKKKKKR/GLF, c = PEIPKRKKR/GLF.

[c]Shed 3: sufficient sera for the H7 HI test (AV11SS) were obtained from only 8 of the 20 birds sampled.

There was a high H7 seroprevalence in Sheds 1 and 3. Shed 4 was positive for H7 rRT-PCR, with one bird positive on HI test.

Clinical signs and description of the affected population

The affected premises was a site containing free-range laying chickens that were housed in four separate sheds containing 25 000 pullets aged 16 weeks when placed. At the onset of the disease, sheds 1, 2, and 3 each contained 3000 birds aged 30 weeks, and shed 4 contained 16 000 birds aged 29 weeks. On 22 May, the birds in shed 1 appeared lethargic, and an inspection of the affected birds by the private veterinary surgeon revealed carcass congestion and organomegaly together with severe egg peritonitis, multifocal hepatic necrosis, multifocal splenic hemorrhages, and mucoid sinusitis. Following treatment with chlortetracycline there was no significant improvement, and similar clinical signs were observed in sheds 2 and 3 on 27 May. By 30 May there was a noticeable mortality and egg drop in sheds 3 and 4, but by 2 June there was a rapid escalation of mortality and significant egg drop in sheds 3 and 4. In fact, mortality in shed 4 was approaching 100%. Following the escalating mortality on 2 June, formal restrictions were served on the premises, and samples were taken for laboratory examination on suspicion of notifiable avian disease [11].

Initial diagnosis

The diagnosis of H7N7 HPAIV was based on rRT-PCR [54], HA0 cleavage site sequencing, virus culture, and subtype identification, together with the IVPI test in chickens (IVPI score = 3.0). Interestingly, several HPAIV HA0 cleavage site motifs were detected consistent with ongoing evolution of the virus (PEIPKKR*GLF, PEIPKKKKR*GLF, PEIPKKKKKKR*GLF, and PEIPKRKKR*GLF). Furthermore, serology in sheds 1–4 revealed interesting proportions of H7 antibody reactors (see Table 10.3). All diagnostic procedures were performed according to international standards [40].

The serology results indicate that there were seropositive birds in each shed, and the pattern of distribution of seropositive birds was supported by clinical and production records, which were consistent with the introduction of a putative H7 low-pathogenic avian influenza (LPAI) virus (LPAIV) infection into shed 1 at least 2 weeks prior to the formal disease report. Subsequently the virus spread to shed 3, 7 days before the formal report, and 3 days later it spread to shed 2. The lower mortality in sheds 1, 2, and, 3, whilst associated with the HPAIV, was detected following confirmation of disease and was of less consequence, with milder clinical signs and much lower mortality than in shed 4, indicative of a partially immune population in these epidemiological units.

Outbreak development and associated surveillance; control measures

Following the formal imposition of control measures on 2 June [24], a 3-km protection zone and a

10-km surveillance zone were implemented. There were 63 premises within the protection zone, five of which did not contain any susceptible poultry species. Only three of these premises were commercial. The remaining premises were non-commercial and typically contained between 1 and 36 birds. All of these premises were visited and clinically assessed with a comprehensive analysis of the production records, looking for any possible manifestation of AI. Limited sampling was conducted within the protection zone, involving the three commercial premises, all of which yielded negative results. There were 71 commercial premises in the surveillance zone, all of which were clinically inspected, and no evidence for spread of infection was found [25].

Clinical evidence from the farm records supports virological data indicating that the HPAI infection derived from a pre-existing low-pathogenicity AI H7 virus present on the premises. Laboratory investigations provided support for this hypothesis. H7 viral RNA was detected from fecal samples collected from beneath sheds 1 and 2. Further molecular analysis yielded from one of these samples an identical hemagglutinin cleavage site sequence to that of an unrelated H7 influenza virus from 1976 in Australia, which has been shown by *in-vivo* testing to be an LPAIV [11].

One potential hypothesis for the source of the outbreak that was investigated was an unidentified AI at a domestic premises in the UK, associated either by proximity or by potential contact. Introduction through wildlife in contact with the infected premises was also considered. A significant finding of these investigations was that a population of mallards (*Anas platyrhynchos*) had been introduced onto a pond on the premises in 2007, and was seen to be mixing with the poultry. Although laboratory testing was conducted on a small proportion of samples from these birds, the results were negative. However, there is still significant uncertainty as to the true AI status of the mallard population on this site. Furthermore, the pond was in closest proximity to the paddock associated with shed 1, which was the first house that showed evidence of infection, leading to the conclusion that the mallards were a highly plausible and likely source of the infection.

A further interesting finding of the investigations was the presence of five pigs on a different part of the site, which were not in close contact with the poultry. The pigs were clinically inspected and sampled. They were found to be clinically normal, and laboratory testing confirmed the absence of active infection with type A influenza virus and exposure to infection.

Analysis of the clinical data and laboratory results indicated that at the time of the initial report on 2 June 2008, there was serological and clinical evidence that infection with a presumed H7 LPAIV had been present on the IP for at least 12 days in shed 1 prior to sampling. Active H7 infection through the presence of infectious virus was detected in sample birds in sheds 3 and 4. At culling, random sampling of a larger number of birds in all four sheds confirmed widespread seroconversion in both sheds 1 and 3, using virus from the infected premise as the antigen for serology tests. Exposure to the putative progenitor LPAIV in shed 2 together with the presence of H7 active infection with HPAIV in all four sheds was demonstrated. The mortality rate was highest in shed 3, although more birds died in shed 4 due to the larger population size. The low proportion of seropositive birds and the high mortality rate in shed 4 are consistent with the introduction of an H7 HPAIV infection into a fully susceptible poultry population, resulting in an acute and rapid escalation in mortality.

The rapid escalation in the severity of clinical disease in shed 3 is consistent with the occurrence of at least one mutation of the presumed H7 LPAIV to high pathogenicity in the presence of a partially immune poultry population. The events that led to the eventual acquisition of genotypes and phenotypes of high pathogenicity occurred 7–10 days after the initial clinical presentation in shed 1. Moreover, following the acquisition of the HPAI genotype in shed 3, approximately 50% of the birds died over a 5-day period, which suggests that these members of the poultry population in shed 3 were naive and had not seroconverted to prior H7 LPAIV infection at that time. Immediate spread of the H7N7 HPAIV in shed 3 then occurred into a fully susceptible population in shed 4, resulting in an acute and severe clinical deterioration (with a putative HPAIV incubation period of 48 hours). Genetic analysis of the viruses suggested that there was onward spread of the HPAIV back into sheds 1 and 2 at this time [11].

Throughout the outbreak there was no evidence of human infection. All of the staff involved in the culling and the control effort on the infected premises were pre-screened by public health officials, vaccinated, and issued with Tamiflu packs. Full personal protective equipment was used during procedures on the infected premises.

The costs of the outbreak have not been precisely evaluated, but we would estimate a cost of less than £1 million associated with the control of the infected premises, depopulation, field inspection, sampling for laboratory examination of the source and spread investigation and the premises, and laboratory tests.

H7N7 high-pathogenicity avian influenza in Spain in 2009

On 13 October 2009, an outbreak of avian influenza caused by H7N7 HPAIV was confirmed in laying hens from a premises in Guadalajara, Spain (Table 10.4). Following the rapid implementation of control measures, infection was confined to a single premises and did not spread further. Analysis of the laboratory and clinical data led to the hypothesis that the virus had entered the site as an LPAIV in the days leading up to the clinical presentation, mutated following transmission within the site in the different epidemiological groups, and this ultimately led to very high mortality at some of the sites (A and B). No human cases were reported, and the financial cost of the outbreak has not been quantified.

Clinical signs and description of the affected population

The affected premises consisted of a site containing four sheds (A, B, C, and D) that housed laying hens, with a total of around 309 000 birds. In addition, some distance away from these four sheds, but on the same site, there was a breeding house. There was a noticeable egg drop in all four sheds in the week beginning 5 October. Egg drop in the first week was 56% in shed A (from 485 000 eggs), 72% in shed B (from 264 000 eggs), 28% in shed C (from 358 000 eggs), and 46% in shed D (from 351 000 eggs). In addition, in sheds A and B there was an elevated mortality rate (8% in shed A and 1% in shed B). On 9 October, formal restrictions were served on the premises and samples taken for laboratory examination on suspicion of notifiable avian disease [52].

Table 10.4 Summary of H7N7 HPAI infections in Spain in 2009.

Location (country; state/province)	Spain; Guadalajara
HPAI subtype	H7N7
Outbreak duration and dates[a]	30 days: 9 October 2009 – 10 November 2009
Date resolved[a]	27 January 2010
Number of positive premises (by production species)	1 (chicken layers)
Birds affected (number and species)	308 640 chicken layers Wider pre-emptive cull of 2.06 million birds in control zones
Human infections	None reported
Direct costs	Not known
Control measures[a]	Stamping out, movement control inside country, screening, zoning, disinfection of infected premises and establishments
Source of virus	Introduction of virus as LPAI progenitor from wild birds

[a]Information obtained from the OIE (World Organisation for Animal Health).

Initial diagnosis

Diagnosis of H7N7 HPAIV was made on the basis of rRT-PCR, cleavage site sequencing (PELPKGTKPRPRR*GLF), virus culture, and subtype identification according to standard methods [40]. In addition, serology testing was performed on sera collected from sheds A and B. It was found that 20% of the birds in shed A contained H7 antibody, and 90% of the sampled birds in shed B contained H7 antibody [52].

Outbreak development and associated surveillance; control measures

Following the imposition of control measures on 9 October [26], a 3-km protection zone and a 10-km surveillance zone were established. As a precautionary measure, a total of 860 000 birds on a total of two farms within the 3-km protection zone were culled, together with 1.2 million birds on seven farms within the 10-km surveillance zone. All depopulation was completed by 18 October. All premises within the zones were

visited and clinically assessed, and no evidence of AI infection was found. A single farm containing pigs was tested by PCR and serology for H7 virus, with negative results. The premises were sampled twice in order to obtain evidence of freedom from infection, and to demonstrate the absence of any progenitor LPAIV using rRT-PCR [27]. Further investigations performed on samples (swabs from birds, and environmental samples) collected from the infected premises revealed the presence of a low pathogenic virus in sampled birds in sheds C and D, with a cleavage site motif of PELPKGR*GLF, and a high proportion of serological reactors in shed D (shed C was not tested), consistent with a low pathogenic infection in this shed. In addition, environmental samples collected from all of the sites revealed the presence of HPAIV in sheds A, B, and C, and LPAIV in sheds C and D. The HPAIV contained a unique 18-base-pair insertion into the cleavage site region, and was responsible for the acquisition of virulence. The precise mode of origin of this insertion is uncertain, but it could be derived from host ribosomal RNA. Phylogenetic analyses of the HA gene placed the virus in a cluster with contemporary European H7 viruses, with the closest similarity (98.3% homology) to A/swan/Slovenia/53/09.

Table 10.5 Summary of high-pathogenicity avian influenza H7N3 outbreak in Mexico.

Location (country; state)	Mexico; Jalisco State
HPAI subtype	H7N3
Outbreak duration and dates[a]	**1** 155 days: 13 June 2012 – 16 November 2012 **2** 422 days: 3 January 2013 – 1 April 2014
Date resolved[a]	**1** 16 November 2012 **2** No official reports
Number of positive premises (by production species)	**1** 44 (chickens) **2** 14 (chickens)
Number of birds affected	**1** 11.4 million birds were depopulated or died. A further 11 million birds were pre-emptively slaughtered
Human infections	Two cases of conjunctivitis
Direct costs	> US$1 billion
Control measures[a]	Stamping out, quarantine, movement control inside country, screening, zoning, vaccination, disinfection of infected premises
Source of virus	Not definitively identified

[a]Information obtained from the OIE (World Organisation for Animal Health).

Highly pathogenic avian influenza H7N3 outbreak in Mexico

First wave of the outbreak: 2012

Clinical signs, initial diagnosis, outbreak development, and control measures

On 18 June 2012, poultry producers from the Los Altos region of Jalisco State reported to animal health authorities (by telephone) an increased mortality in layer hens (Table 10.5). On the same day, samples from three farms in two municipalities were submitted by local animal health officials to the Central Laboratory of the Mexico–United States Commission for the Prevention of Foot-and-Mouth Disease and Other Exotic Animal Diseases (CPA). The samples were processed, and the initial diagnosis was H7 HPAI obtained on 21 June and reported to the World Organisation for Animal Health (OIE) early on 22 June [28]. Complete characterization of the three viruses was obtained 3 days later by the neuraminidase inhibition test (N3), with IVPI scores in the range 2.5–3.0, and sequencing of the cleavage sites compatible with HPAIV. Therefore the viruses were classified as HPAI subtype H7N3, which was the causal agent of the increased mortality observed in the region. The animal health authority reported the updated results to the OIE on the same day [29].

Between 19 and 21 June, three private diagnostic laboratories provided the veterinary authorities with 8 allantoic fluid samples derived from further cases containing H7N3 virus and confirmed by the CPA laboratory [20]. One of these was from a case sampled on 28 May 2012, potentially indicating that the infection might have been present before the index case was identified. This may have accounted for the cleavage site identified in the virus that had an insertion from poultry 28S rRNA [20]. There is therefore not conclusive evidence that the precursor LPAIV circulated in the poultry flocks in the region.

The putative index case corresponded to three layer farms of different ages. Neighboring farms

were also sampled in order to identify the potential spread of infection. A focal area of approximately 5 km was established around the infected farms, with a perifocal zone of 10 km, surrounded by a zone of 40 km. Intensive surveillance of the neighboring farms and of the farms in local municipalities was initiated in order to identify all infected farms and implement the control measures. Surveillance investigations in general used 30 serum samples, 30 cloacal swabs, and organs from daily mortality cases on farms, depending on the number of poultry houses [49].

On 25 June, a quarantine zone for 8 municipalities was established. All positive farms were under definitive quarantine, with complete restriction on movements of poultry and poultry products. Measures included culling of the entire population, destruction of carcasses by incineration, burying animals on the farm, composting or destruction in authorized rendering plants within the zone, and destruction of litter and poultry products, including eggs. Farms were required to implement the protocols authorized by the animal health authorities for washing and disinfection. In addition, following complete sanitary emptying, sentinel birds were placed, and if after 21 days they were negative on the basis of both serology and virus isolation, the repopulation of the farm was authorized by the animal health authorities [49].

Between 25 June and 5 July, 8 movement control posts were established in the control zone, for movement of poultry and poultry products. Control posts were coordinated by animal health officials, to verify documentation. Movements were authorized by official veterinarians, using information on the origin, transport conditions, and final destination of the poultry and poultry products in movement, to ensure compliance with control regulations. In addition, laboratory tests (PCR and serology) were conducted *in situ* if necessary, under the control of the Federal Police and the Mexican Army. Following surveillance investigations, a further 24 positive farms had been identified using virus isolation by 2 July [5], rising to 31 positive farms by 10 July [30]. On 2 July, SAGARPA (Secretariat of Agriculture, Livestock, Rural Development, Fisheries and Food), a unit of the Federal Government of Mexico, published the National Animal Health Emergency System with the aim of controlling and eliminating the H7N3 HPAI outbreak, setting out clear conditions for all activities implemented under the National Animal Health Law [45].

During the following weeks, up until September 2012, a total of 44 infected farms were identified in an area covering more than 19 500 km^2 of the control zone. Around 22.4 million chickens in total were culled during this event, with direct and indirect costs calculated by the National Poultry Association (UNA) to be more than US$650 million [50].

Regulations with regard to the movement of poultry and poultry products were established for each area (i.e. focal, perifocal, and buffer zones). Surveillance was conducted across the whole country. This involved the monitoring of 2069 productive units and the analysis of around 158 000 samples. In Jalisco State, a total of 712 farms were surveyed, representing a total population of 117.3 million chickens. Of these, 44 commercial farms (38 layer farms and 6 breeder farms) located in eight municipalities of Los Altos region tested positive between June and September. No positive backyard farms were identified, but there were additional isolations of H7N3 HPAIV from dead wild birds – three from common grackle (*Quiscalus quiscula*) and one from barn swallow (*Hirundo rustica*). The importance of these birds in virus dispersion is not known.

Jalisco State provides 55% of all eggs [5] and 11% of all broilers produced in Mexico. The Los Altos region contains more than 80 million layers in production, with more than 30 million rearing pullets and 12 million broilers in cycle. Mexico is the largest egg consumer in the world, with an average consumption of 22.9 kg per capita per year. Due to these challenges, a strategic plan to reduce virus circulation through vaccination was considered.

During the surveillance of wild birds in Mexico in 2006, an H7N3 LPAIV was isolated from a cinnamon teal (*Anas cyanoptera*) in sampled water lands in Mexico State [49]. Genomic analysis showed that this virus and the Jalisco poultry viruses had a nucleotide similarity of 90.5–98.1% for all segments studied [9, 18]. Given this high level of similarity, the duck virus was selected as master seed for production of vaccine. Through the support of pharmaceutical laboratories with experience in AI vaccine preparation, the first batch of 10 million doses was released under the

strict control of the National Food Quality, Food Safety and Health Service (SENASICA), on 26 July, after the basic standards had been satisfied. The vaccine was applied for protection and security, using ring vaccination within perifocal and buffer zones for longer-lived chickens. The vaccine was maintained in continuous production, and by 28 August more than 120 million doses had been administered to chickens in the risk area (a total of 165.9 million doses including booster vaccination with a vaccine bank of a further 70 million doses). In addition, poultry farmers were encouraged to improve biosecurity on farms, and control of the movements of live birds, litter, and poultry products was continued [5].

Clinical signs observed in the affected layer flocks included ruffled feathers, lethargy, anorexia, prostration, swollen head and face, and hemorrhagic lesions in combs, wattles, legs, and feet. At necropsy, laryngeal and tracheal edema was reported. In addition, tracheal mucus (in some cases with blood exudate), pulmonary edema, and in some cases petechial hemorrhages in the parenchyma were observed. Yolks in the abdominal cavity with hemorrhages on organ surfaces were also frequently noted.

The last isolation of the virus during 2012 was in September, and the animal health authorities informed the OIE on 12 December that the outbreak of H7N3 HPAI had been eliminated and that surveillance activities would continue in accordance with the OIE Code [46].

Second wave of the outbreak: 2013–2014

Clinical signs, initial diagnosis, outbreak development, and control measures

On 3 January 2013, a producer from Aguascalientes State (north of Jalisco, in the 2012 control zone) reported to the animal health authorities an increase in mortality among layer hens in production. Samples were collected and tested, and the presence of H7N3 HPAIV was confirmed. A second positive farm belonging to the same company was identified on 5 January [41]. A vaccination program was initiated on 16 January for all layer farms in the State and for broiler farms in risk areas [51]. On 12 January, two positive layer farms were identified in Los Altos, Jalisco, following a report by veterinarians of increased mortality and the observation of gross lesions consistent with HPAI. On 12 February, chickens on a large breeder farm in Guanajuato State (West Jalisco) also showed clinical signs of HPAI, and during surveillance in the focal zone another four breeder farms, one broiler farm, and five layer farms were confirmed as virus positive. Spread of virus infection occurred in Guanajuato State and Jalisco State during the following weeks. In March, an outbreak occurred in Tlaxcala State (580 km from the control zone), in a backyard flock that was eliminated immediately. On 16 April the first case of H7N3 HPAI was identified in Puebla State (650 km from the control zone). Immediate control measures were implemented in these zones. By 31 August 2013 there were 4 cases in Aguascalientes State, 37 cases in Jalisco State, 34 cases in Guanajuato State, 3 cases in Puebla State, and 1 case in Tlaxcala State [34]. In all cases, vaccination was implemented in the affected zones, and in 8 additional States prophylactic vaccination in layers and breeders was also implemented.

SENASICA confirmed that there were 14 outbreaks in the first 6 months of 2014 (Juan Garcia, personal communication), although these have not been reported to the OIE. Country-wide surveillance is being maintained in order to detect early potential reintroductions of the virus. UNA has estimated that the cost of the outbreak up to 2013 was in excess of US$1 billion. Mexican exports of eggs and poultry meat decreased as a result of trade restrictions, but are now recovering, although a full return to previous export levels is not expected to occur until the outbreak is considered to be eradicated and this has been verified by the importing countries [15].

Highly pathogenic avian influenza H7 in Australia during 2012–2013

H7N7 HPAI in New South Wales in 2012

Clinical signs and description of the affected population

On 14 November 2012, H7 AI was confirmed in a semi-free-range flock of layer hens on a property in New South Wales (NSW) (Table 10.6) [31]. Subsequent testing confirmed an H7N7 HPAIV. Staff at a semi-free-range chicken layer enterprise

Table 10.6 Summary of H7 HPAI infections in Australia during the period 2012–2013.

Location (country; state)	**1** Australia; New South Wales **2** Australia; New South Wales
HPAI subtype	**1** H7N7 **2** H7N2
Outbreak dates[a]	**1** 9 November 2012 – 20 December 2012 **2** 8 October 2013 – 21 November 2013
Date resolved[a]	**1** 22 December 2012 **2** 26 November 2013
Number of positive premises (by production species)	**1** 1 (chickens) **2** 2 (chickens)
Birds affected (number and species)	**1** 50 000 chickens **2** 490 000 chickens
Human infections	None reported
Direct costs	Not known
Control measures[a]	Stamping out, movement control inside country, screening, zoning, disinfection of infected premises and establishments
Source of virus	Introduction of virus as LPAI from wild birds (in both outbreaks)

[a]Information obtained from the OIE (World Organisation for Animal Health).

near Maitland observed sudden death in about 1% of 12 500 birds in one of four similarly populated sheds on 9 November 2012. Initially, fowl cholera was suspected, and the attending veterinarian prescribed antibiotic treatment, but also submitted samples to the NSW State Veterinary Diagnostic Laboratory (SVDL), requesting bacterial culture and avian influenza exclusion. Two days later mortality started to increase in a second shed, and reached 10% by day 5, while daily mortality in the first shed steadily decreased from 2% to 0.3% by day 5. The birds were 46 weeks or more in age, and in full lay. A drop in egg production was noted in a third shed, but no increase in mortality was evident before the birds were destroyed.

Initial diagnosis

The initial submission was a pool of cloacal swabs. Testing gave a positive result for the influenza type A TaqMan assay, a negative result for the H5 TaqMan assay, a positive result for the H7 TaqMan assay used at NSW SVDL, but a negative result for the H7 TaqMan assay used at the Australian Animal Health Laboratory (AAHL). Virus was isolated from the sample after inoculation of 9- to 11-day-old SPF embryonating eggs.

HA sequence subtyping using pan-HA primers [14] confirmed the presence of H7 HPAIV with the cleavage sequence motif PEIPRKRKR*GLF. The sample gave conventional RT-PCR product for NA sequence analysis using N7-subtyping primers [13]. Blast analysis of the NA gene sequences showed the highest similarities to N7-subtype avian influenza A virus, aligned against A/duck/Tsukuba/700/2007 (H7N7) as the reference sequence. The neuraminidase subtype was confirmed by the neuraminidase inhibition assay.

Outbreak development and associated surveillance

There were a number of deficiencies in biosecurity on the farm, including uncovered or poorly covered water tanks that were accessible to wild waterfowl, and feed spillage from a leaking silo. Infection was assumed to have occurred through contact with wild birds. No other farms were affected. AIVs are known to circulate in migratory and waterbirds in Australia, and monitoring of circulating AIVs in waterbird populations is ongoing. A number of influenza A viruses, including H7 LPAIVs, are regularly detected in waterbirds.

Phylogenetic analysis based on the HA gene showed that the H7N7 virus from the poultry outbreak in New South Wales in November 2012 belongs to the Australian sub-lineage of H7 AIV. However, there was no previously identified H7 HA sequence in GenBank that was identical to the current poultry outbreak virus, indicating some level of genetic drift both from previously identified Australasian H7 AIVs circulating in wild birds, and from previous HPAI outbreak viruses in Australia.

The sequence of the HA gene provided insight into the discrepancy between the results of the H7 TaqMan assays obtained at NSW SVDL and at AAHL. There were two mismatches in the probe sequence and four in the reverse primer. Both laboratories were using the same primer/probe sets, but the probe chemistry (BHQplus) used in NSW SVDL proved more tolerant of the mismatches in this instance. Once the genetic drift in the Australian-lineage H7 gene had been recognized, retrospective testing of wild bird samples was undertaken using modified assays. Samples from

Anseriformes collected in NSW in May 2012 tested positive in modified assays, but had previously tested negative in the unmodified assay.

The health of farm staff and other individuals involved in dealing with the outbreak was monitored by public health authorities. There were no reports of illness.

Control measures applied

Only one farm was affected, but 23 farms were monitored in the surveillance zones, or as contacts. In total there were around 50 000 birds on the affected farms, of which around 5000 died and around 45 000 were destroyed [35]. The costs of the outbreak are not known.

H7N2 HPAI in New South Wales in 2013

Clinical signs and description of affected population(s)

On 15 October 2013, H7N2 HPAI was confirmed on a free-range and cage layer chicken premises in NSW (1IP) (Table 10.6) [36]. The premises had two poultry enterprises, namely a six-shed caged layer facility containing 275 000 birds, and an eight-shed free-range enterprise containing 160 000 birds. The two facilities operated independently with a split workforce, and were approximately 700 m apart. There was also a feed mill on the property that serviced not only 1IP but also external pig and poultry enterprises in NSW and Victoria. Both enterprises were located on undulating cleared land with a westerly aspect. Clinical disease was first detected on the index property on 8 October 2013. Mortality was in the range 0.2–2% among the sheds. Total mortality peaked at approximately 0.9% per day (1400 out of 160 000 birds) in the free-range enterprise, compared with usual mortality rates of around 0.012% (30 out of 160 000 birds).

Surveillance and tracing activities commenced immediately as a result of infection at the 1IP site, and on 23 October 2013 a second infected premises (2IP) was detected, and infection with H7N2 HPAI was confirmed. The 2IP site was approximately 33 km west of 1IP. The topography consisted of undulating to flat mainly broadacre cropping land. The site was geographically isolated from other poultry facilities. There were two caged layer sheds of similar size and design, situated 30 m apart, with a cooled egg-storage facility attached to the northern shed. The 2IP site had approximately 55 000 layer hens in two sheds, which contained 74-week-old (35 000) and 54-week-old (20 000) birds, respectively.

Increased mortality in one shed at the 2IP site was first noted by farm staff on 22 October 2013. The first deaths were noted in a focal area in the middle of the southern shed in a single bank of birds, with about 100 deaths occurring on 23 October 2013. The staff noted the deaths, which then radiated from that focus over a period of several days until almost the entire area of the shed was involved in the rapidly accelerating daily mortality figures, which were in the region of 100 initially, then 400, and finally over 1000.

Initial diagnosis

The initial submission from 1IP was six cloacal swabs. All of these gave positive results for the influenza type A TaqMan assay, negative results for the H5 and H9 TaqMan assays, but a positive result for the H7 TaqMan assay. Virus was isolated from all samples following inoculation of 9- to 11-day-old SPF embryonating chicken eggs. HA sequence subtyping using pan-HA primers [14] confirmed the presence of HPAI of the H7 subtype, with the cleavage sequence motif PEIPRKRKR*GLF, in all samples. Five of six samples gave conventional RT-PCR product for NA sequence analysis using N2-subtyping primers [13]. Blast analysis of the NA gene sequences showed the highest similarities (94–95%) to N2-subtype avian influenza A virus.

Outbreak development and associated surveillance

Free-range birds on 1IP were potentially exposed to waterbirds. A dam is located close to the free-range poultry enterprise, and ducks were reported on the property. This is supported by the pattern of infection seen on 1IP. Deaths first occurred in the free-range birds, approximately 4 days before clinical signs were seen in the caged birds, suggesting that the free-range layers were infected before the caged layers. AIVs are known to circulate in migratory birds and waterbirds in Australia, and monitoring for circulating influenza viruses in waterbird populations is ongoing. A number of influenza A viruses, including H7 LPAIV, are regularly detected in waterbirds.

There appeared to be a point-source introduction of low-pathogenicity virus by a wild bird, followed by adaption, serial passage, and (in this case) selection of a virus with high pathogenicity to chickens. Further spread between the free-range and caged layer facility is likely to have been mechanical, through contaminated cardboard egg cartons. Follow-up sampling of those birds that may have been the first to be infected and sampling from wild birds in the area failed to confirm the putative LPAI source.

Although feed for the 2IP site was sourced from the 1IP site, it was considered unlikely that this was the route of transmission, as feed deliveries were made to multiple properties during the relevant time period, but these premises remained disease-free. The disease pattern at the 2IP site also suggested focal introduction rather than disease being interspersed through the flock. The reuse of cardboard trays (egg cartons) at both sites was the most likely route of transmission from 1IP to 2IP, and between the enterprises at 1IP. The cardboard trays that were used to transport the eggs were returned to each farm at the end of processing. The used trays were not decontaminated before leaving the processing factory. It is highly likely that transmission to the caged layer enterprise and to the 2IP site occurred through contamination of cardboard trays from the free-range enterprise on the 1IP site.

Phylogenetic analysis based on a near-complete HA gene sequence showed that the H7N2 virus from the poultry outbreak in October 2013 in NSW belongs to the Australian sub-lineage of H7-subtype AIVs. The HA of the current outbreak of H7N2 virus is of Australian lineage and related to the H7 AIVs currently seen in circulation in wild waterfowl as LPAIVs and the most recent poultry case of H7N7 HPAIV (in 2012 in NSW).

The health of farm staff and other individuals involved in dealing with the outbreak was monitored by public health authorities. There were no reports of illness.

Control measures applied

A total of two farms were affected, and 36 farms were monitored in the surveillance zones, or as contacts. In total there were around 490 000 birds on the affected farms, of which 18 620 died and 471 380 were destroyed. Clinical signs were first noted on 8 October 2013, and the final report to OIE was dated 21 February 2014 [42]. The costs of the outbreak are not known.

H5N2 high-pathogenicity avian influenza in Chinese Taipei in 2012

In 2012, an H5N2 HPAI outbreak was reported in Chinese Taipei as two distinct disease events [32, 37]. The first case was reported in Liou-Jia District of Tainan County, based on the presence of clinical signs of suspected disease in an abattoir [33]. The Local Disease Control Center (LDCC) traced the chickens to the original broiler breeder farm, which had experienced a mortality rate of 16.6%. Additional outbreaks were identified in native chickens ($n = 4$) and layers ($n = 1$) on five premises in Chang-Hua, PingTung, Yunlin, and Penghu Counties, involving a total of 47 151 chickens (Figure 10.2 and Table 10.7) [32, 37]. The average mortality rate for chickens on the affected farms was 12% (range 1.28–24.9%). No human cases were reported, and the financial cost of the outbreak is not known.

Diagnosis

The diagnosis of H5N2 HPAIV was based on RT-PCR assay, hemagglutinin (HA) cleavage site sequencing (PQRRKR*GLF), and the IVPI test in chickens (IVPI score = 2.91).

Clinical features

The trigger for suspect case investigation was abnormal mortality identified by the farmer or veterinarian, or clinical signs seen in the abattoir.

Outbreak development and molecular epidemiology

An H5N2 LPAIV was first reported in Chinese Taipei in December 2003, and was associated with cases on a total of 21 chicken farms, resulting in the culling of affected chickens [8]. The outbreak was declared to have ended in March 2004, but additional cases of the same virus lineage were reported in October 2008, October 2009, between December 2012 and July 2013, between August and September 2013, in November 2013, and in April 2014 [8, 19, 43, 44]. These H5N2 LPAIVs were reassortants with HA and neuraminidase

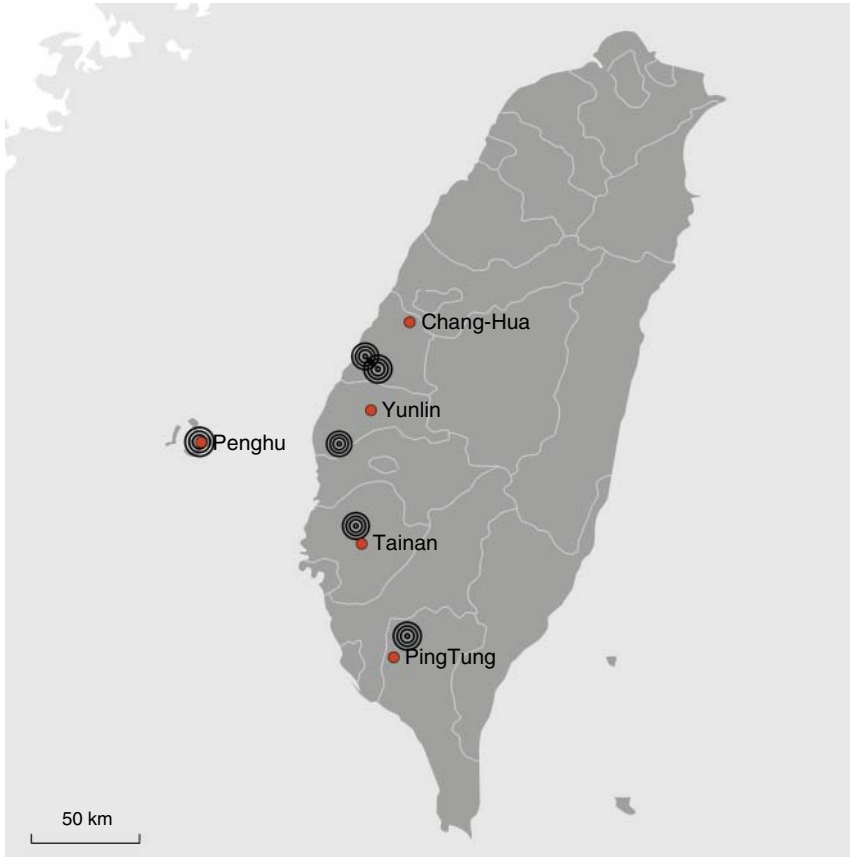

Figure 10.2 Distribution of H5N2 LPAI-affected premises in Chinese Taipei during 2012. Courtesy of the World Organisation for Animal Health. *See Plate section for color representation of this figure.*

(NA) genes derived from the North American lineage that was most closely related to the 1994 Mexican vaccine seed strain, and the remaining six gene segments were from an H6N1 LPAIV of the Eurasian lineage that has circulated in poultry in Chinese Taipei since 1997 [8, 19]. Other H5 LPAIVs have been isolated from migratory waterfowl in Chinese Taipei, but such viruses were of Eurasian lineage and not the source of the HA and NA gene segments of the outbreak H5N2 LPAIVs [8]. The 2003 H5N2 LPAIV had an HA cleavage site with three basic amino acids (i.e. PQREKR*GLF), and an IVPI score of 0, which was similar to other H5 LPAIVs [55]. However, the 2008 H5N2 LPAIV had an HA cleavage site sequence with four basic amino acids (i.e. PQRKKR*GLF), which was consistent with other reported H5 viruses with a phenotype of high pathogenicity, but the Chinese Taipei virus had intermediate *in-vivo* virulence in chickens (IVPI = 0.89) [8]. More detailed testing indicated that the risk of mortality was age dependent, with higher mortality observed in 6-week-old (IVPI = 1.86) than in 8-week-old (IVPI = 0.68) chickens [55]. However, four and eight passages of the 2008 H5N2 virus in chickens resulted in the emergence of high-pathogenicity phenotypes in 8-week-old chickens (IVPI = 1.85 and 2.36, respectively) [55].

It is not known how the Mexican H5 and N2 gene segments entered AIVs in Chinese Taipei, but movement by wild birds is unlikely, as wild bird surveillance detected only Eurasian H5 and N2 AIV gene segments, and not North American ones [8, 19]. The presence of HA and NA gene segments from 1994 Mexican H5N2 LPAIV plus reassortment with internal genes of endemic Chinese Taipei LPAIVs suggests that the H5 and N2 genes were introduced

Table 10.7 Summary of H5N2 HPAI infections in Chinese Taipei in 2012.

Location (country; counties)	Chinese Taipei; Tainan, Chang-Hua, PingTung, Yunlin, and Penghu Counties
HPAI subtype	H5N2
Hemagglutinin cleavage site	PQRRKR*GLF
Outbreak duration and dates[a]	289 days: 7 February 2012 – 22 November 2012
Date resolved	28 March 2013
Number of positive premises (by production species)	6: native chickens (4), broiler breeder (1), layer chicken (1)
Birds affected (by number)	47 151 susceptible, 5697 deaths, 41 454 culled, none slaughtered
Human infections	None reported
Direct costs[a]	Not known
Control measures	Stamping out, quarantine, movement control inside the country, screening, zoning, disinfection of infected premises and establishments, vaccination prohibited, no treatment of affected animals
Source of virus	Mutation of H5N2 LPAIV that had circulated in poultry since 2003

[a]Chinese Taipei reported two separate epidemiological events with regard to H5N2 HPAI. However, the viruses were of the same lineage, indicating that the two events were a single epizootic, with surveillance unable to detect the virus between the two outbreak periods (i.e. between 7 May 2012 and 17 November 2012).

via poultry, most probably as an escape from an illegal vaccination program [8].

Control measures

Clinical and epidemiological investigations of the surrounding farms were conducted within a 3-km radius in order to identify additional cases. Stamping-out strategies (Table 10.6) were used on the infected cases and on any additional cases identified in the 3-km surveillance zone. Other measures that were implemented included quarantine, movement control inside the country, screening, zoning, and disinfection of infected premises or establishments (Table 10.6). Vaccination was prohibited, and no treatment was allowed for affected animals.

Table 10.8 Summary of H7N7 HPAI infections in Italy in 2013.

Location (country; region)	Italy: Emilia-Romagna region
HPAI subtype	H7N7
Outbreak duration and dates[a]	28 days: 10 August – 8 September 2013
Date resolved[a]	8 September 2013
Number of positive premises (by production species)	6: layer chickens (4), turkey (meat) (1), turkey (rural) (1)
Birds affected (by number)	952 658 susceptible, 5676 deaths, 946 982 culled, none slaughtered
Human infections	3 mild cases of conjunctivitis
Direct costs	Approximately €7 million
Control measures[a]	Stamping out, movement control inside country, screening, zoning, disinfection of infected premises and establishments
Source of virus	Inconclusive, but likely to be an LPAI progenitor virus introduced by wild birds

[a]Information obtained from the OIE (World Organisation for Animal Health).

H7N7 High-pathogenicity avian influenza in Italy in 2013

Clinical signs and description of the affected population

On 14 August 2013, an H7N7 HPAIV outbreak was confirmed in a holding that housed 135 000 layers in five sheds (numbered 1, 2, 4, 5, and 7) located in the province of Ferrara, Emilia-Romagna Region, Italy (Table 10.8). Sheds 1 and 7 were the outer ones and had external areas for free-range hens. In the last week of July 2013, an increase in mortality was observed, from 0.2% to 0.7% in the outer sheds, and from 0.2% to 0.9% in the inner sheds. From 7–12 August, the mortality peak reached 8.9% in shed 2, and decreased to 5% in the same shed on August 12th. Samples collected on 9 August tested positive for H7 by RT-PCR in sheds 2 and 5, and an H7N7 HPAIV was subsequently confirmed by sequencing and the IVPI test. In sheds 1 and 7 the virus was not detected. However, seroconversion to H7 was demonstrated [7].

Outbreak development and associated surveillance

Enhanced surveillance was implemented and five additional epidemiologically linked holdings tested positive for the H7N7 virus between 19 August and 5 September. The index farm is located in the delta of the Po River, and the presence of wild waterfowl in proximity to the free-range hens was documented. The epidemiological and laboratory investigations described above suggest that it is very likely that the virus was introduced into sheds 1 and 7 as an LPAIV progenitor and through contact with wild birds.

Initial diagnosis

Diagnosis on the index farm and in the subsequent cases was based on RT-PCR and sequencing, followed by virus isolation in SPF embryonating chicken eggs and the IVPI test (for the index virus only) according to the standard techniques [12]. Notably, in the same shed (shed 2) of the index farm, two H7N7 HPAIVs were isolated with distinct multibasic cleavage sites, both indicative of HPAIVs (PKRKRR*GLF and PKRRERR*GLF). Both viruses had an IVPI value of 3. Only the motif PKRRERR*GLF was revealed in the viruses isolated in the subsequent cases. Phylogenetically, the Italian H7N7 HPAIVs clustered together with H7 LPAIVs sporadically detected in poultry in the Netherlands and Germany between 2010 and 2012, and they were also genetically related to H7 viruses circulating in wild birds in Italy and in Eurasia from 2009 to 2013. This is consistent with the epidemiological data, which suggest a possible introduction of an LPAIV through contact with wild birds. The Italian H7N7 HPAIV was distantly related to previous H7N7 HPAIV outbreaks that occurred previously in the UK and the Netherlands, as well as to other HPAI outbreaks that occurred in Europe and other areas worldwide [7]. Notably, among the workers employed at the infected farms, three cases of H7N7 infection were confirmed. These three affected individuals were also involved in the culling and/or cleaning and disinfection operations during the outbreak. They developed mono- or bilateral mild and self-limiting conjunctivitis with no respiratory signs. They were isolated at home without specific antiviral treatment, and recovered within a few days. The viruses that were isolated from these patients were identical in their HA and NA gene segments to those isolated from the poultry outbreak. No mammalian host adaptation markers and mutations associated with adamantine resistance were detected in the virus genomes. Genetically and phenotypically the viruses were susceptible to neuraminidase inhibitors [48].

Control measures applied

All of the infected farms were depopulated, and more than 900 000 birds were culled (Table 10.8). As soon as the index case was confirmed, the eradication measures outlined in the Directive 94/2005/CE were implemented, and an intense monitoring program was carried out on a regional and national scale [38]. The total direct cost, including culling of infected animals and those with suspected infection, disposal of infected birds and premises, and cleaning and disinfection, was estimated to be approximately €7 million.

H7N7 high-pathogenicity avian influenza in the UK in 2015

On 13 July 2015 an outbreak of H7N7 HPAI was confirmed in a commercial chicken laying flock in Lancashire, northern England [6]. The infection was confined to a single premises, and did not spread further. The virus had been introduced as an LPAIV several weeks previously. There was a moderate and progressing clinical presentation characterized mainly by a drop in egg production and an increasing mortality rate. However, mortality levels were moderate as a result of prior immunity in a substantial proportion of birds before mutation of the virus to high pathogenicity. Detailed analysis revealed that, following primary introduction, the virus had spread among different epidemiological groups within the infected premises, during which time it mutated to a high-pathogenicity strain. The site was complex, and contained both free-range and caged laying birds, with a total of 170 000 birds at the time of confirmation. Detailed investigations revealed that

wildfowl present on the ponds on the premises were the most likely source of the introduced LPAIV, which spread to the free-range birds as a result of indirect contact. Although the infected premises was located in an area with a relatively high density of poultry farms, there was no lateral spread, and the outbreak was quickly resolved.

Genetic characterization revealed that the virus was closely related to, but differentiated from, contemporaneously circulating strains in wild birds and poultry in Northern Europe. The index virus possessed an unusual HPAI cleavage site motif (PEIPRHRKGR/GLF). However, the virus was most probably derived from a genetic reassortment event in nature of two or more progenitor strains, and could be clearly differentiated from an LPAI H7N7 virus that was associated with an outbreak in broiler breeders in England in February 2015, thereby constituting a separate unrelated introduction. No human cases were reported in association with the outbreak.

H7N7 high-pathogenicity avian influenza in Germany in 2015

On 26 July 2015 an outbreak of H7N7 HPAI was confirmed in laying hens on a farm in Lower Saxony, Germany. Infection was confined to a single premises and did not spread. Clinical signs included a reduction in egg production, reduced feed intake, and increased mortality. The infected premises containing 10 000 birds was next to a neighboring holding where H7N7 LPAIV had been detected in June 2015, and although testing at the time revealed that this premises was negative, the possibility that a common or direct link existed could not be excluded. Using conventional strategies for zoning and culling of birds on the infected premises, the outbreak was quickly resolved even though the farm was located in an area of relatively high poultry density. Genetic characterization of the virus revealed that, although related to strains that were contemporaneously circulating in European wild birds and poultry, the virus was distinguishable from these and the outbreak had occurred as a result of an independent introduction. The HA cleavage site motif of the virus was PEIPKRKRRGLF (T. Harder, personal communication). It was deduced that the virus was derived by spontaneous mutation of an introduced H7N7 LPAIV [16]. No human cases were reported.

Addendum

Since this chapter was completed in mid-2015, two additional outbreaks of HPAI have occurred. The first one involved H5Nx non-Gs/GD-lineage HPAIV in multiple poultry flocks in France at the end of 2015, through to 2016. The second outbreak involved H7N8 HPAIV in a single turkey flock in Indiana, USA, in January 2016, and there was associated infection of several adjacent turkey flocks with the progenitor H7N8 LPAIV.

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11 Low-pathogenicity avian influenza

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Introduction

The family Orthomyxoviridae includes six genera – Influenzavirus A, Influenzavirus B, Influenzavirus C, Isavirus, Thogotovirus, and Quaranjavirus [181]. Only viruses in the genus Influenzavirus A are known to infect birds [6]. Aquatic birds (belonging to the orders Anseriformes and Charadriiformes) are considered to be the natural hosts and reservoir for influenza A viruses (IAVs), whereas gallinaceous birds (poultry) are not considered to be natural hosts of IAVs [6]. Based on antigenic differences on the two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), IAVs are further divided into subtypes. At present, avian-origin IAVs belonging to 16 HA subtypes (H1–H16) and 9 NA subtypes (N1–N9) have been isolated from birds [6]. Although the host barriers that prevent IAVs from the natural reservoir from crossing to poultry may be lower than those that prevent them from crossing to other animal species, the spread and perpetuation of these viruses in poultry are expected to lead to adaptive changes that influence virulence, transmission, and host range. From a disease management and reporting perspective, the term "avian influenza" is defined by the World Organisation for Animal Health (OIE) *Terrestrial Animal Health Code* as an infection of poultry caused by any high-pathogenicity avian influenza virus (HPAIV), and by H5 and H7 subtypes with low pathogenicity (H5/H7 LPAIVs), whereas infections caused by other subtypes (H1–H4, H6, and H8–H16) are simply referred to as influenza A [203]. For simplicity, in this chapter the term LPAIV is used to refer to avian IAVs of low pathogenicity regardless of subtype, and with an intravenous pathogenicity index (IVPI) of less than 1.2. It must be noted that many countries around the world have neither sufficient resources in place nor the regulatory authority to investigate and report the presence and prevalence of LPAIVs other than H5 and H7 viruses. Thus the list is likely to be incomplete and biased towards the notifiable subtypes, H5 and H7. In this chapter the main focus will be on those LPAIVs which have caused outbreaks in poultry that have escaped control measures and have become endemic and/or significant public health risks. In particular we shall discuss the Asian H9N2 and H7N9 and the Mexican H5N2 LPAIVs.

Low-pathogenicity avian influenza virus (LPAIV) in natural and agricultural host systems

Wild birds as the natural reservoir and introduction source for agricultural systems

Replication of IAVs in wild waterfowl and shorebirds occurs primarily in the intestinal tract and occasionally in the respiratory tract [57, 240, 241, 291]. A duck can shed virus for 30 days, and can excrete 10^8 mean embryo infectious doses per mL (EID_{50}/mL) of feces [292], contaminating surface water, sloughs, and shore lands. Transmission is thought to occur efficiently by the fecal–oral route through virus excretion in the water. Fecal–oral transmission and movement of wild waterfowl and shorebirds are the mechanisms that enable the survival and dispersal of IAVs in nature [58, 240, 241]. Other orders of birds may be infected, but IAVs do not appear to be naturally perpetuated in them [290]. However, it is important to recognize that these birds may act as both biological and mechanical vectors for the movement of IAVs between the waterfowl reservoir and domestic birds. In the

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Netherlands, outdoor housing of commercial layers has been shown to increase the risk of introduction of an LPAIV by more than 10-fold compared with indoor-layer farms [77]. Thus the waterfowl and shorebird reservoir, the environment that these birds occupy, and any object or living organism (including humans) that shares this environment may be a source of IAVs for domestic poultry.

Perpetuation of avian influenza in unnatural hosts: role of live poultry markets

The most important man-made reservoirs of LPAIVs are live poultry markets [24, 272]. Combined with village poultry production systems with multiple species and non-confinement, live poultry markets play a major role in the emergence and maintenance of LPAIVs (and HPAIVs) [215]. In poultry, LPAIV infection may affect either the intestinal tract or the respiratory tract, or both. Initially, LPAIV transmission may not be as efficient as in aquatic birds, but access to shared feeders and waterers, where there is opportunity for contamination from both feces and respiratory secretions, facilitates transmission within poultry flocks [91]. Live poultry markets with a high density of birds and intense human activity provide ideal conditions for LPAIVs to become fully adapted to and thrive in poultry [5, 50, 137, 193, 238, 255, 272, 312].

An agricultural system conundrum: integrated fish farming

Centered on the fishpond, integrated fish farming is a system of producing fish in combination with other agricultural and livestock farming operations. Integrated fish farming is perhaps the most ecologically friendly farming system on the planet. The principle of integrated fish farming involves farming of fish along with livestock and/or agricultural crops. In this system, the waste products of one biological system serve as nutrients for a second biological system. In integrated systems such as these, subsystems are beneficially interlinked to each other in a limited area, minimizing the production costs but resulting in diversified outputs of farm products, including (but not limited to) fish, meat, eggs, vegetables, fruits, fuel wood, and fodder. Complex combinations of multiple systems exist. In Asia, fish farming systems commonly integrate aquatic and land-based poultry as well as pigs in close proximity with each other and in contact with wildlife, including aquatic birds. Such integration creates a major conundrum, namely how to maintain an efficient agricultural system without increasing the risks of introducing emergent or zoonotic pathogens [230]. Although disputed by some reports, LPAIVs are the epitome of pathogens that can find an ideal environment for host switching [80, 81]. Movement of live animals and/or contaminated equipment in and out of these systems and into live animal markets adds to the complexities that promote the emergence of novel pathogens with zoonotic potential [238].

H9N2 influenza A viruses: the paradigm of live poultry market-adapted LPAIVs

From the initial introduction of the H9N2 viruses into poultry in China, no other LPAIV has shown such resilience and spread to domestic bird species across many countries in Asia, Middle East, and Africa. Two distinct phylogeographical lineages of H9N2 have been described, namely the North American and Eurasian lineages (Figure 11.1). From these two major lineages, further clusters can be identified, including a South American cluster in wild birds [303], and two major clusters of poultry-adapted viruses in Asia [47].

The first description of an LPAIV of the H9N2 subtype dates back to an outbreak in turkeys in February 1966 in northern Wisconsin, USA [100, 245]. Low mortality, coughing, sneezing, and a marked decrease in egg production and hatchability characterized the outbreak. H9 viruses have not established stable lineages in poultry in North America, although as many as 16 outbreaks of H9 subtype-associated disease in turkeys were documented between 1981 and 1996 [90]. Details of these and other LPAI outbreaks in poultry in the USA, particularly in the main turkey production states of Minnesota and Wisconsin, were highlighted in the previous edition of this book [89]. The emergence of the HPAI H5N1 viruses and subsequent human infections in 1997 led to changes in range production practices in parts of the USA. In Minnesota, turkey range production essentially ceased from 1998 onward, accounting for less than

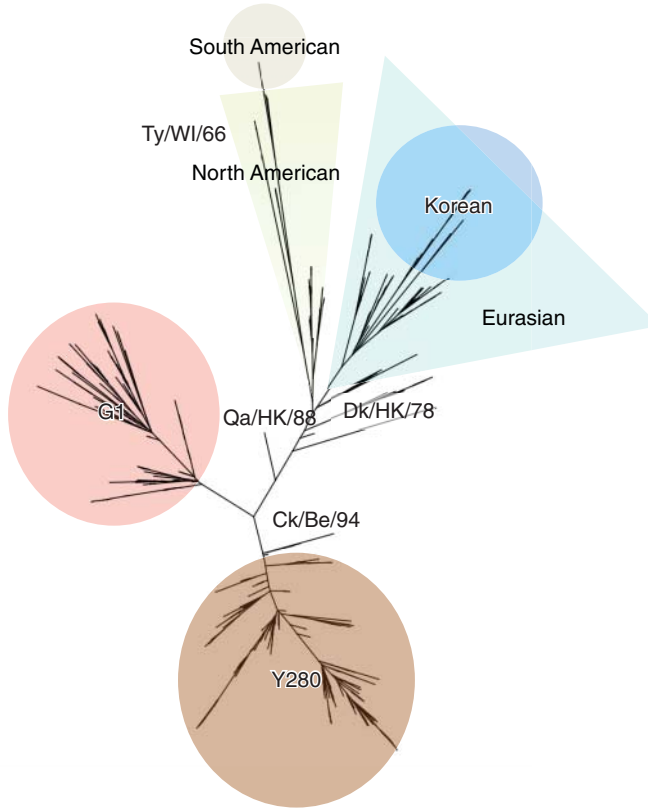

Figure 11.1 H9 HA phylogenetic tree showing major phylogenetic groups. Phylogenetic analyses were performed using online tools available at the Influenza Research Database (www.fludb.org). The unrooted tree was generated using the Archaeopteryx software tool as described elsewhere [337]. Labeling and colors were added using PowerPoint software (Microsoft, Inc.). *See Plate section for color representation of this figure*.

0.5% of the turkey flocks in the state. The effect of such change has been a significant decline in LPAI outbreaks. At the time of writing, prevention of LPAI outbreaks is largely achieved through prevention of exposure to influenza viruses by avoiding direct or indirect contact with waterfowl and shorebirds and their environment, and only sporadic isolations of the H9 subtype and other LPAIVs have been obtained from poultry in the USA. In contrast, H9 viruses are often found in wild ducks and shorebirds in North America [89].

In Asia, H9-subtype viruses (mostly in combination with the N2 NA subtype) have caused disease outbreaks and have established stable lineages in chickens and other land-based poultry, such as quail, pheasant, partridge, and other minor domestic poultry species [82, 85, 157]. Phylogenetic analysis of Asian H9N2 viruses suggests that they have been transmitted from aquatic birds to poultry on multiple occasions [214]. Interestingly, the natural avian reservoir of H9 viruses in China has not been identified, and H9N2 viruses were detected only in apparently healthy domestic ducks in limited surveillance studies of live poultry markets and farms in Hong Kong between 1975 and 1985 [175, 236]. In 1988, three H9N2 viruses were isolated from dead quail in one farm in Hong Kong (40 000 birds), where there was an outbreak of respiratory disease that lasted for around 3 months [214]. These isolates were the first evidence of H9 viruses in land-based poultry in Asia. The available evidence suggests that H9 viruses did not appear in chickens in China until the early 1990s [82]. The first outbreak of H9N2 LPAI in mainland China was described in Guangdong Province, where it lasted from November 1992 to May 1994, and

affected 17 chicken farms and two rare bird farms. Mortality in broilers was 10–40%, and laying rates dropped by 14–75% [258]. By the late 1990s, H9N2 viruses were being detected in most provinces in China, associated with live bird market activity throughout the year, and with an incidence that ranged from 0.2% to almost 5% depending on the market and the time of year [41, 141, 152, 164, 304, 305]. Since then, H9N2 LPAIVs have expanded their geographic range. A combination of legal and illegal poultry trade, as well as possible spillover in wild birds, has contributed to the spread of H9N2 viruses across country borders. This Eurasian lineage of H9N2 LPAIVs has been associated with outbreaks and remains endemic in live bird markets and commercial poultry operations in many countries, including (among others) Bangladesh, China, Egypt, Germany, Israel, India, Iran, Lebanon, Pakistan, and Vietnam [13, 15, 48, 51, 92, 114, 187, 189, 190, 196, 207, 232, 259, 271]. It must be noted that the H9N2 outbreaks that occurred in Germany in 2013–2014 and in Poland in 2013 were not incursions of the virus from poultry populations in Asia, based on the lack of relationship between HA and NA gene sequences of the Polish isolates and viruses circulating in the Middle East or Far East of Asia [243, 244]. Phylogenetic analysis showed that the Polish turkey-origin H9N2 LPAIVs represent independent introductions to poultry from the wild-bird reservoir, perpetuating in Europe from 2006 to 2013.

Perhaps the most important factor that has contributed to efficient spread of the H9N2 viruses relates to their multiple modes of transmission in poultry, including air droplets, fomites, feed, and water. In the laboratory, and often in the field, H9N2 viruses do not cause obvious clinical signs of disease in poultry, although viruses can replicate at high levels in the respiratory tract. Various studies have shown that different poultry species vary in their susceptibility to infection with LPAIVs. Japanese quail appear to be more susceptible to lower doses of virus than chickens and turkeys, but only in turkeys are respiratory signs of disease readily observed [6, 12, 59, 99–102, 128, 169, 192, 198, 214, 237, 239, 274, 296].

When H9N2 outbreaks occur in poultry, they have typically been associated with secondary bacterial infections that occasionally result in high morbidity and mortality [23, 109, 114, 118, 126, 197, 199, 237, 244]. At necropsy, affected birds typically show lesions of local pulmonary consolidation from caseous plugs, petechiae in the throat, trachea, and/or intestine, and mucus obstruction of the bronchi and trachea [198, 237]. Interestingly, H9N2 viruses have occasionally been found in co-infections with H5N1 HPAIVs in apparently healthy birds in poultry flocks in Egypt [9, 185]. Such observations highlight the impact of H9N2 viruses due to their ability to mask and promote transmission of HPAIVs in poultry. Vaccination to prevent disease, typically in the form of an inactivated virus, is effective against H9N2 viruses but has failed to control their spread. At least one vaccine-linked H9N2 virus of the North American lineage was also detected in China (Figure 11.1), although it is not known if the virus has become extinct since then. As H9N2 LPAIVs are not included in the OIE's list of notifiable transboundary diseases, and surveillance systems to track them are often absent, it is likely that their geographic range and endemic roots are much more significant than is generally realized.

The H9N2 viruses from the Eurasian lineage have been broken down into three major sub-lineages, namely G1-like, Y280-like (also known as Beijing/94-like), and Korean-like (Figure 11.1) [85]. The HAs of H9 viruses in the Korean-like lineage are closer to their primordial ancestors than those in the G1- and Y280-like lineages [132, 134–136, 138, 154]. Viruses in the G1-like lineage have been found across Asia and Europe, whereas the Y280-like and Korean-like lineages appear to be generally more confined to Asia [51, 69]. More recent phylogenetic studies suggest further sub-lineage divides among the G1- and Y280-like lineages [69, 156]. Exhaustive phylogeographical and host-dependent evolutionary analyses have suggested alternative and more sophisticated clade names for H9N2 viruses [42, 47, 69, 233, 259], but the overall topology of the phylogenetic tree is essentially as shown in Figure 11.1.

At present, spillover of poultry-adapted H9 LPAIVs to wild birds has been documented in China, but it is unclear whether these, or perhaps other avian or mammalian species, have become permanent hosts [321, 333]. Plateau pikas

at Qinghai Lake in China have been shown to possess serum antibodies against H9N2, and also to be susceptible to experimental infection with a prototypical H9N2 virus, but it is not clear whether they represent bona fide vectors for the spread of these viruses under natural conditions, or whether they were exposed, infected, and became dead-end hosts. Only a few H9-subtype viruses have been found in Eurasian wild birds that do not belong to lineages found in poultry, and evidence of rare intercontinental reassortment among H9N2 viruses from the North American and Eurasian lineages has been described in China [336].

Public health risks of H9N2 influenza A viruses

Human and swine infections with H9N2 LPAIVs have been reported, with all cases restricted to Asia (Figure 11.2). The first two human isolates of H9N2 were recovered in 1999 in Hong Kong from two patients who reported mild respiratory symptoms [209]. Studies showed that these isolates were genetically and antigenically related to the G1-like sub-lineage [147]. Subsequent isolates from sporadic human cases have been from the G1- and Y280-like lineages [26, 27, 40, 79, 84, 226]. All human cases reported to date (just over a dozen) have been mild and have resolved without clinical complications or sequelae, except for one H9N2-positive case in an immunocompromised patient with a history of post-bone-marrow-transplant chronic graft-versus-host disease and bronchiolitis obliterans that developed into respiratory failure [40]. The relatively small number of human cases appear to have contracted the virus from direct contact with infected birds, and to date there has been no evidence of human-to-human transmission [276]. In 1998, the first swine H9N2 virus was isolated in Hong Kong. Swine infections, without overt signs of disease, appear to be far more common than human infections [45, 46, 225, 301]. Swine infections have also been more common with viruses from the Y280-like lineage. In addition, there is evidence that H9N2 viruses of the Korean-like lineage have infected pigs [45, 225, 307]. Under experimental conditions, some isolates were also reported to exhibit increased pathogenicity in mice without adaptation [21, 52, 107, 143, 148, 158, 159, 225, 268, 285, 298, 299, 328]. In 2011, H9N2 virus infections in dogs (and a single isolation from a horse) were detected in Guangxi, China [7, 257, 324]. In a longitudinal study, dog serum samples from the same location showed significantly high seropositive rates against H9N2 virus of 20.21% in 2010, 28.98% in 2011, and 44.85% in 2012 [257]. It must be noted that serological studies such as these should be accompanied by side-by-side comparisons using serum samples from areas in which neither H9N2 viruses nor other poultry-adapted viruses are known to circulate, in order to eliminate false-positive results. Experimentally, the canine H9N2 virus was able to infect dogs and cats, but transmission was limited to cats [324]. Thus it is possible that dogs and other mammals which are commonly present in markets act as intermediary hosts and could extend the host range of H9N2 viruses [7]. In recent years, H3N2 LPAIVs in Korea and China and equine H3N8 IAVs in the USA have become established in dogs, adding a potentially new host that can act as a mixing vessel for novel IAVs [95, 130, 142]. In fact, an H3N1 canine/human reassortant virus was isolated from a dog in Korea during routine surveillance [247], indicating that dogs can indeed act as an intermediary host for influenza.

Human serological studies suggest that levels of human exposure are high among poultry workers in commercial and live poultry markets and in individuals in contact with poultry where H9N2 viruses are present. Studies in Cambodia, Egypt, India, and Vietnam have revealed significant exposure of humans to H9N2 viruses [22, 108, 160, 208, 277, 332]. The number of human seropositive samples expressed as a percentage of H9N2 virus has been in the range of around 1–10% (and even as high as 40% in one study from Iran), occasionally with relatively clear distinctions between populations at risk (those in contact with poultry) and the general population. However, caution is needed, as other studies have reported that cross-reacting antibodies against H2 might explain the elevated number of antibodies against avian H9N2, especially among those born before 1968 [195, 254]. Overall, serological studies need to bear in mind that some degree of cross-reactivity will be present in some individuals with a history of previous human influenza infection and/or vaccination.

Figure 11.2 H9 HA phylogenetic tree showing major phylogenetic relationships of H9N2 viruses isolated from various animal species. Phylogenetic analyses and editing were performed as described for Figure 11.1. H9 HA position 226 in the receptor-binding site (site) with leucine is shown in red, with glutamine is shown in black, and with mixed virus populations carrying leucine and/or glutamine is shown in green. Note that a single virus isolate from a human case with methionine 226 is shown in blue. Light red box corresponds to G1-lineage viruses, and light brown box corresponds to Y280-lineage viruses. *See Plate section for color representation of this figure.*

The contribution of H9N2 influenza A viruses to the diversity of avian influenza viruses that pose a public health risk

Homosubtypic and heterosubtypic H9N2 reassortants have been isolated from many poultry species in many live bird markets across Asia [3, 45, 284, 314, 316]. H9N2 viruses in Asia have been efficient donors and recipients of gene segments from wild bird and poultry IAVs. The remarkable ability of H9N2 viruses to reassort has resulted in the establishment of a plethora of influenza subtypes in poultry, some with a broad host range. Novel HA and NA subtype combinations have emerged with internal gene constellations derived from H9N2 viruses. Some of these viruses have shown an ability to infect mammals, including humans. The list of virus subtypes that are typically found in poultry, particularly in live bird markets in China and South-East Asia, although extensive is likely to be incomplete due to insufficient surveillance data. It must be emphasized that animal surveillance systems in many under-developed countries do not have the resources to track the presence of LPAIVs, and therefore such viruses remain unreported. To demonstrate the full significance of H9N2 LPAIVs it is important to mention their role as the donors of the internal gene segments to the H5N1 HPAIVs that emerged in poultry in 1996–1997 and re-emerged in 2003–2004. H5N1 HPAIVs spread to other countries in Asia, Africa, and parts of Europe, and have caused human infections [228]. In confirmed human cases of H5N1 HPAI, the level of lethality has been remarkable, at around 60%. Between 2003 and July 2014, the World Health Organization (WHO) reported 667 human cases of H5N1 HPAI, with 393 fatalities (www.who.int/influenza/human_animal_interface/avian_influenza/en/). Countries such as Cambodia, China, Egypt, Indonesia, and Vietnam, in which H5N1 HPAIVs remain endemic, continue to report occasional human infections [170, 293]. More recently, human infections with LPAIVs of the H7N9 and H10N8 subtypes have also been reported [30, 270, 326]. Both of these viruses share internal gene segment constellations derived from poultry-adapted H9N2 LPAIVs circulating in China [64, 72, 219]. Only two cases of infection with H10N8 LPAIV have been reported, both in Jiangxi Province, China [34, 327]. One of those cases was a 73-year-old woman who presented with fever, was admitted to hospital on 30 November 2013, and died 6 days later due to multiple organ failure. She had visited a live poultry market 4 days before the onset of illness. The virus contained a mixture of PB2 gene segments encoding both E627 and K627, and it was sensitive to neuraminidase inhibitors [34].

Human infections with either LPAIV or HPAIV of the H7 subtype have occasionally been reported, and most of these cases have been associated with mild conjunctivitis [2, 17, 29, 56, 66, 163, 275]. Until 2013, the only exception occurred in 2003, during an H7N7 HPAI outbreak in commercial poultry in the Netherlands. The H7N7 HPAIV was also detected in 88 humans who had conjunctivitis or mild respiratory symptoms, and in one person who died of pneumonia and acute respiratory distress syndrome [66]. All of the human cases had been in contact with poultry during the outbreak, with potentially 30 human-to-human transmission events but no sustained transmission among humans [54, 122]. Of more concern is the current situation with regard to LPAIV of the H7N9 subtype in Asia. Between March 2013 and October 2014 [28, 191], 453 laboratory-confirmed cases of human infection with H7N9 LPAIV, including 175 deaths, were reported to the WHO (www.who.int/influenza/human_animal_interface/influenza_h7n9/en/). These cases were reported from East China, and were associated with live bird market activity during the winter months in the provinces of Anhui, Fujian, Guangdong, Guangxi, Hebei, Hunan, Jilin, Jiangsu, Jiangxi, Shandong, and Zhejiang, and in the municipality of Beijing [33, 105]. In addition, 10 cases were reported from Hong Kong SAR [37, 269], four cases from Taipei, Taiwan [31, 146, 162, 173], one case from a Chinese traveler in Malaysia, and two cases from the Xinjiang Uyghur Autonomous Region [330], which borders Russia, Mongolia, Kazakhstan, Kyrgyzstan, Tajikistan, Afghanistan, Pakistan, and India. Patients with confirmed H7N9 virus infection at hospital admission displayed one or more clinical signs that included high fever, non-productive as well as productive cough, shortness of breath, dyspnea, and hypoxia. On X-ray imaging, compromised lower respiratory tract disease with opacities, consolidation, and infiltrates was typically reported.

Severe cases of H7N9 virus infection have included septic shock, respiratory failure, acute respiratory distress syndrome, refractory hypoxemia, acute renal dysfunction, multiple organ dysfunction, rhabdomyolysis, and encephalopathy. In addition, secondary bacterial and fungal infections have been reported, sometimes associated with multi-drug-resistant bacteria [36, 71, 106, 117, 150, 165–167, 286–288, 295, 310, 313, 319, 322, 330, 331]. With the exception of a small number of H7N9 virus infections that presented with uncomplicated upper respiratory illness and mild fever, both in children and in adults most other cases have been severe. The median time from illness onset to hospital admission has been approximately 4.5 days, with many of these patients requiring intensive care. The time from illness onset to death has ranged from 7 to 20 days. Systemic high-dose steroid use appears to be associated with an increased risk of prolonged viral shedding and the emergence of antiviral resistance [106]. The emergence of H7N9 virus highlighted once again the unpredictable nature of IAVs. No evidence of H7N9 virus activity was observed in poultry before the human cases were reported, although these viruses appear to have become more ubiquitous in live bird markets since that time [63, 116, 127, 249, 320]. H7N9 viruses do not appear to support sustained human-to human transmission. In contrast, it is important to note that chickens and other poultry do not appear to show noticeable signs of disease on infection with H7N9 viruses, but transmit the virus readily [206]. When chickens, quail, pigeons, and various duck species were experimentally infected with a prototypical H7N9 virus strain, efficient virus replication and transmission were readily observed in chickens and quail, but not in pigeons [115, 206]. In these and other studies, ferrets inoculated with human isolates of H7N9 virus showed significant levels of replication, with a level of transmission intermediate between that of prototypical human and other avian influenza viruses (AIVs) [18, 222, 334]. Regardless of their pathotype for poultry, a common feature of H7 virus infections in ferrets is their potential spread to the brain [19, 115, 263]. Unlike other AIVs, in general H7 viruses do not require a switch in receptor specificity in order to infect humans, and they can cause infection and be transmitted, albeit partially, in mammalian models while still maintaining an avian-like sialic acid α2,3-Gal-receptor-binding preference [18, 20, 222, 248, 263, 334]. As the source of H7N9 viruses has been neither fully characterized nor controlled, further human cases are expected [206]. Although the number of cases decreases dramatically in the summer months, the most recent cases were reported from Xinjiang Uyghur Autonomous Region, which had not previously reported cases, and is not adjacent to previously affected areas, indicating that the virus is continuing to circulate and to expand its geographic domain. Closure of live bird markets, disinfection, rest days, and culling of birds have been effective in controlling human infections, but appear to have had little effect on containing the re-emergence and spread of HPAIVs and LPAIVs in Asia [67, 68, 97, 186, 278, 297, 315]. The complex dynamics of integrated farm practices and their intimate connection with live animal market systems make the prevention, control, and eradication of LPAIVs a daunting task.

Molecular markers of the host range of H9N2 and H7N9 LPAIVs

It is important to recognize that molecular markers of host switching are poorly defined for IAVs from the natural reservoir (i.e. aquatic birds) to poultry. With few exceptions, most efforts are aimed at understanding interspecies transmission of IAVs from birds to mammals, but not transmission among different bird species. It is also important to emphasize that a significant number of studies have identified mutations that increase the virulence of LPAIVs (and HPAIVs) in the mouse model, but the role of these and other mutations in infection of other mammals is still unclear.

Hemagglutinin

Receptor-binding site

Numerous poultry H9N2 isolates contain leucine at amino acid 226 (L226) in the receptor-binding site (RBS) of the HA (H3 numbering, 216 in the mature H9 HA), and show preferential binding to analogs of receptors with sialic acid (SA) linked to galactose by α2,6 linkage (SA α2,6Gal) [177, 281]. Both of these traits are typical of human

IAVs. In contrast, H9N2 viruses that contain glutamine at amino acid 226 (Q226) in HA show an increased preference for SA α2,3Gal [281]. Depending on the context of other amino acids in or near the RBS, Q226-containing viruses may also display dual receptor specificity [281]. The acquisition of L226 in H9N2 viruses has occurred in land-based poultry [41, 141, 304], consistent with the observation that both SA α2,3Gal and SA α2,6Gal receptors are present in the respiratory tract of some of these species [70, 83, 119, 282]. Quail, pheasants, guinea fowl, and turkeys have been shown to possess both types of receptors in the respiratory tract and intestinal tract, whereas ducks and geese contain almost exclusively SA α2,3Gal SA α2,3Gal [119, 318]. The available evidence suggests that L226 in the HA of H9N2 viruses promotes occasional infections of mammals, particularly humans (Figure 11.2). L226-containing viruses have been shown to grow more efficiently than Q226-containing viruses in human airway epithelial (HAE) cultures maintained at the air–liquid interface (ALI) [281]. However, viruses containing Q226 have also been identified from pigs and occasionally from humans (Figure 11.2). In experimental inoculation of Rhesus macaques, infection with H9N2 viruses led to a biphasic febrile response with virus replication detected in the upper and lower respiratory tracts [323]. In addition, under experimental conditions the presence of L226 in HA allows for efficient replication and transmission in ferrets and guinea pigs via direct contact, although airborne transmission to indirect contacts is either absent or less efficient compared with human IAVs [144, 283]. The ferret is considered the gold standard for studying transmission of human IAVs, as it is susceptible, transmits the virus by the airborne route, and displays clinical signs similar to those observed in humans. H9N2 viruses containing L226 are compatible for reassortment with human seasonal H3N2 (sH3N2) and 2009 H1N1 pandemic (pdm) viruses [120, 121, 220, 251, 260, 283]. Many of these reassortants show increased infectivity and direct contact transmissibility in ferrets. In one study, a reassortant virus that had surface genes from an avian H9N2 virus and internal genes from a human seasonal H3N2 virus (sH3N2) transmitted only to direct contact ferrets. This lack of airborne transmission occurred despite the fact that the H9N2 avian/human reassortant virus caused clinical disease signs and lung pathology in ferrets in a manner similar to the human sH3N2 virus [283]. Further adaptation by serial passage in ferrets of the H9N2 avian/human reassortant virus resulted in minimal amino acid changes compatible with airborne transmission in these animals. Two amino acid changes on the H9 HA, namely T189A in the HA1 region and G192R in the HA2 region, were essential for airborne transmission [251]. Transferring this HA gene segment in the background of the 2009 pdm strain (H9N1, 1+7 reassortant) also resulted in efficient airborne transmission [121]. The contribution of these small changes on the HA was also observed in alternative H9N1 avian/pdm reassortant viruses (3+5 and 5+3 viruses) [120], highlighting the impact of small amino acid changes on the virus transmission phenotype. The T189A mutation (179 in the H9 HA sequence) is not unique to the ferret-adapted H9 virus. Although the majority of natural isolates contain threonine, strains containing alanine, asparagine, isoleucine, proline, and serine have been identified. Natural H9 isolates with the G192R (182 in the HA H9 sequence) mutation are rare, with only a single sequence having been reported, whereas few display the G192D substitution.

The HA of H7N9 viruses that caused human infections in Asia carries molecular changes predictive of recognition of human-like SA α2,6Gal receptors. The mutations Q226L and G186V (H3 numbering is 217 and 177, respectively, in the mature H7 HA) provide some level of human-like receptor-binding recognition. However, H7N9 viruses do not replicate well in epithelial cells of human trachea, and maintain significant binding to avian-like receptors [53, 235, 267, 300, 306].

Proteolytic cleavage site

With regard to other molecular markers, the presence of serine, instead of alanine, at the P5 position in the cleavage site of H9 HA (P**S**RSSR/GL) has been shown to improve cleavage efficiency and to increase replication in chickens and mice [261]. *In vitro*, the HA of H9 viruses can be cleaved by extracellular proteases of the respiratory tract, such as human transmembrane protease, serine S1 member 2 (TMPRSS2) and human airway trypsin-like protease (HAT) [16]. Likewise, the HA of H7N9

viruses is recognized by TMPRSS2, the activity of which appears to be essential for the life cycle and pathogenesis of the virus [227, 265]. Interestingly, H9 HAs with the cleavage site sequence RSSR/GL or RSRR/GL can be cleaved by matriptase, a protease that is widely expressed in most epithelia. Matriptase is abundantly expressed in the kidney, and may in part explain the nephrotropism of some H9N2 viruses observed in chickens [16]. Under special circumstances in which there is loss of a glycosylation site, the H9 HA can be cleaved by furin. Cleavage by furin is atypical for the HA of LPAIVs, but may influence the ability of H9N2 viruses to spread in poultry [273]. The question of whether the HAs of H7N9 viruses are the substrate of similar proteases or mechanisms merits further attention.

Neuraminidase

Many H9N2 strains carry deletions in the stalk region of NA, which is typical of IAVs that become adapted to respiratory tropism in poultry [3, 4, 14, 132, 147, 153, 155, 246, 307, 325]. Laboratory studies have shown that serial passage of wild bird IAV isolates in chickens or quail leads to the development of strains that are better adapted to poultry carrying NA stalk deletions [74, 104, 250]. Many naturally occurring Eurasian H9N2 isolates from poultry carry a 3-amino-acid deletion in the NA stalk involving positions 62–64, which also appears to increase their virulence in mice [85, 148, 158]. An alternative 2-amino-acid deletion involving positions 38–39 was observed in two of the human strains from Hong Kong in 1999 [147]. The N2 NA with the 38–39 amino acid deletion has not become fixed in the virus population, and has not been reported since 1999, except from two H9N2 viruses from Japan that were isolated from parakeets imported from Hong Kong [176]. No direct link between stalk deletion and transmission from poultry to humans or pigs has been established. No evidence of increased resistance to NA inhibitors (NAI) has been found in H9N2 viruses; none of the more than 850 N2 NA sequences analyzed contained the R292K or H274Y NAI resistance substitution. The NA of H7N9 viruses from human cases and those from poultry parental strains carry a deletion involving amino acids 68–72. At least 3 out of more than 180 sequences show the R292K mutation that would confer resistance to neuraminidase inhibitors [86]. The R292K mutation does not appear to affect virus fitness and virulence [86].

Polymerase complex

PB2

Within the internal gene segments, PB2 proteins with mutations indicative of improved replication in mammals (E627K or D701N) [93, 94, 129, 168, 256] are unusual among H9 viruses. The majority contain the typical avian-like E627, D701 profile. Only four (out of more than 700) naturally occurring H9N2 strains described to date display the K627 mutation in PB2, and there is no evidence that such mutation is fixed in the virus population. Few adaptation studies in quail and mice also show H9 viruses with PB2 segments containing the K627, indicative of improved lower respiratory tract virus replication [21, 104, 143, 148, 158, 159, 225, 285, 298, 299, 328]. Interestingly, none of the natural H9N2 isolates obtained from swine or humans contain the PB2 E627K mutation. A cluster of H9N2 viruses from Israel, that later transferred to Egypt, contains the PB2 E627V mutation [1, 8, 9, 13, 48, 49, 216, 217], which also appears in two isolates obtained from chickens in Hong Kong in 2011. The PB2 E627V mutation appears fixed in H9N2 viruses circulating in Israel and Egypt, but not in Hong Kong. Unusual mutations at position 627 in PB2 have been reported in two different chicken isolates, one containing the E627G substitution [152] and the other with the E627A mutation [49]. In sharp contrast, a third (63 out of 186) of the H7N9 PB2 sequences available contain the E627K substitution. The H7N9 isolates with the E627K mutation are typically obtained from humans but not from poultry [183]. The PB2 D701N mutation is present in a single H9N2 isolate obtained from an immunocompromised patient, which would suggest that under certain conditions H9N2 viruses might acquire human-adapted mutations (40). In the laboratory, additional mutations in PB2 of an H9N2 strain, namely D253N and Q591K, have been associated with increased polymerase activity, improved replication in ALI HAE cells, increased TNF-α expression in human macrophages, and enhanced pathogenicity in mice [184]. The PB2 D253N and Q591K mutations are highly unusual among H9 LPAIVs, and probably reflect the selection of the virus for a large plaque

phenotype in tissue culture (MDCK) cells. Further analysis of available PB2 sequences from H9 and H7N9 strains has yielded no natural isolate with the D253N mutation, whereas the Q591K mutation is found in only three avian H9N2 and six H7N9 isolates. Interestingly, the PB2 D253N mutation is found in an avian/human H9N1 reassortant after serial adaptation and airborne transmission in ferrets, suggesting a potential role in mammalian adaptation [120]. At position 591, the mutation Q591R was shown to increase virulence for mice of a prototypical H5N1 HPAIV [308]. The same Q591R mutation also modulates virulence of the 2009 pdm H1N1 virus [182, 308]. In the PB2 591 position of H9 and H7 viruses it is common to find either glutamine or leucine, but none have been identified with arginine. In a separate study, the mutation M147L in PB2, in combination with E627K, was shown to increase pathogenicity in mice [285]. The PB2 147 position in H9 strains shows some plasticity with strains that carry methionine, isoleucine, threonine, or valine. However, leucine has not been described to date in naturally occurring strains. The PB2 of H7N9 strains contains isoleucine at position 147. More recently, the PB2 F404L mutation has been shown to increase polymerase activity and virulence in mice against the background of an H9N2 virus as well as H5N1 HPAIV and 2009 pdm strains [158, 159]. PB2 segments from both H9N2 and H7N9 strains show a high degree of conservation of phenylalanine at position 404. Alternative mutations obtained during adaptation in ferrets of avian/human H9N1 and H9N2 reassortant viruses produced mutations at positions T58I and L374I, respectively [121, 251]. PB2 T58, located in a region that overlaps the PB1 and NP binding sites, is highly conserved among H9 viruses, only a few other isolates having alanine (A58), but none having isoleucine. In a separate study in which an H7N1 HPAIV was adapted to ferrets, a similar threonine to isoleucine mutation was observed in the same PB1/NP binding region of PB2, but at position 81 (T81I) [263]. The PB2 T81I mutation is common among H9 viruses, which also accommodate methionine and alanine. Likewise, the PB2 L374I mutation is common among H9 viruses. Finally, the PB2 A707T mutation has been observed in an alternative avian/human H9N1 virus that was transmitted by the airborne route in ferrets [120], but such a change is unusual for H9 viruses, with sequences having either alanine or serine but not threonine. Other mutations in PB2 that have been shown to modulate the virulence of the 2009 pdm H1N1 virus in mice include E158G/A, H357N, I504V, T588I, and G590S [110, 111, 161, 224, 329, 335]. It is important to note that the virulence of 2009 pdm H1N1 is strongly affected by changes on the surface gene segments, and thus the effects of other mutations appear to be more strain-specific and the result of direct virus adaptation to mice [311]. In the PB2 of H9N2 and H7N9 viruses, E158, H357, and V504 are the highly conserved variants, whereas position 588 corresponds mostly to alanine. The G590S mutation is not seen among H9 viruses, but 8% of the H7N9 viruses do show such substitution. The significance of these epistatic changes for interspecies transmission and mammalian adaptation of LPAIVs has yet to be elucidated.

PB1

No direct link has been established for PB1 mutations involved in the host range of H9N2 and H7N9 viruses. None of the sequences analyzed to date contain the H99Y mutation, but many contain the I368V mutation, both of which combined were shown to be important for airborne transmission of an H5N1 HPAIV strain in ferrets [98, 149]. Mutations L473V and L598P present in an H5N1 HPAIV could enhance the virulence in mice of the laboratory-adapted A/WSN/1933 strain carrying a PB2 E627 mutation [302]. PB1 V473 predominates among H9 and H7N9 strains, but P598 is highly unusual, and only three naturally occurring H9N2 strains contain the L598P mutation (out of more than 600 sequences). H9N2 strains from Asia, like other LPAIVs and HPAIVs from the same region, show some degree of variation in the N- and C-terminal ends of the PB1 protein. The N-terminal end of PB1 contains the PA binding domain, which is highly conserved among IAVs [96, 212, 213]. The effect of mutations in this region is not yet known. A significant number of Asian LPAIVs, including H9N2 and H7N9 strains, encode an additional amino acid at the C-terminal end, which contains the PB2 binding site [78, 202, 218, 221] and in which the typical sequence 753-LRRQK-757 is instead found as 753-LRRQKQ-758 or 753-LGRQGK-758. Minor variants on these three consensus sequences also

exist. Alternative mutations in PB1, D120N and D439E, and S261N were observed in H9 viruses that were adapted for airborne transmission in ferrets [120, 121, 251]. The biological significance of such polymorphisms remains unexplored.

PA

In PA, two mutations have been independently assigned a role for virulence in mouse models. The PA K185R substitution was shown to decrease virulence of an H5N1 HPAIV [61], whereas the T97I mutation was shown to increase the virulence of an H6N1 LPAIV [38]. Most H9 strains, and all of the H7N9 strains available, contain PA gene segments with R185 and T97. The PA position T97 shows some variation in H9N2 viruses, with some strains containing either A97 or N97, but not I97. An additional mutation, K26E, within the endonuclease site of PA was described in one of the avian/human H9N1 viruses mentioned earlier [120]. However, analysis of PA sequences from H9 and H7N9 viruses suggests that E26 is favored over K26, and therefore the K26E mutation might represent selection for a more stable residue that improves overall polymerase activity regardless of the host environment. Additional mutations in PA that have been shown to modulate virulence in mice and/or polymerase activity, particularly in the context of the 2009 pdm H1N1 strain, include F35L, A36T, A70V, T85I, P224S, L295P, E298K, L336M, and I550L [25, 111, 161, 224, 231, 262, 311, 335]. It must be noted that some of these mutations do not appear to affect infections of humans with AIVs. Perhaps more relevant is the finding that in H7N9 viruses that have infected humans, the PA S409N mutation appears to improve viral fitness [309]. Although both serine and asparagine 409 are present in H9N2 and H7N9 viruses, the former appears to be more favored, which could lead to fewer opportunities for infecting mammals. The significance of these polymorphisms in PA for host range and virulence warrants further studies.

Nucleoprotein gene

Despite the early recognition of NP as a host range factor, the changes in NP associated with interspecies transmission are still poorly characterized. The NP A184K leads to increased virulence of a prototypical H5N1 HPAIV in chickens, associated with increased virus titers and elevated nitric oxide levels in tissues, as well as up-regulation of IFN-α, IFN-γ, Mx1, and iNOS, among others [289]. Similarly, in a different study the NP I109T mutation increased neurotropism of an H5N1 HPAIV in chickens [264]. More recently, the N52Y substitution in NP was shown to increase sensitivity of the prototypical human H7N9 virus to human Mx, indicating that this residue is a determinant of Mx resistance in mammals [223]. Analysis of more than 800 NP sequences from H9N2 and H7N9 viruses revealed a high degree of conservation of isoleucine and lysine at positions 109 and 184, respectively. Only minor variants are found in H9N2 viruses, with position 109 carrying serine, threonine, or valine, and position 184 carrying arginine or methionine. However, NP position 52 shows more sequence variation, with viruses having asparagine, glutamine, histidine, or tyrosine (one H9N2 isolate and one H7N9 isolate contain serine). No direct evidence of NP mutations affecting interspecies transmission has been described.

Matrix protein gene

H9N2 and H7N9 strains resistant to adamantanes are common due to the S31N mutation in the M2 proton channel pump, characteristic of such phenotypes. Less frequent is the V27A mutation related to a second site in M2 involved in adamantane resistance.

Mutations in M1 associated with host range or virulence of H9N2 and H7N9 viruses have not been described. The M1 T139A mutation is related to increased virulence in mice and increased virus yield in tissue culture of an H1N1 human virus [242], and it is commonly found in H9N2 viruses along with the T139N substitution. In a separate study, the introduction of N30D and T215A substitutions in an H5N1 HPAIV backbone conferred increased virulence in mice [60]. However, both D30 and A215 are highly conserved among H9N2 and H7N9 viruses, so their role in the virulence of these and other IAVs with increased host range needs to be further elucidated [270].

Non-structural protein gene

One of the most studied influenza virus proteins is NS1, which contains an N-terminal RNA-binding domain and a C-terminal effector domain [55, 87,

88, 145, 172]. In wild birds, two NS gene segment alleles have been described – A and B. Allele A is the most extensively characterized, because it is the one found in human and other mammalian influenza viruses. The NS1 protein varies in length, with strains encoding a 217-amino-acid protein, a 230-amino-acid protein, fewer strains encoding intermediate-length proteins, and some encoding a 237-amino-acid protein. The overall effect of NS1 is to counteract the antiviral responses mounted by the host cell upon infection [11, 123, 125, 253, 266]. Details of the NS1–host factor interactions and modulation of these activities are beyond the scope of this chapter. Virulence due to NS1 has been ascribed to the presence of at least one of the following markers: P42S, E92D, I106M, and the C-terminal sequence ESEV/EPEV. Of more than 1000 sequences that are available for the NS1 protein of H9 viruses, the majority contain S42 and a small proportion encode A42, but none encode proline. There are 200 full-length sequences available for the NS segment of H7N9 viruses, all of which encode S42. NS1 E92 is highly unusual in H9N2 and H7N9 viruses, and most strains encode D92. The NS1 I106M restores binding to CPSF30 and modulates the virulence in mice of an Asian-origin H7N9 virus [10], the internal gene segments of which are derived from an H9N2 virus. In nature, the NS1 with either I106 or M106 is common, and therefore it can be speculated that naturally occurring H9N2 and H7N9 viruses differ in their potential to infect and/or cause disease in mammals. The ESEV/EPEV sequence corresponds to a PDZ-binding domain [76, 88]. Cellular factors with PDZ motifs have been identified that interact with NS1 via the ESEV/EPEV domain, including Scribble, Dlg1, PDlim2, and MAGI-1, which ultimately result in modulation of interferon and apoptotic signals in influenza-infected cells [62, 76, 124, 151, 317]. H9N2 and H7N9 strains, like many other AIVs, encode a 230-amino-acid NS1 protein that contains the ESEV/EPEV sequence. The ESEV/EPEV motif has been shown to be a virulence factor in a mouse-adapted H1N1 [112], but of limited effect in an H5N1 HPAIV in mice [252]. Approximately 50% of the H9 NS1 sequences available represent truncated versions of 217 amino acids, which is consistent with the notion that truncations in NS1 have a limited impact on the replication and spread of IAVs in birds [252].

Miscellaneous changes

Among the small protein components of IAVs, NS2/NEP [200], and PB1-F2 [35], different roles have been assigned to virulence through modulation of various viral and cellular processes. In addition, other viral protein products have been recently discovered, the functions of which have yet to be elucidated, particularly in the context of AIVs. These are PB1-N40, PA-X, PA-N155, PA-N182, M42, and NS3 [65, 113, 188, 234, 279, 294].

The most well-characterized function of NS2/NEP is as a viral factor responsible for nuclear export of M1–vRNP complexes by linking them through the host factor hCRM1 and from it to the cellular nuclear export machinery [194]. However, NS2/NEP is also involved in the control of virus replication, and early studies showed that a single amino acid mutation (I32T) controlled the tendency of the virus to produce defective interfering particles [201]. Just a few H9N2 viruses show the I32V substitution (out of more than 1000 sequences). No natural H9N2 or H7N9 isolate contains threonine at position 32 of NS2/NEP. More recently it has been shown that the mutation M16I greatly enhances the polymerase activity of an H5N1 HPAIV in human cells in a concentration-dependent manner. NS2/NEP M16 and I32 are highly conserved in H9N2 and H7N9 viruses.

PB1-F2 is an alternative translation product of the viral PB1 segment [32, 35, 75, 179]. It was initially characterized as a pro-apoptotic mitochondrial virulence factor. The full-length PB1-F2 open reading frame is 87–90 amino acids in length, and is highly conserved in AIVs. It has also been present in all human influenza pandemic virus isolates obtained during the twentieth century, but appears to become lost evolutionarily over time as the new virus becomes established in humans. A full-length PB1-F2 is needed for a prototypical H5N1 HPAIV to display full virulence in ducks [229]. In mammals, sequence variation in PB1-F2, particularly the N66S substitution, modulates viral pathogenesis in the context of the 1918 H1N1 Spanish influenza

virus and H5N1 HPAIV [43, 44]. Interestingly, deletion of the PB1-F2 open reading frame of an H5N1 HPAIV results in increased virulence in chickens [139, 140]. Additional mutations in PB1-F2 have been identified that modulate virulence, but it must be noted that PB1-F2 activity is context and strain dependent [174, 178, 180, 210, 211]. H9N2 and H7N9 PB1-F2 proteins mostly contain 87–90 amino acids. However, deletions are observed as well as significant sequence polymorphisms, including many strains that encode the N66S substitution. It remains to be determined whether such deletions and polymorphisms play a role in virulence and transmission in poultry and mammals.

In summary, the epistatic nature of molecular changes requires constant mining of genetic and phenotypic information in order to better define the features that lead to interspecies transmission of influenza viruses [171]. In this regard, H9N2 and H7N9 viruses have acquired mutations predictive of increased host range and pandemic potential.

The other side of the coin: perpetuation of LPAIV H5N2 in commercial poultry production systems: the experience in Mexico

Mexico's poultry industry positions the country as the fourth largest producer of chicken meat in the world, and the seventh largest egg producer. In March 1994, H5N2 LPAIV was detected in commercial poultry operations [280]. The virus was phylogenetically linked to other H5 viruses of the North American lineage, but was distinct from the H5N2 virus responsible for the HPAIV outbreaks in Pennsylvania, USA, in 1983–1984 (Figure 11.3) [103]. The extent of H5N2 virus spread was then analyzed by serology, which showed that positive flocks without apparent clinical signs, both from commercial operations and from backyard bird populations, spanned half the country. A standstill period and repopulation program was instituted that included emptying of barns, thorough cleaning, and disinfection of facilities. However, by November 1994 the virus had mutated to a highly pathogenic form affecting 2 million layers in Puebla State, a densely populated area with approximately 25 million layer chickens on over 100 farms. By January 1995, a similar virus had appeared in Queretaro State, affecting 20 million broiler chickens and 400 000 breeders. Sequence analysis revealed that the cleavage site HA gene segment of the virus had mutated from the avirulent PQRETR/G to the virulent PQRKRKTR/G sequence form [73, 103]. An eradication campaign was implemented that included depopulation of affected farms, cleaning and disinfection of facilities, standstill periods, use of sentinel birds before repopulation, and control of movement of poultry and poultry products across state boundaries. Vaccination of all birds was also incorporated to control spread of the disease. The decision to implement a vaccination program in Mexico was based on the realization that the affected farms were in high-density production areas, which made it difficult to diagnose and cull birds rapidly enough. In addition, poultry was only produced for domestic consumption, not for export. Between January and December 1995, approximately 380 million vaccine doses were administered to both affected and at-risk bird populations, covering approximately 55% of the country and 70% of the commercial poultry population. Vaccination of long-cycle layer farms was performed in states where LPAIV cases were detected. May 1995 marked the last isolation of H5N2 HPAIV in Mexico, and by December 1995 the Mexican government had declared the country to be free of the virus, although the H5N2 LPAIV has continued to circulate. No human cases were reported, and no evidence of human infections was detected. The estimated cost of the emergency program was US$49 million, covering the operation, biosecurity, vaccination, and the value of dead and destroyed birds [1]. Since June 1995, H5N2 LPAIV has continued to circulate in Mexico and has spilled over to poultry in Guatemala and El Salvador. Evidence of antigenic drift associated with vaccine usage, possibly from inadequate or incorrect vaccination, has been observed, which may have the unintended effect of producing strains with host switching capability [133]. More importantly, Mexican-origin H5N2 strains, perhaps intended for vaccine usage, caused two separate outbreaks in Japan and Taiwan (Figure 11.3) [39, 131, 204, 205]. In Taiwan, the H5N2 virus emerged as a reassortant carrying the surface gene segments of a Mexican-origin H5N2 virus and internal segments from a Eurasian-origin H6N1 virus that have been circulating in poultry in the country.

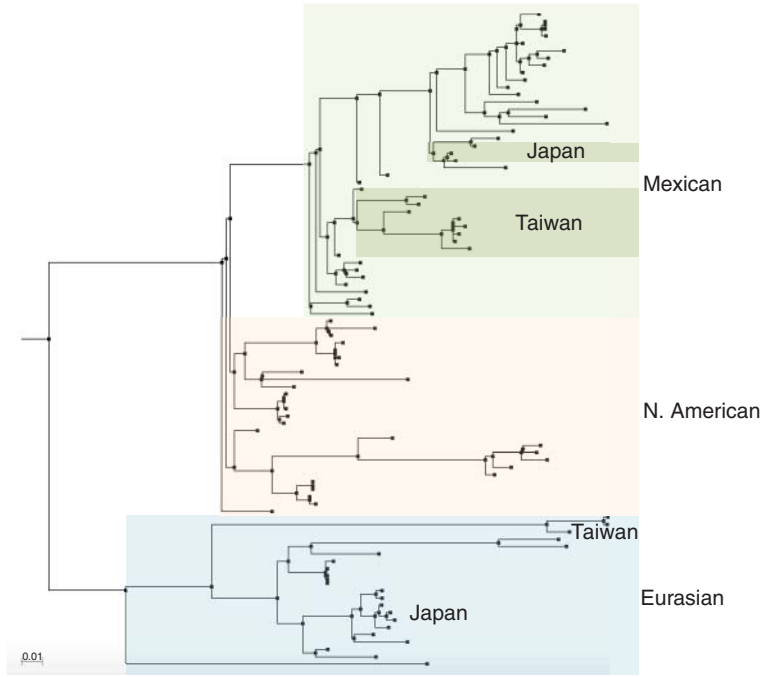

Figure 11.3 H5 HA phylogenetic tree showing major phylogenetic relationships of H5N2 viruses of North American (light orange) and Eurasian (light blue) lineages. Phylogenetic analyses and editing were performed as described for Figure 11.1. The Mexican viruses (shown in light green) form an independent evolutionary path stemming from an ancestor in the North American lineage. Mexican H5N2-vaccine-derived viruses were isolated from independent outbreaks of LPAI in Japan and Taiwan. In Taiwan, Mexican-derived H5N2 surface gene segments have reassorted with Taiwanese LPAIVs, and their endemic nature remains uncertain. *See Plate section for color representation of this figure*.

The reassortant H5N2 continues to circulate in Taiwan, adding to the diversity of LPAIVs in the region.

Summary

As poultry production continues to expand, there is an increasing possibility that pathogens of poultry will find opportunities to experience host switching. The zoonotic potential of LPAIVs is well known. The past 25 years have seen a systematic increase in the number of LPAIVs (and HPAIVs) that remain endemic in poultry populations and, concomitantly, have seen unprecedented geographic spread. A combination of agricultural, environmental, social, and economic factors has led to the emergence of LPAIVs with pandemic potential. Future endeavors need to reconcile sustainable production systems, cultural practices, and traditional and novel intervention strategies to decrease the disease burden of LPAIVs (and other pathogens in poultry), which in turn will minimize the zoonotic and public health risks.

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12 Epidemiology of avian influenza in agricultural and other man-made systems

Leslie D. Sims, John Weaver and David E. Swayne

History of humans and birds

In order to understand the ecology and epidemiology of avian influenza (AI), a review of human activities related to poultry production, especially since the late nineteenth century, is necessary. It is also helpful to have an understanding of the role of different avian species in human life as religious symbols, pets, and for entertainment and food within different cultures. The manner in which birds for these enterprises are reared, transported, and sold contributes significantly to the transmission and persistence of AI virus (AIV).

Chicken

The domestic chicken (*Gallus gallus domesticus*) has origins in various species of wild jungle fowl from South-East Asia. No living wild ancestor exists today, and debate continues as to the exact ancestral origins [8, 40]. The major contributor is believed to be the red jungle fowl (*Gallus gallus*), but there may be additional contributions by the other three jungle fowl species. The earliest archeological evidence of domesticated chickens came from 6000 BC in China, but the original date and site of domestication was probably earlier in South-East Asia, with subsequent human migration northward, moving the domesticated chickens with them. By 2500 BC, chickens were found in Europe and Western Asia. Chickens were initially used for religious purposes and were not utilized as a food source until later. Today, birds still play a role as religious symbols, as typified by the release of captive passerine birds by Buddhists in parts of Asia [65], and poultry sold in live poultry markets (LPM) in the Caribbean Islands for Santeria and other rituals. A primary early activity of bird rearing was for entertainment through cock fighting [179], an activity that still plays an important role in some countries, such as Thailand, and which was associated with fatal exposure of humans to H5N1 high-pathogenicity avian influenza (HPAI) virus (HPAIV). The Egyptians were the first to focus on the chicken as an important agricultural commodity, and developed the first artificial-heat egg incubators. Some of these incubators held 10 000–15 000 eggs, and would have required large flocks of chickens to support such agricultural endeavors. Chickens were widespread in Greek civilization, but it was the Romans who brought them into the center of life in Western civilization and developed much of the science and husbandry for use in everyday life. The husbandry principles established by the Romans were used until well into the nineteenth century.

In modern European culture, the keeping of pure breeds began in the eighteenth century and peaked in the 1800s in both Europe and North America, where poultry rearing became a craze for the purposes of exhibition, personal amusement, and keeping the birds as pets. This period has been called "chickenmania" or "poultrymania", with widespread rearing of chickens by everyone from common people to nobility. During this time, national, regional, and international poultry exhibitions became popular, thus eliminating the geographic isolation of poultry, and subsequently that of their diseases. Although eggs and sometimes meat were a by-product of the "poultrymania",

Animal Influenza, Second Edition. Edited by David E. Swayne.
 Published 2017 by John Wiley & Sons, Inc.

the primary functions of poultry were for entertainment and showmanship. Poultry exhibitions were still important in the early twentieth century, and were responsible in at least one case for widespread transmission of HPAI in Europe [93]. They are still held today at agricultural fairs and shows.

Commercial era

The organization of poultry for food production in the modern era began in the late 1800s in Europe, first to provide eggs and later as a source of meat [40, 179]. These early endeavors were very modest by today's standards. The increasing importance of poultry as a food source for all people is evident in the 1910 census for the USA, which reported 280 million chickens on 5.5 million farms, averaging 51 birds per farm. Around 80% of all farms kept poultry [179]. At this time most flocks were small, on average containing 50–200 birds, and were reared with outdoor access. This is similar to the situation in many developing countries in the first part of the twenty-first century. For example, in Vietnam in 2005 there were estimated to be more than 7.9 million households engaged in traditional extensive poultry production, with an average flock size of 32 birds, representing about 94% of all poultry producers and around 70% of total poultry production [84].

In the USA, the trend toward indoor production began in the late 1800s in California with the development of the first commercial hatchery for egg-laying chickens, which shipped White Leghorn chicks throughout California. This was the beginning of the trend for large-scale movement of day-old chickens over long distances, including the transcontinental movement that is a feature of the industry today. By 1905, the value of poultry in the USA was US$500 million, and by 1910, poultry was second only to corn as a revenue crop. In 1913, the Petaluma area shipped 100 million table eggs per year, and the area had over 1 million chickens. Production reached 450 million eggs in 1918. One hatchery produced 150 000 chicks in 3 weeks. In 1940, the largest chicken farm was a 250 000-bird layer farm in Petaluma, California. This trend towards industrial-scale production resulted in large, highly susceptible populations of genetically similar poultry that were housed in close confinement. Biosecurity measures taken to prevent entry of pathogens reduced the risk of introduction of AIV to these housed flocks, but if the measures were breached the consequences for the farms were dire. In most countries there was little planning of the way that the poultry sector developed, with many farms developing around sources of inputs and close to urban markets, creating large concentrations of farms that could facilitate transmission of disease if they were not managed carefully [26, 176]. Some of these farms were located in areas that attract migratory birds, such as northern Italy and the Fraser Valley in Canada. This has resulted in outbreaks of AI due to the transmission of virus from wild birds to poultry, followed in some cases by spread between farms [26, 137].

Meat production lagged behind egg production, with meat-producing birds first being a by-product of the laying industry (i.e. culls, young cockerels, and capons) [179]. However, genetic and nutritional efforts in the 1950s led to the development of meat-type chickens with increased feed efficiency, and rapid growth through the crossing of Cornish and White Plymouth Rock stocks [40, 179]. In the USA and Europe in the mid-1900s, a large flock of egg or meat chickens would contain 3000–4000 birds. Flocks of similar size are still common today in many developing countries, but the trend in some places is for larger flocks, and this was exacerbated by outbreaks of H5N1 HPAI as integrated companies reduced their reliance on small-scale contractors. At the time of writing, farms containing over 1 million head of poultry exist, in which the birds are segregated into multiple houses and reared under conditions of high biosecurity. Nevertheless, farms of this type have been infected with AIV [73], including HPAIV.

Globally, chickens are the primary poultry species reared for food, for both eggs and meat. Chicken numbers have increased dramatically in many parts of the world over the past 30 years. For example, between 1980 and 2010 there has been a three- to fivefold increase in poultry numbers in parts of Asia and in Brazil [55]. The significance of this increase from an epidemiological perspective depends on many factors, including the type of bird (including species and degree of genetic uniformity), and the manner in which poultry are reared, transported, and sold, as well as the concentration of farms. In some countries, such as the Netherlands, the

density of poultry farms has increased dramatically, and this has implications for transmission of avian influenza in places where farm density is very high [189].

The increase in the poultry population in China has been accompanied by a reduction in the number of small-scale producers, especially in the eastern seaboard provinces where alternative employment is available. In these areas there is less reliance on household poultry for financial and food security, but such reliance remains the case in other parts of China, and in countries such as Vietnam, Indonesia, and Egypt. Between 1996 and 2005 the number of poultry farms in China decreases from 104 million to 34.6 million. Between 2007 and 2009 around 2 million small-scale broiler producers ceased production, and this trend is expected to continue [96, 177, 217]. In other countries, such as Thailand, much of the increase in poultry production has been in large industrial-scale farms, but poultry are still reared in village households, not only for home consumption, but also for social, recreational, and cultural reasons [53].

In many countries, biosecurity standards on some farms are not at levels commensurate with the threat posed by avian influenza and other pathogens [60, 133, 183]. Many factors influence whether farmers implement these measures, including threats from other diseases, appetite for and perceptions of risk, and the cost of upgrades to facilities, especially in locations where access to capital for investment is limited. In addition, in countries where H5 Goose/Guangdong-(Gs/GD)-lineage viruses remain entrenched, a significant proportion of poultry is still sold through LPM, many of which have poor hygiene and biosecurity measures in place. There has been marked improvement in markets in some of these countries since the outbreaks of H5N1 HPAI and, more recently, human cases of H7N9 LPAI in China. However, AIVs continue to circulate in some markets, and it is challenging to bring about change in the behaviors of traders, despite improvements in knowledge and the occurrence of cases of zoonotic disease directly related to live poultry in markets [126]. A comprehensive control program resulting in improved surveillance as well as better LPM facilities and management eliminated H7N2 LPAIV that had persisted for 13 years in the New York City LPMs, and reduced the likelihood of poultry in these markets being infected with AIVs [214].

The types of chicken that are raised vary, with the vast majority of poultry being supplied by major international breeding companies. In some parts of Asia there is a preference for slower growing chickens, usually a cross between a native breed and a faster growing bird such as the Israeli Kabir that retains the rich flavor and yellow beak, feathers, and fat of the native breed [112]. In others regions, such as Indonesia, small cheap commercial broilers are preferred [30].

Chicken-rearing systems in many developed countries have now gone full circle from rearing all animals outdoors to fully enclosed production, and then a swing back towards increased free-range production, in line with consumer demands. This has resulted in large flocks of poultry once again being reared outdoors. Unless care is taken in the way that animals are supplied with feed and water, the risk of infection with AIV from wild aquatic birds on these farms can increase [42, 63].

Vertical integration and large company ownership are now widespread in the commercial poultry sector globally. In-house testing is often undertaken, and although this can speed up testing for disease, it can also result in non-reporting or delayed reporting of AI to public veterinary authorities. Cases of H5N1 HPAI were occurring in a number of countries in Asia, were recognized as such, but were not reported when the virus first emerged in South-East Asia in 2003 [174].

The modern broiler chicken has been bred for intensive production, and its response to antigenic stimulation differs from that of layer chicks [100]. The reduced active immune response in broilers has potential implications if poultry are being reared and sold under suboptimal conditions. Industrial meat chickens also have a very short lifespan, which makes it difficult to implement vaccination programs for AI unless vaccination in the hatchery using vector vaccines can be developed and applied effectively.

In 2013 it was estimated that there was a standing population of around 21 billion chickens globally, with more than 50% (11.9 billion) of these reared in Asia [55]. China is the world leader in terms of poultry numbers, with a standing population of over 5 billion chickens. It is noteworthy that the

majority of countries in which H5 Gs/GD-lineage HPAIVs remain endemic are in Asia, where the increase in poultry populations has not always been accompanied by appropriate biosecurity measures.

Turkeys

Domestication of turkeys occurred in Central America, probably in Mexico between 200 BC and 700 AD, and arose initially from Mexican subspecies (*Meleagris gallopavo gallopavo*) [40]. These small domesticated turkeys were carried from the New World to Europe by the Spanish, beginning in the early sixteenth century, and returned back to the New World as early as 1607 in the north-eastern USA, where they were hybridized with the larger eastern wild turkey (*Meleagridis gallopavo silvestris*) to produce a larger more vigorous bird. Selection for the broad-breasted trait was initiated by Jesse Throssel in Canada and further developed in the USA in the early 1920s. Initially, production utilized traditional outdoor rearing methods with birds primarily being raised for seasonal holiday markets. Beginning in the late 1950s, concentrated production began following the development of controlled-environment houses and chemotherapeutic methods to control the protozoal disease known as blackhead. Industrial production has mainly been in developed countries. One of the drivers for housing of turkeys in the USA was the occurrence of avian influenza. Globally in 2013 there were around 460 million turkeys, with over 75% of these reared in the Americas. The USA had a standing population of approximately 250 million, whereas Asia had a turkey population of only 12 million [55].

Waterfowl

Domestic ducks comprise two different species, namely domestic or mallard-type ducks, including Pekin and Indian Runner types (*Anas platyrhynchos*), which originated in Asia and Europe, and the Muscovy duck (*Cairina moschata*), which originated in the tropics and subtropics of central and northern South America [40]. Mallard-type ducks represent the vast majority of domestic ducks reared. The mallard was domesticated in two independent events, first in South-East Asia several thousand years ago, and again in Europe during the Middle Ages. Domestication almost certainly occurred after the emergence of AIVs, so it is likely that some populations of domestic ducks have been infected with AIVs from that time [172]. Domestic ducks are a minor poultry species in Europe, but are an important poultry species in parts of South-East and East Asia, where they are reared for both meat and eggs. Around 81% of the world's standing duck population is in Asia, with China and Vietnam accounting for 65% of the world's total. Most of the production is outdoors, but industrial production does exist for meat birds, mostly in Europe and North America, but also more recently in Asia. Ducks often graze on rice paddies, and may be transported over long distances between fields, which can result in transmission of AIVs over relatively long distances. The Pekin duck was developed on Long Island, USA, in the mid-1800s, and intensive production of ducks was occurring there in the early part of the twentieth century. The manner in which ducks are reared has played an important role in the persistence, evolution, and spread of H5N1 HPAIV and other AIVs. Ducks that are reared outdoors share the same environment as wild aquatic birds, providing opportunities for two-way transmission of AIVs.

The Muscovy duck was taken from the Americas to Europe, primarily France, and to Africa by the Spanish and Portuguese explorers, but it was also transported to Asia, where it adapted well to the hot climates [40]. It has remained a minor poultry species, with the greatest commercial production located in France, Eastern European countries, Taiwan, and South-East Asia, primarily as a meat source. It is frequently found in village households in Asia, and is used to brood eggs of other species. Production in other parts of the world, especially Africa and South America, is primarily as village poultry and in low-production, subsistence farming for eggs and meat. A sterile hybrid of the domestic duck and Muscovy duck, known as the mulard, is commercially important in South-East Asia, especially in Taiwan, and in France.

There are two types of domestic geese. Eastern breeds, such as Chinese and African, were derived from the wild swan goose (*Anser cygnoides*) in China around 4500 years ago. Western breeds, such as Embden and Toulouse, were derived from the wild graylag goose (*Anser anser*) in Egypt (around 1500 BC) and possibly in Germany [40]. The goose

is primarily reared in China, with smaller numbers in Eastern Europe, with most geese being raised outdoors, through a combination of indoor and outdoor production or in village settings, although there is some industrial production. In other developed countries, goose production is a minor industry, and few geese are raised in developing nations.

Duck and goose production accounts for 7.5% of world poultry production [146]. Based on estimates by the Food and Agriculture Organization of the United Nations (FAO), China is the number one producer of both ducks (standing population 685 million head in 2013) and geese (standing population 285 million), accounting for 58% and 84% of the global standing population (1.18 billion ducks and 340 million geese) of these species, respectively. Other major duck-producing countries are Vietnam (standing population 82.9 million), Indonesia (50.9 million), Malaysia (51 million), and Bangladesh (46.5 million). Eastern European countries are home to around 19 million domestic geese [55]. In addition to meat, duck eggs, duck liver, and duck and goose feathers are significant exported products. Feathers are used for stuffing coats, sleeping bags, pillows, etc. Uncontrolled trade in these products can pose a risk for AIV transmission.

Other minor poultry

Various other species of poultry are raised for meat, eggs, feathers, and hides throughout the world, but are minor contributors to agricultural production. However, in some countries with wet market systems, certain minor species are important contributors to livelihoods. These include ratites, especially ostrich (*Struthio camelus*) and emu (*Dromaius novaehollandiae*), and also Japanese quail (*Coturnix coturnix japonicus*), bobwhite quail (*Colinus virginianus*), ring-necked pheasant (*Phasianus colchicus*), chukar partridge (*Alectoris chukar*), guinea fowl (*Numida meleagris*), and pigeons (*Columba livia*). South African ostriches are reared outdoors, and it has proved very difficult to maintain these flocks free from infection with LPAIVs of the H5 subtype [131].

There is considerable global trade in poultry and poultry products. The vast majority of live poultry that are sold across international borders are day-old chicks. These generally pose a low-level risk of transmission of AIV, especially if OIE guidelines on trade are followed. However, occasional cases have been reported in which day-old chicks may have played a role in AIV transmission, including one case in Lao PDR [231]. Poultry meat and eggs represent a low risk of AIV transmission. Meat from birds infected with HPAIV can contain high levels of virus. Eggs can be contaminated with HPAIV if they are from infected flocks, and occasional cases have been reported in which the contents of the egg contained virus. Outbreaks of AI (as defined by OIE) are usually followed by the introduction of trade restrictions on poultry and poultry products, thereby reducing the risk posed by these commodities through legal trade. Smuggling of live spent layer hens and young chicks is recognized as a risk factor for the introduction of H5 Gs/GD HPAIVs into Vietnam.

General ecology and epidemiology of influenza A viruses

The ecology and epidemiology of influenza A viruses (IAVs) are complex, involving various free-living, captive-raised, and domestic bird hosts as well as various wild and domesticated mammalian hosts in diverse environments (Figure 1.1). IAVs belong to the family Orthomyxoviridae, genus *Influenzavirus A*, and contain eight gene segments [38]. IAVs found in birds are classified by their surface glycoproteins into 16 different subtypes of hemagglutinin (H1–16) and nine different subtypes of neuraminidase (N1–9). Two additional H and N types (H17N10 and H18N11) have been found in bats [119].

Free-living aquatic birds as primordial reservoirs

Free-living birds should not be viewed as a single entity of "wild birds" occupying one ecosystem with equal risk for infection with LPAIV. Rather, birds are a genetically and phenotypically diverse group of animals that occupy a variety of habitats and ecosystems. Specifically, the class Aves contains 29 orders, 187 families, over 2000 genera, and over 9600 species [62]. LPAIVs have been shown to naturally infect more than 105 avian species,

representing 12 orders [132, 187, 188, 228]. However, the number of naturally infected avian species is likely to be higher [6].

The majority of the LPAIVs have been isolated from aquatic birds from the orders Anseriformes (ducks, geese, and swans) and Charadriiformes (e.g. shorebirds [turnstones and sandpipers], gulls, terns, murre, and guillemots) [188]. Such birds are considered to be the main LPAIV reservoirs, with the most frequent isolation of AIVs from Anseriformes (predominantly subtypes H3, H4, and H6) and from Charadriiformes (predominantly subtypes H3, H9, H11, and H13) (Figure 1.1, Table 1.1) [3, 4, 6, 116, 184]. In addition, LPAIVs have been infrequently isolated from other aquatic birds in the orders Ciconiiformes (herons and ibis), Gaviiformes (loons), Gruiformes (coots), Pelecaniformes (cormorant), Podicipediformes (grebe), and Procellariiformes (shearwater). On rare occasions, LPAIVs have been isolated from non-aquatic birds in the orders Piciformes (woodpecker), Passeriformes (perching birds, e.g. sparrows, starlings, mynahs, finches, and weaverbirds), Columbiformes (doves and pigeons), and Galliformes (pheasant and partridge) [187, 188]. However, non-aquatic species are not considered to be reservoirs of LPAIVs, and infection in these species is thought to occur via spillover from infected domestic poultry [187]. Infections by LPAIVs in free-living birds typically produce asymptomatic infections, and such viruses are passed within and between species of birds that occupy the same ecosystem [205]. Most free-living avian species are neither exposed to nor infected with LPAIVs, especially upland game or terrestrial birds of the order Galliformes (jungle fowl, wild turkeys, Bobwhite quail, etc.), because of habitat utilization and behavior [139].

In contrast to LPAIVs, HPAIVs had until 2002 been less frequently isolated from free-living birds [218], and the existence of a long-term wild bird reservoir had not been demonstrated [154]. However, since 2002, H5 Gs/GD HPAIVs have been isolated from various dead captive and free-living birds, and in live birds spatially associated with outbreaks of disease in wild birds or poultry. In addition to birds in most of the orders listed above, H5 HPAIVs have been isolated from birds of the orders Falconiformes (falcons, eagles, hawks, buzzards, and Old World vultures), Phoenicopteriformes (flamingoes), Strigiformes (owls) [218], and a broader range of Passeriformes, including crows and magpies. In some cases these wild bird infections have resulted from exposure to infected poultry, and many of these free-living birds had scavenging or carnivorous feeding habits which could result in transmission from consumption of infected carcasses of poultry or wild birds.

From 2003 onwards it has been apparent that wild birds are capable of transmitting H5N1 HPAIVs over long distances. This was first evident when cases of disease associated with H5 Gs/GD HPAIV were detected in Japan in 2003–2004. The pattern of outbreaks was consistent with wild bird introductions. Subsequently there have been at least four incursions of different but related H5 Gs/GD-lineage HPAIVs into the Republic of Korea and Japan.

The role of wild birds in long-distance transfer of H5 Gs/GD HPAIVs was settled in 2005 when wild birds were found to be dying from this disease in remote lakes, visited by migratory birds in Mongolia and Russia, in places with no poultry. Virus was subsequently transferred to the Middle East and Europe, with multiple viral incursions into the Danube Delta and western Black Sea. Virus was also transmitted to West Africa and Egypt, with wild birds being considered by some to be the most likely means of introduction. However, a long-term reservoir status of this H5N1 HPAIV lineage in free-living aquatic birds has not been demonstrated, with no single H5 clade of Gs/GD-lineage HPAIV being found in wild bird populations for longer than 3 years (see Chapter 9).

In the winter of 2014–2015, wild birds were again implicated in the long-distance transmission of H5 Gs/GD-lineage HPAIVs with, for the first time, viruses from two different clades (clades 2.3.4.4 and 2.3.2.1c) being detected in Russia at the same time, and subsequently in North America and Europe (clade 2.3.4.4) and West Africa, Europe (Bulgaria, Romania) and India (Clade 2.3.2.1c). The precise routes of introduction of these viruses have yet to be established for some of these incursions, but the molecular evidence strongly suggests wild bird introductions, despite large gaps in known migratory pathways for the European introductions of clade 2.3.4.4 viruses.

The major reservoirs of infection with H5 Gs/GD-lineage HPAIVs and propagators of these viruses are domestic ducks in parts of Asia and

Egypt. Poultry moving through poorly managed LPMs and traders' yards also play a role within these regions [87, 192] and, in some countries, survival of virus in village poultry and large poorly managed multi-age farms may be possible.

Historically, HPAIVs have arisen from LPAIVs after circulation in gallinaceous poultry, and are the result of mutations at the proteolytic cleavage site of the hemagglutinin protein [199]. However, the H5 Gs/GD-lineage HPAIVs have remained highly pathogenic and have continued to circulate in poultry and wild birds since they were first identified in geese in Guangdong in 1996. A detailed discussion of AI and free-living birds is presented in Chapter 7.

Influenza A viruses in mammals

IAVs (of most subtypes) have caused infections in a variety of mammalian species; these viruses or some of their genes have their origins from AIVs maintained in the free-living aquatic bird reservoirs (Figure 1.1). Typically, IAVs are not promiscuous, unlike many enteric bacteria (which jump easily between host species). Instead, in many instances they exhibit some host adaptation, thus requiring long periods of time (years or decades) to adapt to a new host species and become endemic. However, cross-species transmission from birds to mammals, including humans, can occur, with some AIVs capable of infecting mammals without prior adaptation [122]. Onward transmission of these zoonotic viruses from infected mammals is still rare, demonstrating that additional adaptation or reassortment between various IAV genes is required for sustainable transmission.

Serological evidence of infection with a range of AIVs has been detected in humans [86, 98]. Viral factors that facilitate transmission of viruses from birds to mammals have been reviewed, and there are still considerable gaps in our knowledge. We do not yet have a comprehensive understanding of the conditions that facilitate interspecies transmission, despite decades of research in this area [122]. Some of the changes that facilitate infection of mammals can arise during replication in domestic poultry, such as changes in receptor specificity, whereas in other cases these changes are already present in viruses from wild birds [122].

Once H5N1 Gs/GD-lineage HPAIVs had emerged as a cause of severe disease in poultry and humans in 1997, concerns were raised that these viruses might develop the capacity to transmit efficiently between mammals, producing a novel, severe, human influenza pandemic. Respiratory transmission of H5N1 HPAIV has been reproduced experimentally in ferrets in "gain of function" studies [76, 91], but so far only limited chains of onward transmission have occurred in humans with field strains of zoonotic influenza virus [229].

IAVs originally derived from birds are responsible for infections in mammals in three broad situations:

1. endemic infections with established, host-adapted viruses such as swine influenza, equine influenza, most human influenza strains, and, recently, canine IAVs
2. sporadic infections, limited to epizootic infections such as in mink, seals, whales, and some pig and some human cases with LPAIVs of free-living bird or poultry origin, including H9N2 and H7N9 subtype viruses
3. recent sporadic infections with H5 Gs/GD-lineage HPAIV, such as have occurred in tigers, lions, leopards, house cats, dogs, Owston's civets, stone martins, (rarely) pigs, and around 450 reported human cases.

Endemic infections with host-adapted viruses

Endemic IAV infections have been established in pigs, horses, dogs, and humans, causing common upper and lower respiratory tract infections and frequent disease (Figure 1.1). These host-specific viruses mainly became established by reassortment of gene segments from AIVs and host-adapted IAVs to produce a hybrid or reassortant virus. For horses, the classic IAV strain was the H7N7 subtype, first described in the early 1950s, but not detected since the 1970s. It has been displaced by the H3N8 strain [211]. Furthermore, in the early 2000s, a strain of virus derived from an equine H3N8 strain was identified in racing greyhounds in Florida exhibiting severe respiratory disease, and subsequently the H3N8 influenza has become a common etiologic agent of kennel cough for dogs in the USA (Figure 1.1) [27, 39]. Another influenza virus of the H3N2 subtype derived from an LPAIV has also become established in dogs in Asia [136, 223]. Through reassortment, some strains of this virus have acquired the M gene from the 2009 human H1N1 pandemic virus [85].

For swine, the first reports of influenza-associated respiratory disease occurred during August 1918 in Illinois, USA, which followed the spring wave of H1N1 Spanish flu in humans in 1918, suggesting an initial human-to-swine transmission of the H1N1 virus [46, 208]. An H1N1 IAV was isolated in 1931 from pigs with respiratory disease, and was determined to be the etiologic agent of swine influenza [167]. This H1N1 virus is the source of the classic H1N1 swine influenza present in North America today [99, 101]. In 1979, an H1N1 AIV appeared in European swine, and is the predominant influenza A virus in swine in Europe [46]. In the mid-1980s in Europe and in the mid-1990s in the USA, H3N2 influenza appeared in swine, and these viruses are still detected. Reassortant strains between H1N1 and H3N2 have been isolated from swine, including the H1N2 and H3N1 subtypes [215]. Genetic evidence from viral gene sequences suggests that swine were the source of virus for the human H1N1 influenza pandemic in 2009, although originally a number of the genes in this virus were derived directly from AIVs [178]. Considerable reassortment has also been detected in swine between human pandemic and swine influenza viruses [148].

In humans, endemic influenza A usually produces a self-limiting respiratory disease, but can result in significant mortality in the elderly, in pregnant women, and in immunocompromised individuals. Periodically, new subtypes have emerged, resulting in pandemics and displacement of or co-circulation with previous endemic IAV subtypes. For example, the 1918 pandemic probably resulted from either the introduction and slow adaptation of an H1N1 virus of avian origin, or the reassortment of genes between an AIV and existing human IAV genes [150, 151, 207, 233]. Sequence analysis of IAV genes has shown that the 1957 (H2N2) and 1968 (H3N2) human pandemic IAVs resulted from reassortment of three (HA, NA, and PB1) and two (HA and PB1) AIV genes with five and six human IAV genes, respectively [95, 149, 158, 160]. The H2N2 displaced the H1N1 subtype in 1957, and the H3N2 displaced the H2N2 subtype in 1968, but the H1N1 subtype reappeared in 1977, and H1N1 and H3N2 strains are currently co-circulating worldwide, although the H3N2 subtype is predominant. This equilibrium was altered when the 2009 H1N1 pandemic virus emerged from swine. This was the first time that a human influenza pandemic had been caused by a virus of the same subtype as a circulating endemic strain. It came about because of the major antigenic difference between swine and human H1N1 viruses, and the presence in this virus of a combination of internal genes that facilitated transmission between humans [178].

Disease caused by pandemic viruses can affect different age groups to those affected by endemic viruses, depending on pre-existing immunity to viruses and, potentially, on the first strain of influenza virus to which each person is exposed (antigenic imprinting) [233].

Details of endemic host-adapted infections by AIVs can be found in Chapter 5 (public health implications for humans), Chapters 16–19 (swine influenza), Chapters 20 and 21 (equine influenza), and Chapter 22 (canine influenza).

Sporadic infections in mammals with LPAIVs

Some LPAIVs have caused sporadic respiratory disease in mink (H10N4 and H10N7) [51], seals (H7N7, H4N5, H3N3, H3N8, pH1N1, and H10N7) [15, 23, 61, 79], and whales (H1N1, H13N2, and H13N9) [81, 114]. Infections have largely been limited to individual animals, but some epidemics have been reported. There has been a lack of evidence for these becoming and remaining endemic [23, 50, 61, 81, 108, 114, 226]. Self-limiting, sporadic infections have been reported in swine with H1N7, H4N6, and H9N2 LPAIVs [19, 94, 129, 234], and there has been considerable infection with H9N2 virus in pigs in China. Infections with LPAIVs in humans with complete recovery have also been recorded, including cases only detected by serology. Most of the strains involved have not become established, but sporadically some H7N2 and H9N2 LPAIVs have caused limited numbers of individual human cases [28, 138]. The emergence in 2013 of H7N9 LPAIV capable of crossing the species barrier once again demonstrated that AIVs do not follow a fixed set of rules. Viruses of this subtype emerged as a cause of severe disease in humans in China in 2013. The vast majority of cases have been linked to LPMs where the virus is circulating in poultry. This is a low-pathogenicity virus in poultry, and experimental infection in chickens does not result in any signs of disease [135]. This virus may have been able to more readily cross the species barrier

as a result of changes in the NP gene that confer increased resistance to Mx proteins [152].

Details of sporadic mammalian infections by AIVs can be found in Chapter 23.

Sporadic human infections with HPAIVs

A few HPAIVs have caused infection and deaths in mammals, including humans, but these viruses have not become established as endemic, and mammal-to-mammal transmission has been limited. Since 1997, the H5N1 Gs/GD-lineage HPAIV has caused sporadic cases of infection and death in large felines (tigers and leopards), house cats, dogs, Owston's palm civets, a stone martin, domestic pigs [35, 97, 104, 153, 181, 236], and humans. An H7N7 HPAIV caused infections without disease in pigs during the 2003 Dutch outbreak [113], and a single case of H5N2 virus infection was reported in pigs during the 1983–1984 outbreak in the USA [21]. Human cases of infection with H7N7 and H7N3 HPAIVs have also been reported. Most of these cases have involved close contact with infected birds, and in the case of H5N1 viruses, specific modes of contact include preparation of sick and dead poultry for consumption, or consumption of raw products such as duck blood or uncooked infected poultry carcasses, or visits to LPMs [28, 58].

Details of human infections by AIVs can be found in Chapter 5.

Poultry and captive birds

Humans have created new niches for birds outside of their natural environment through captivity and domestication [198]. In some cases these new environments have favored transmission, adaptation, and perpetuation of AIVs outside the free-living aquatic bird reservoirs to other avian species, including gallinaceous poultry which are not natural hosts of AIVs [195]. Outdoor rearing and lack of biosecurity measures favor the introduction, adaptation, maintenance, and spread of AIVs in captive birds and domestic poultry. Infections of domestic poultry have been reported with H1–13 subtypes of LPAIVs and H5 and H7 HPAIVs, but the most frequently reported infections have been from H1, H5, H6, H7, and H9 subtypes.

In general, LPAIVs have been isolated from domestic poultry, most frequently (in descending order) in turkeys, ducks, and chickens. These viruses have also been isolated, although less frequently, from captive wild birds kept as pets, or in quarantine stations, private collections or reserves, and zoological parks [3, 6]. However, the incidence and distribution vary greatly according to geographic region, species of bird, age of bird, time of year, and the environmental or agricultural system occupied [205]. The remainder of this chapter will focus on the epidemiology of AI in domestic poultry and captive birds.

Concepts for understanding pathobiology

Definitions of several pathobiological terms and explanation of some disease concepts are essential in order to understand the complex ecology and epidemiology of AIVs (Figure 12.1 and Table 12.1.) [203]. Exposure to an AIV can initiate the infection process, but control of virus exposure can prevent infection in domestic and captive animals. For example, exposure is prevented for animals within an AI-free country, zone, or compartment (CZC), or if the animals are in an AI-affected CZC in places where biosecurity measures are sufficient to keep AIV off farms and other premises. However, if virus exposure does occur, infection will only follow if the exposure route is appropriate, the exposure dose is above the infection threshold, adequate immunity is not present in the host, and the AIV strain is sufficiently adapted to the specific host species. The outcome of such infections may range from infection without clinical signs, to mild disease, to severe disease with high mortality. For AIVs with low adaptation to the host, infection may require a high exposure dose or secondary factors to increase host susceptibility. Even then infection may only result in virus replication and shedding without disease. By contrast, AIV strains with high adaptation to a host species usually require low exposure doses to produce infection [204]. Generally, an AIV strain is optimally adapted for a single host species, although closely related host species may also be susceptible even though the virus may have arisen in other species. For example, H5 Gs/GD-lineage HPAIVs evolved in Asia where few turkeys are reared, yet turkeys require a lower infectious dose of these viruses than chickens or

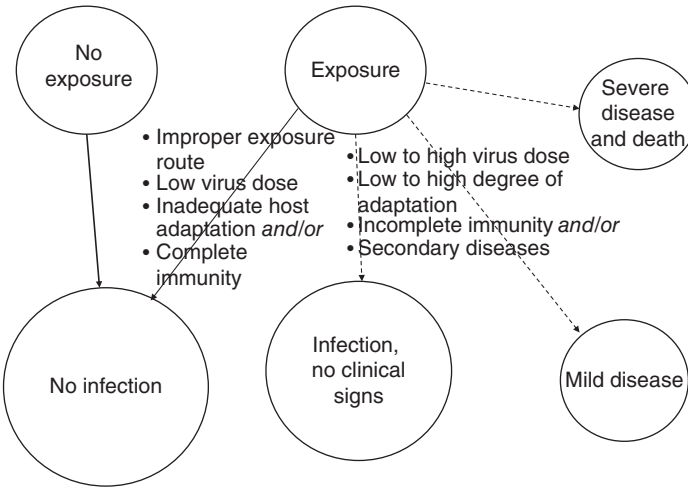

Figure 12.1 Pathobiology concepts for understanding AI in poultry. Source: D. Swayne, U.S. Department of Agriculture/ Agricultural Research Service.

ducks [2]. In other cases, other species may be susceptible and become infected when exposed, but the AIV strain will exhibit a lesser degree of adaptation as evident by lower replication titers, resulting in no or minimal onward transmission. In most free-living aquatic birds, optimally adapted LPAIVs replicate in the alimentary tract and are shed in the feces. Such infections are not associated with pathobiological changes (i.e. there is no disease). However, when these viruses are passed to domestic poultry through exposure and progressive adaptation, the results can vary. In many cases in poultry, as the LPAIV replication titers increase, so does the severity of pathobiological changes such as gross and microscopic lesions in the respiratory, alimentary, and reproductive systems. However, this is not always the case, as has been shown with H7N9 LPAIVs in China from 2013 onward that replicate to high titers in chickens but do not produce disease. The most pathogenic virus strains cause major cell damage and death. With HPAIVs, the replication is systemic and can result in severe damage to critical organs with resulting high death rates in the exposed population. The extent of disease expression may also be determined by the genetics of the host, its immune status, and the presence of concurrent diseases. Generally, an AIV that is transmissible within a given population of a particular species implies sufficient adaptation to allow natural host-to-host spread from infected to naive, susceptible host of the same or closely related species. Transmission is dependent on multiple complex factors, including the following:

1. magnitude, route, and duration of virus shedding
2. host species, immune status, population density, and husbandry methods
3. environmental conditions that increase virus survival
4. opportunities for mechanical spread of the virus by humans, birds, or equipment [21].

Exposure and transmission

In places where AIV is circulating, exposure is the first step in transmission and initiation of infection. Conceptually, an AIV can be introduced into a poultry flock via direct or indirect contact with virus. There are six different means of introduction (Figure 12.2):

1. direct exposure to AI-infected birds
2. exposure to equipment or materials that are contaminated with AIV, usually from respiratory secretions, feathers, or feces
3. movement of people with AIV on their shoes or clothing
4. AIV-contaminated water or feed
5. AIV moved in air
6. flies and rodents.

The most efficient means of introduction is through direct contact with infected birds that shed large quantities of the AIV into the common environment through their respiratory secretions

Table 12.1 Pathobiological terms.

Term	Definition
Adaptation	Progressive genetic changes in a virus, resulting in increasing efficiency of replication
Exposure	Access of the host to the virus
Fomites	Inanimate objects, such as clothes, shoes, equipment, and supplies, that can be contaminated with AIVs and thus have a role in their transmission
Incubation period	Time from exposure to appearance of clinical signs
Infectious or patent period	Time from first detection of the virus from excretions or secretions to the point when the virus is no longer detected
Infectivity	Ability of the virus to bind, replicate, and be released from host cells (i.e. ability to produce infection)
Pathobiological changes	Abnormal physiological and anatomic changes that occur as a result of virus replication within the cell, tissue, and/or organ
Pathogenicity or virulence[a]	Disease-producing capacity of the AIV
Latent period	Time from exposure to the virus to the point when virus is produced and detected in excretions or secretions
Transmissibility	Natural host-to-host spread

[a]The terms "pathogenicity" and "virulence" are used interchangeably, but some regard pathogenicity as a qualitative trait (i.e. capacity to cause disease) and virulence as a quantitative trait in a particular host system.

and feces (i.e. birds acting as biological vectors). The infected birds can be free-living birds or domestic poultry. However, birds can be exposed to the AIV through indirect means, such as equipment or materials (fomites) that have been contaminated by respiratory secretions, feces, or dust from infected birds (i.e. the equipment or materials acting as mechanical vectors). Humans have been a source of indirect exposure to AIV through shoes, clothing, and hands contaminated with virus when individuals have been in direct contact with infected birds or their secretions or excretions (i.e. humans acting as mechanical vectors). In addition, birds have been exposed to AIVs through contact with contaminated water. Exposed birds have included wild aquatic birds, domestic ducks, chickens, and possibly turkeys [77, 117], but could include any type of poultry kept in LPMs once drinking water or the environment has become contaminated. Some evidence exists for exposure to AIVs through contaminated dust particles or water droplets within air, and movement by wind. High-volume air sampling outside affected poultry premises during the 1983–1984 H5N2 HPAI outbreak in the USA detected AIV in samples taken up to 45 meters from houses, but not at greater distances [21]. During the Canadian H7N3 HPAI outbreak, $10^{2.4}$ mean tissue culture infectious doses ($TCID_{50}$)/m^3 were detected in low-volume air sampling inside barns with infected poultry, and low concentrations of AIV nucleic acids were found outside the affected barns [147].

The dispersion of AIV through aerosols, dust, and feathers was proposed as a means of transmission from affected farms in the Canadian H7N3 HPAI outbreak, especially during depopulation activities which generated dust and aerosols that were dispersed by wind [18, 130]. Also, a few flocks in the 1983–1984 H5N2 HPAI outbreak in the USA appeared to have been infected by spreading of non-composted contaminated litter or manure on adjacent fields [21]. However, in other outbreaks, such as the H7N2 LPAI outbreaks in Virginia, USA, during 2002, the spatial distribution of affected farms was not consistent with windborne spread, but suggested that movement of fomites by humans was the primary means of farm-to-farm spread [1]. Experimental studies have suggested that the airborne route is not a primary mode of virus transmission [11, 57, 82, 124, 125], although some viruses of the H9N2 subtype can be transmitted by air over short distances between poultry, and modeling of the 2003 Dutch H7N7 HPAI outbreak suggested that wind-mediated spread accounted for 18% of the outbreaks [237]. Airborne transmission of H5N1 HPAIV to chickens and ferrets has been replicated in a simulated market in which infected chickens were butchered and dressed (D. E. Swayne, unpublished data), and airborne transmission has been postulated as one means of introduction of virus to farms in North America during the 2014–2015 H5N2 epidemic.

Influenza viruses from other species can also be transferred to poultry, as was reported for human pandemic influenza H1N1 viruses in turkeys as a result of contamination during artificial insemination [134].

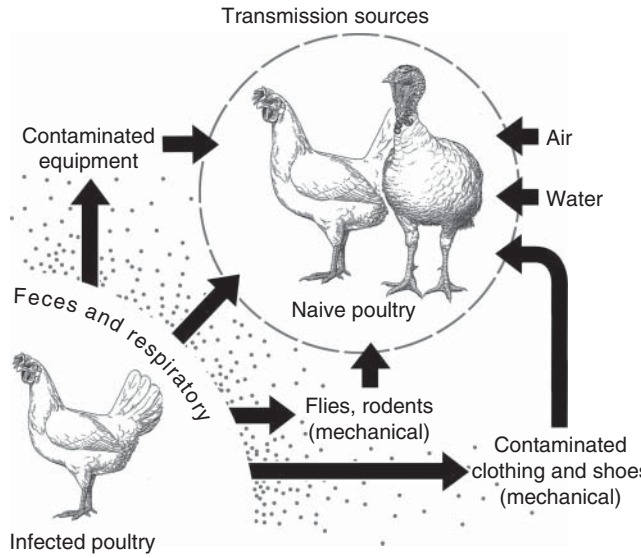

Figure 12.2 Six means or modes by which AIVs are introduced into poultry. Source: K. Carter, University of Georgia, and D. Swayne, U.S. Department of Agriculture/Agricultural Research Service.

Although there is much evidence of spread of AIVs by horizontal means, demonstration of vertical transmission is lacking [10, 21, 45]. However, HPAIVs do produce systemic infections in hens, and the last few eggs laid before death by hens with such infections have HPAIV on the eggshell surface and within the internal contents of the egg [10, 13, 25]. Because AIVs kill embryos, incubation of infected eggs has not yielded viable young [10]. However, cleaning of fecal material off the egg shell surfaces and disinfection may be necessary to prevent hatchery-associated dissemination of AIVs if the eggs originate from AIV-infected flocks [21]. No studies of the fate of AIVs on the surface of eggs during incubation have been conducted to date. The temperatures reached during incubation would be expected to reduce the concentration of residual virus on shell surfaces during the 21-day incubation period, but may not eliminate all virus. Most LPAIVs and HPAIVs cause reduction and cessation, respectively, of egg production, further limiting the potential for vertical transmission of AIV.

Special mention must be made of LPMs and traders' yards, which if not managed correctly can remain infected, posing a risk to any bird entering the market or yard. Transmission in these places can be via any of the routes described above, and includes contact with contaminated transport and holding cages.

Adaptation and transmissibility

IAVs exhibit varying degrees of host adaptation, which has an impact on infectivity and transmissibility. The ease of transmission of individual AIV strains is to some extent affected by how close the hosts are genetically, and the degree of host adaptation expressed by individual virus strains. Following exposure, transmission of IAVs occurs most frequently and readily between individuals of the same host species to which the virus strain is highly host adapted (i.e. intraspecies transmission) [198]. Interspecies transmission does occur, and has been most frequent between individuals of closely related species, especially within the same taxonomic family – for example, between chickens, turkeys, quail, pheasants, and guinea fowl (all belonging to the order Galliformes, family Phasianidae). Interspecies transmission has also been reported between less closely related birds, from different orders – for example, from free-living mallard duck (order Anseriformes) to turkey (order Galliformes) – but such transfers have been less common than those between closely related host species [198]. A number of outbreaks of HPAI have been preceded by introduction of an LPAIV from wild birds, or in some cases by direct introduction of

an HPAIV, as has occurred with H5 Gs/GD-lineage HPAIVs since 2002. Such transfers of AIVs from wild aquatic birds to poultry are a continuing concern. For example, many AIVs have emerged in East and South-East Asia, have evolved in domestic ducks, and have then infected terrestrial poultry. This appears to be the case with LPAIVs of the H7N9 subtype. Mixed-species marketing in LPMs probably facilitates cross-species transmission, and this could also occur in mixed-species farming and household flocks. A wide range of AIVs are present in terrestrial poultry in Asia. Interspecies transmission has occurred between species from different phylogenetic classes. For example, transmission from chicken (class Aves) to human (class Mammalia) has been reported [198], although this does not occur readily, as there is frequent exposure of humans to infected poultry, especially in LPMs, but with little transmission to and infection of humans. An exception to the rarity of interclass transmission has been the ease and frequency of transfer of swine H1 and H3 IAVs to turkeys when the two species were raised on the same farm or within close geographic proximity [121, 196, 198, 206]. Other factors that may increase AIV cross-species transmission and the frequency of infections include intermixing of species on the same premises (e.g. intermixing of domestic ducks and geese with chickens and turkeys), the presence of young birds which are more susceptible to infection, a high density of birds (which increases the opportunities for exposure through increased viral loads and rates of contact), and humid weather and cool temperatures (which increase environmental survival of the virus, especially in the presence of organic matter) [198].

Factors that allow transmission

One example of adaptation of the AIV strain and its impact on transmission was the outbreak of H7N2 LPAI in Virginia in 2002. In the outbreak zone, a higher proportion of turkey farms than chicken farms were affected by the H7N2 LPAIV. This is partially explained by experimental data which showed that turkeys required 100–250 times less virus to cause infection than chickens, indicating that this H7N2 LPAIV was better adapted to, and more contagious for, turkeys than chickens [111]. Similarly, a greater susceptibility for turkeys compared with chickens has also been noted for LPAIVs from free-living aquatic birds, which explains why turkey flocks in North America have been more frequently infected with AIVs from free-living aquatic birds than have chickens [145] Japanese quail and pheasants, too, may have greater susceptibility than chickens to LPAIVs from free-living birds [89, 107, 141, 204]. It has also been demonstrated for H5N1 HPAIV that turkeys are susceptible to infection with a dose 100 times lower than that required for chickens [2]. However, testing of H5N8 and H5N2 Gs/GD-lineage outbreak viruses from the north-western USA, which were isolated during December 2014, demonstrated that these viruses were waterfowl adapted, as 10^2 EID_{50} of virus resulted in 100% infection in mallards, but 200–5000 times more virus was required to infect chickens and turkeys. Contact transmission was slightly greater in turkeys than in chickens. The mechanism of species adaptation is poorly understood, but is probably associated with multiple genetic and biochemical factors, such as hemagglutinin receptor-binding affinity, efficiency of release by neuraminidase, and the efficiency with which AI viral polymerase genes are expressed and their ability to take over the cell-based systems that are required to produce the AIV [103].

Species adaptation of AIVs has been shown to be a multi-step process when transferring between free-living aquatic birds and domestic gallinaceous poultry [70, 139]. For example, in Minnesota during the 1980s and 1990s, the index case of AIV in turkeys began as an asymptomatic infection in range-raised birds during the early autumn, with detection of infection by seroconversion in a few birds at slaughter. The turkey infections were preceded by 6 to 8 weeks by AIV infections in sentinel ducks placed among free-living ducks [67]. Such newly introduced AIVs were passed through multiple turkey flocks over several months before being optimally adapted to turkeys. The turkey-adapted AIVs produced infection in a high percentage of birds within the affected flock, produced clinical signs such as respiratory disease, drops in egg production, and mortality, and interflock transmission of the viruses became much easier [70, 139]. However, the transfer of AIVs from free-living waterfowl (order Anseriformes) to domestic ducks and geese has been much easier than transmission to turkeys

[204], requiring minimal adaptation because of the close genetic relationship between the wild and domestic duck species, and the more frequent contact between outdoor-reared domestic ducks and geese, and free-living aquatic birds. Many AIVs from free-living shorebirds and gulls (order Charadriiformes) do not replicate in intranasally inoculated domestic ducks (order Anseriformes), indicating that transfer of AIVs between some wild aquatic bird species is more difficult and may require multiple steps for adaptation [78, 187, 228]. This information indicates that AIVs express various degrees of host adaptation for different avian species, and that labeling such IAVs from different birds as "AIVs", implying they have equal ability to infect all birds, is a misnomer. It would be equally inaccurate to categorize equine, human, and swine IAVs as "mammalian IAVs", as they have disparate abilities to infect different mammalian species. The relative rarity of successful species jumps of AIV followed by adaptation suggests that adaptive responses are complex and are affected by factors other than exposure [103]. The evolution of H5N1 Gs/GD-lineage HPAIVs also demonstrates the complexity of viral evolution. The strain of virus that was detected in Hong Kong in 1997 was poorly adapted to domestic ducks, but by 2000, reassorted strains of virus that were capable of infecting domestic ducks were circulating in China and had been detected in imported domestic ducks in Hong Kong SAR. It is considered likely that the re-adaptation of this virus to ducks was a crucial step in the epidemiology of this disease, was one of the reasons for the virus becoming endemic, and probably played a role in the transmission of virus to wild birds through shared habitats. It is noteworthy that, as of early 2016, Chinese H7N9 LPAIVs isolated since 2013 are poorly adapted to domestic ducks and, unlike H5 viruses, have not been detected in wild migratory birds or transferred to other countries by migratory birds.

Incubation and infectious periods

With HPAIVs in chickens, the incubation period (i.e. time from exposure to appearance of clinical signs) ranges from a few hours in intravenously inoculated birds, to 24 hours in intranasally inoculated chickens, to 3 days in naturally infected individual birds. The incubation period for naturally infected chicken flocks can be up to 14 days [41, 45]. However, shedding of the virus occurs earlier than the appearance of clinical signs, and may last longer than the clinical disease. The infectious period extends from the time when the virus is first shed by a bird to the time when the virus is no longer present in the oropharyngeal and cloacal swabs, or in or on feathers [205]. The pre-patent or pre-infectious (latent) period indicates the time lag between exposure and the shedding of virus by the birds. The use of the infectious period is crucial for disease investigations and for preventing transmission and initiating disease control. The incubation period is of limited use, as many species, such as domestic ducks infected with some strains of H5N1 HPAIV, may not show illness when infected, but still shed the virus. It is also important to consider the pre-patent (latent) period (i.e. the time from exposure to virus shedding), as this is crucial for tracing infection, especially when potentially infected birds have been moved off the farm after being exposed to virus.

The lengths of the latent, incubation, and infectious periods are dependent on the dose of virus, the route of exposure, the species exposed, the immune status of exposed birds, and various environmental factors [45]. Experimental studies conducted with a Gs/GD-lineage H5N1 virus demonstrated that this virus had a very short latent period [17]. This has significant implications for progression of outbreaks, which is affected by both the latent period and the basic reproduction number (R_0) for the disease. R_0 is a measure of infectivity, and has been estimated for a number of AIVs [17, 155]. For a spreading epidemic, R_0 must be greater than 1; for disease control, R_0 must be less than 1. R_0 is specific to the disease agent and the contact rate between susceptible animals. It varies according to many host factors, including species or breed, production and husbandry system, and age, resistance, and immune status, as well as duration of survival of infected hosts. R_0 for LPAIVs is generally much higher than for HPAIVs because the virus is fatal for the latter, reducing opportunities for viral transmission [155]. Estimates vary depending on the methodology used and the poultry production systems employed. For Gs/GD-lineage H5 viruses, R_0 has been estimated to be in the range 1.95–2.68 [210, 224], although these calculations are based mainly on infection in

village poultry production. An R_0 value of this magnitude indicates a moderately infectious disease, but is low compared with many other infectious agents. However, even with this relatively low R_0, the disease can still spread rapidly within a flock, as has been recorded in the field [210], presumably because the latent period following infection with this virus is short [17].

Experimental systems designed to measure R_0 cannot fully replicate the complex environment and contact structures in farms, and only provide a guide to real values. For example, the effective contact rates between infected and uninfected poultry housed in cages would be expected to increase if drinking water is contaminated by infected birds, and birds in each row of cages share common water troughs. R_0 can also be used to determine the level of protection required from vaccination to stop transmission of a virus in a given population. As R_0 increases, the proportion of a flock that needs to be vaccinated effectively to prevent transmission also increases, and is equal to $1 - 1/R_0$ [159].

Maintenance of AIVs in populations

Typically, the majority of individual birds shed virus for only 7–10 days, and AIVs do not persist or produce latent infections in individual birds, as has been shown to occur with some avian viruses, such as the herpesvirus of infectious laryngotracheitis. Occasional cases in which virus can be shed for an extended period in individual birds have been reported [88]. However, AIV can be maintained for much longer time periods within large populations of birds, such as are found in village poultry production, live poultry markets, or commercial poultry operations, because the initial exposure and acute phase do not cause an immediate 100% infection rate. As the number of susceptible birds decreases in a closed population, the rate of viral transmission slows down. There is still remarkably little information on the course of AIV circulation in poultry populations, and this may also depend on the environment in which the birds are reared, which affects the stability of the virus outside the host.

AIVs have re-emerged from previously infected flocks after a significantly stressful event, yet no long-term carrier status has ever been defined, or they have re-emerged following the introduction of naive susceptible birds into the affected population. LPAIV has been recovered for up to 36 days after known exposure time from a chicken (tracheal sample) [7] and 22 days from a turkey [106], but in turkeys, when the time of exposure was not certain, the virus was recovered up to 72 days after the beginning of the floor pen experiment [83]. In one field case, H7N2 LPAIV in Pennsylvania during 1997–1998 was recovered from the daily mortality of a clinically normal layer flock 6 months after recovery from the acute LPAI clinical disease, and from another layer flock 8 weeks after the acute LPAI clinical disease but following the induction of a molt [238]. Therefore, once a flock is diagnosed as being infected with AIV, the possibility should be considered that it may be a potential source of virus for the life of the flock, until those birds are eliminated and the farm is properly cleaned and disinfected, and repopulated with AI-free stock. Vaccination has been shown to be effective in reducing the amount of virus shed into the environment and stopping virus transmission [220, 221]. There are examples of successful achievement of virus elimination through the use of selective culling of infected flocks (including controlled marketing) and/or emergency vaccination [47, 73].

The immune response to LPAIVs in ducks differs to that in chickens, and this may play a role in viral maintenance in duck populations. When ducks were experimentally exposed to an H5 LPAIV they developed an immune response, but when they were re-exposed to the same virus 21 days later they were still able to be infected with and shed virus, especially via the cloaca. Chickens that had been previously exposed did not shed any virus when re-exposed to the homologous virus [29].

Shedding of virus into the environment and environmental tenacity

In infected animals, AIVs are excreted from the nares and mouth (respiratory secretions), conjunctiva, and cloaca (feces) into the environment. HPAIVs can also be detected in feathers and feather dander, which could potentially play a role in disease transmission through either allo-preening or dislodged feathers. Waste from defeathering in slaughterhouses represents a potential source of virus. Environmental virus sources are responsible for most exposures, but birds can also be exposed

to AIVs through predation or cannibalization of infected carcasses of dead birds. Experimental studies in chickens showed that HPAIVs were shed in the largest quantity from the oropharynx ($10^{4.2–7.7}$ mean chicken embryo infective doses [EID_{50}]/mL of respiratory secretions), and in a slightly lower quantity from the cloaca ($10^{2.5–4.5}$ EID_{50}/g of feces) [200, 201]. This varied depending on the virus strain, and for some H5N1 HPAIVs, reduced or no cloacal shedding was detected. For LPAIVs in chickens, the environmental shedding was lower for oropharyngeal (swabs, $10^{1.1–5.5}$ EID_{50}/mL) and cloacal (swabs, $10^{1.0–4.3}$ EID_{50}/mL) samples [201], although this was not the case for H7N9-subtype viruses that emerged in China as a serious zoonotic disease [135], and for some H9N2 viruses. In experimental studies, these higher-shedding titers for HPAIVs have translated into greater environmental contamination and greater transmissibility than were found for comparable LPAIVs [219]. Titers in carcasses (meat) vary according to virus strain, tissue type, bird species, and clinical stage of infection:

1 Titers from dead chickens infected with 1983 H5N2 HPAIV from Pennsylvania had $10^{2.2–3.2}$ EID_{50}/g of meat, whereas 2003 H5N1 HPAIV from South Korea had $10^{5.5–8.0}$ EID_{50}/g of meat, but quantities of virus in meat of H5N1 HPAI-infected chickens were sufficient to infect ferrets by consumption of the meat [12].
2 Titers for H5N1 HPAIV in heart of chickens have been reported to be as high as $10^{10.6}$ EID_{50}/g.
3 H5N1 HPAIVs produced different titers in clinically normal ($10^{2.0–3.4}$ EID_{50}/g) and sick ($10^{4.0–6.0}$ EID_{50}/g) domestic ducks [201, 203, 209].

AIVs shed into the environment are protected by accompanying organic material which shields the virus particles from physical and chemical inactivation [45]. In addition, specific environmental conditions, such as cool and moist conditions, increase survival times and have a profound impact on transmission. For example, H5N2 and H5N1 HPAIVs remained viable in liquid poultry manure for 105 days in the winter under freezing conditions, for 30–35 days at 4°C, for 7 days at 20°C, and for 4 days at 25–32°C when kept out of direct sunlight [10, 56, 180, 225]. In experimental studies with H5N1 HPAIV added to poultry manure, no virus was recovered after 24 hours at 25°C and after 15 minutes when maintained at 40°C. Exposure of manure to ultraviolet (UV) light was not effective in killing the HPAIV, probably due to inadequate penetration of the UV light into the manure [36]. For AIVs in water, two H5N1 HPAIVs had a 10^{1} EID_{50} decrease in infectious titer after 4–5 days at 28°C (pH 7.2, and salinity 0 parts per thousand), and no virus was detected after 30 days, but at 17°C, under the same pH and salinity conditions, the two viruses persisted until up to 94 and 158 days, respectively [20]. These H5N1 HPAIVs had shorter environmental survival times compared with H5 LPAIVs obtained from wild waterfowl. For example, LPAIVs from free-living waterfowl were shown to remain infective at 17°C for up to 207 days, and at 28°C for up to 102 days. Increasing water salinity or pH shortened the AIV survival times at both temperatures [185, 186]. These studies and field observations suggest that AIVs could remain infective in water or in moist organic materials maintained at cool temperatures, such as over wintering conditions, to be infective for long periods of time to free-living birds or poultry. To date this has not been demonstrated to occur [232]. Even if H5N1 HPAIVs could survive in ice, they are not causing prolonged cycles of infection, given that genetically identical viruses were not detected in consecutive years in migratory birds from 2005 to 2009. Rather the viruses that were isolated had evolved, suggesting survival within wild bird populations.

Soil conditions also affect survival, as has been demonstrated with H5N1 HPAIV in Cambodia [66].

Inactivation

Following depopulation of an infected farm and disposal of poultry, AIV contaminating the environment must also be properly eliminated and/or inactivated in order to prevent transmission and control field infections. AIVs are very labile and thus susceptible to heat and various disinfectants, including detergents. For enclosed buildings housing poultry, the following has been suggested as an effective program for eliminating AIV from infected premises: heating to 90–100°F for 1 week, followed by removal and proper disposal of manure and litter, cleaning and disinfecting of buildings and equipment, and a 2- to 3-week vacancy period before restocking [69]. If cleaning and disinfection have been conducted thoroughly, the virus is not likely to persist over this time, and the 2- to 3-week period provides a considerable safety margin. In

situations where poultry are sold through LPMs, the turnaround time for transport cages is measured in days. As a result these cages represent a significant risk for transmission of virus, especially if they have been heavily contaminated with AIVs from infected poultry. Even if cages are washed and disinfected, there is still a low risk of residual viral contamination if they have carried infected poultry, given the difficulty of removing all fecal material during cleaning. AIVs in poultry carcasses or litter and manure are effectively killed within less than 10 days when properly composted, or they can be buried or incinerated [163]. On clean surfaces, a variety of disinfectants (including detergents) are effective for inactivating AIVs, such as sodium hypochlorite (household bleach), quaternary ammonium compounds, sodium hydroxide (lye), phenolic compounds, acidified ionophor compounds, chlorine dioxide disinfectants, strong oxidizing agents, and sodium carbonate/sodium silicate combinations [37]. However, organic material must be removed by dry or wet cleaning with detergents before disinfectants can work properly. Guidelines on cleaning and disinfection for avian influenza are available elsewhere [14].

Virulence and pathogenicity

Confusion has arisen from conflicting use of the terms "virulence" and "pathogenicity", which are related to the disease-producing capacity of the AIV as measured by production of clinical signs, or gross, microscopic, and/or ultrastructural lesions [22]. Official reporting of AIVs as LP or HP is based on development of illness and mortality following experimental inoculation into chickens, on the target species, or on the sequence of the hemagglutinin proteolytic cleavage site (see Chapter 1). This official pathotype classification is only specific for the chicken, but has pathobiological application to related galliform species. However, the chicken pathotype is not predictive of the AIV's pathogenicity potential in other unrelated avian species, or in humans or other mammals.

Infections within agricultural and other man-made systems

Human activity has changed the natural ecosystems of birds through captivity, domestication, agriculture, and commerce, beginning thousands of years ago [198]. Thus new niches have been created for AIVs which have changed the incidence and distribution of these viruses and the infections that they cause. Various man-made systems have been developed, and classification schemes vary depending on the perspective of the author. For example, in one classification scheme, five broad categories of man-made systems were identified that could have an impact on AIV ecology and epidemiology [198]:

1. bird collection and trading systems, including captive wild birds and zoological collections
2. village, backyard, and hobby flocks, including fighting cocks and exhibition poultry
3. LPM systems with rural to urban movement of poultry for sale and slaughter
4. outdoor raised commercial poultry, including organic poultry, free-range turkeys, free-grazing ducks, and game birds
5. integrated indoor commercial poultry.

In 2004, the Food and Agriculture Organization (FAO) of the United Nations defined four broad production sectors for the purpose of designing surveillance programs [52]:

- Sector 1 – industrial integrated system with high-level biosecurity, and birds or products marketed commercially (e.g. farms that are part of an integrated broiler production enterprise with clearly defined and implemented standard operating procedures for biosecurity).
- Sector 2 – commercial poultry production system with moderate to high biosecurity, and birds or products that are usually marketed commercially but could be sold through LPMs (e.g. farms with birds kept indoors continuously, largely preventing contact with other poultry or wildlife).
- Sector 3 – commercial poultry production system with low to minimal biosecurity, and birds or products often entering LPMs (e.g. a caged layer farm with birds in open sheds, a farm with poultry spending time outside the shed, or a farm producing chickens and free-ranging waterfowl).
- Sector 4 – village or backyard production with minimal biosecurity, and birds or products consumed locally.

However, consistent categorization of poultry production by any single scheme is not possible because of country-to-country and within-country variations in levels of biosecurity, variations in

marketing schemes, extent of modernization of production systems, and available financial resources. Nevertheless, when considering the risks associated with individual farms or groups of farms, the many contacts upstream and downstream need to be taken into account. For example, farms that sell birds to LPMs may be exposed to transport cages from markets that have not been properly cleaned or disinfected, or to traders who have been in contaminated markets.

From a historical perspective, the definition of a commercial farm has changed over the centuries. For example, from the Roman era to the mid-1800s, most poultry farms had around 50–100 chickens. A large "commercial" farm in northern Italy in the late 1800s had around 1000 chickens, which were used primarily for egg production [93]. Currently, in developed countries, a chicken farm with 1000 birds would be considered a small or hobby farm, whereas a large commercial farm in the USA or China could have several million layers. In many countries, especially developing ones, there is a complex mix of poultry farm types sharing locations. Even backyard producers sell some of their poultry to traders, and poultry workers may keep poultry at home, so there are both functional and spatial links between the various production sectors.

The structure of the poultry sector plays a major role in the dissemination and persistence of AIVs, and appears to be a major factor leading to endemicity for H5 Gs/GD-lineage HPAIVs [54]. In some areas this relates to persistence of virus in domestic ducks, whereas in others the high level of poorly regulated trade in live poultry, including illegal cross-border trade, appears to be more important.

For virus to be maintained in an area, there must be a pool of susceptible birds that allows transmission and propagation of virus, and/or sufficient virus survival in the environment until susceptible birds are present. In some parts of Asia, national land borders cross what have been referred to as eco-zones, resulting in regular reintroductions of virus from within the same eco-zone but from outside the country. Examples of this include the land borders between China and Vietnam, and between Vietnam and Cambodia. In many parts of South-East Asia where H5 Gs/GD-lineage HPAIVs are circulating, climatic and soil conditions do not favor viral maintenance. Yet in the Mekong Delta, clade 1 viruses and their derivatives were maintained from 2003 to 2014 [177, 230]. This indicates that sufficient numbers of susceptible birds must be present to sustain the cycle of infection. Long and porous land borders allow the illegal movement of poultry, and this has been recognized as one of the factors leading to repeated incursions into Vietnam of viruses belonging to novel clades of the H5 Gs/GD-lineage HPAIVs. It has also been proposed that "merit releases" of passerine birds as part of Buddhist ceremonies are a potential means of virus dissemination. H5N8 HPAIVs were introduced to Europe in the autumn of 2014 and winter of 2015 (most probably by wild birds, although it is still unclear how they moved from Asia to Europe without being detected), and managed to penetrate biosecurity defenses on a number of commercial farms in England, Germany, Italy, and the Netherlands; this was also the case for H5N8 and H5N2 viruses detected in North America in the same period.

The sources of introduction of AIVs into poultry operations vary according to the host species, virus strain, husbandry system, and quality of biosecurity practices. For example, AIV has been introduced into turkey flocks in the USA over the past four decades from a variety of sources (Figure 12.3), including the following:

1. free-living aquatic birds to outdoor raised domestic turkeys
2. AIV-contaminated drinking water to indoor raised turkeys when the water was derived from ponds or lakes containing AIV-infected free-living aquatic birds
3. exposure to fomites from an LPM
4. infection to swine H1 and H3 IAVs when pigs were raised on the same or nearby farms.

Similarly, the predominance of AIV infections in turkeys during the autumn in northern Italy coincided with staging of free-living waterfowl prior to southern migration for the winter [144]. H5N2 HPAIV infected turkeys reared indoors in North America in late 2014 and early 2015. These viruses have been detected in wild birds, but the precise mode of introduction of the virus onto individual farms varied, and was not always precisely determined. For example, the first H5N8 HPAIV infections were in backyard flocks of mixed poultry species in the north-western USA that

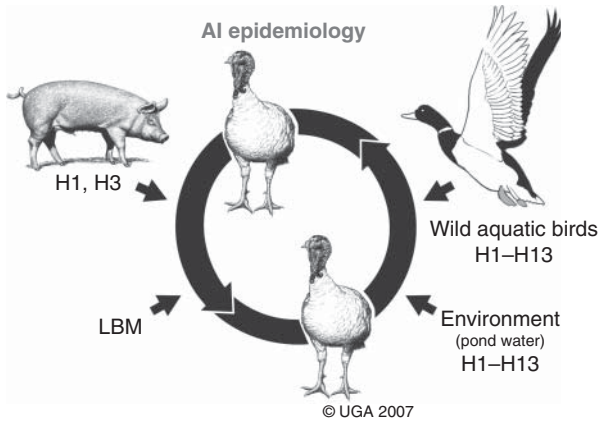

Figure 12.3 Four proven sources responsible for introduction of LPAIV into commercial turkeys within the USA from the 1960s to 2000. Source: K. Carter, University of Georgia, and D. Swayne, U.S. Department of Agriculture/Agricultural Research Service.

were in direct contact with infected waterfowl or captive raptors that hunted infected waterfowl. The first infections on a commercial turkey farm and chicken farm were point source introductions from wild waterfowl, but the exact mode of entry of the virus to each farm and house was not determined. However, water run-off retention ponds containing wild waterfowl were located in close proximity to the affected turkey houses. Later cases of the reassortant H5N2 HPAIV in the Midwestern USA had evidence of point-source introductions from infected waterfowl, but others resulted from secondary spread between farms. Risk factors for HPAIV entry included, among others, the use of lagoon or pond surface water inhabited by wild waterfowl for flushing manure pits, storage of feed grain outdoors with minimal protection from waterfowl, common service personnel and processes including shared equipment, and shared pick-up systems for dead birds.

Introduction to poultry, and adaptation, of AIVs from free-living birds

LPAIVs from free-living aquatic birds have become established in domesticated gallinaceous poultry (primarily chickens and turkeys, but also Japanese quail, guinea fowl, pheasants, partridges, and other species) and domestic waterfowl (primarily ducks and geese) through a two-step process, namely exposure to an infected host, followed by adaptation to the new host (Figure 12.4) [203]. Typically, exposure to the LPAIVs of free-living birds has

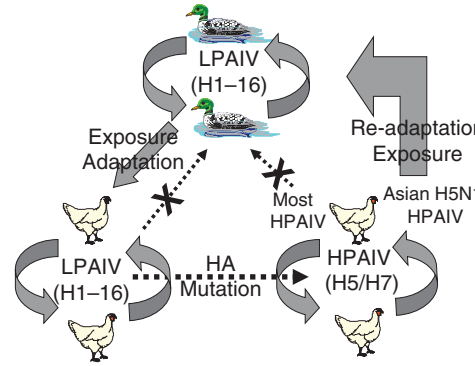

Figure 12.4 Epidemiology of LPAIVs and HPAIVs between free-living aquatic birds and poultry. Source: D. Swayne, U.S. Department of Agriculture/Agricultural Research Service. *See Plate section for color representation of this figure.*

resulted from direct contact with infected birds or indirect contact via fomites. Such exposures have transmitted AIVs from free-living aquatic birds to outdoor-raised village poultry more frequently than to indoor commercially reared poultry. The highest-risk activity for exposure leading to infection has been through direct contact with infected free-living aquatic birds (Figure 12.5), which can be prevented by indoor rearing, temporary confinement from outdoor access when the risk of exposure to infected wild birds is high, or providing poultry outdoor access only in specially constructed areas from which wild birds are excluded by netting. There is a risk of exposure leading to infections through the use of untreated AIV-contaminated surface water from ponds or lakes occupied by

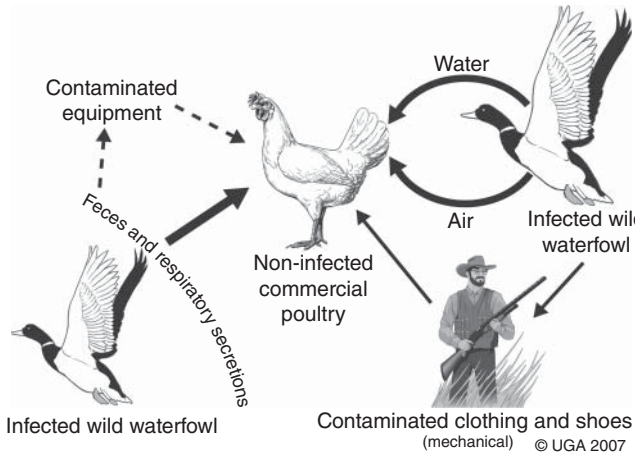

Figure 12.5 Relative importance of different means of initial introduction of AIVs from free-living aquatic birds to poultry. Source: K. Carter, University of Georgia, and D. Swayne, U.S. Department of Agriculture/Agricultural Research Service.

AIV-infected wild aquatic birds, or potentially via wind transmission when such ponds or lakes are located very close to poultry houses. The former risk can be mitigated by treating the water to kill all viruses, or by using untreated water from deep wells. Construction of houses away from waterfowl habitats can reduce the risk of wind-borne transmission. Contaminated clothing and shoes are generally lower risks, as these items contaminated from hunting or sightseeing excursions to waterfowl refuges are unlikely to be worn into poultry flocks, except in remote locations such as those in Siberia, where cases of H5N1 HPAI have occurred in village poultry following hunting of wild birds (in some cases this was due to introduction of hunted birds to village flocks). Similarly, no equipment or supplies should be used for hunting, sightseeing, and working in a poultry flock.

Exposure to AIVs of free-living birds will only result in sustained transmission and efficient replication if the LPAIVs are sufficiently adapted for the poultry host, and the flock of birds is susceptible. Poor virus adaptation to the host will usually result in the virus failing to propagate and transmit between birds, resulting in the virus dying out. On some occasions, sporadic, inefficient transmission within the population will occur until progressive adaptation of the virus to the new poultry species results in emergence of a virus capable of sustained poultry-to-poultry transmission. Once LPAIVs have adapted to the new gallinaceous poultry host, these AIVs have rarely been transmitted back into and sustained in a free-living aquatic bird population, because they are now de-adapted to the original wild bird host (Figure 12.4). As a result, free-living aquatic birds have had a very limited role, if any, in secondary dissemination or farm-to-farm spread of poultry-adapted LPAIVs [80, 127].

When circulating in gallinaceous poultry, some H5 and H7 LPAIVs have abruptly changed to HPAIVs through changes in the cleavage site of the hemagglutinin protein [140]. Others have circulated in poultry for an extended period of time without converting to an HPAIV (e.g. the H7N9 LPAIV that emerged in China in 2013). Historically, these new HPAIVs that originated from LPAIVs were already adapted to gallinaceous poultry, and have typically not gone back into free-living bird hosts, although this may be due in part to the fact that until relatively recently most outbreaks of HPAI were rapidly eliminated, decreasing the opportunity for re-adaptation to wild aquatic birds. Similarly, these historical HPAIVs have had limited or no infectivity for domestic ducks [5, 203]. One exception was the detection of H5N8 virus in ducks on a farm adjacent to an infected turkey farm in Ireland in 1984. Except for H5N3 HPAIV in common terns (*Sterna hirundo*) in South Africa in 1961, only a few isolated cases of HPAIV infections had been detected in wild birds before 2002, thus indicating that HPAIVs have traditionally not been established in wild birds [154, 194, 195]. The ecological situation has changed following the emergence of H5 Gs/GD-lineage HPAIVs. Significant infection and mortality in non-poultry species were first reported in captive waterfowl,

and spatially associated free-living aquatic birds, in two parks in Hong Kong during 2002. These outbreaks were followed by individual reports of mortality in free-living birds in Cambodia and Thailand, and reports of significant mortality in 2005 in free-living waterfowl at Qinghai, China and Lake Erhel, Mongolia (Figure 12.4) [32, 48, 110]. Experimental infectivity trials mirror the changes in H5N1 HPAIV infectivity for wild birds under natural field conditions: a 1997 H5N1 HPAIV strain from Hong Kong SAR was poorly infectious for domestic ducks [143], and many of the 1999–2002 viruses detected in Hong Kong SAR were very infectious but produced asymptomatic infections in domestic ducks [31]. By 2002–2004, the viruses were producing severe illness with some deaths. Some recent strains have caused high death rates in young domestic ducklings and in some cases in older ducks [92, 203]. These field and experimental data suggest that the H5N1 HPAIV has re-adapted back to some free-living aquatic bird species. Occurring at the same time, or probably prior to, the re-adaptation to free-living aquatic birds was the introduction and adaptation of the H5N1 Gs/GD-lineage HPAIV to domestic ducks, causing infection, disease, and eventually death. Both events resulted in changes to the ecology and epidemiology of these viruses compared with earlier HPAIVs. However, the establishment of a free-living aquatic bird reservoir for H5N1 Gs/GD-lineage HPAIVs, at least not a long-term one such as those that exist for LPAIVs, has not been demonstrated.

H5 Gs/GD-lineage HPAI infections in wild birds have been reported, and they have introduced viruses to areas that were previously free from infection. Wild birds are also suspected of playing a role in flock-to-flock spread in village or rural poultry systems, as either biological or mechanical vectors. For example, H5N1 Gs/GD-lineage HPAIV infections of tree sparrows (*Passer montanus*) – birds that are closely associated with human habitation and agricultural buildings – have been reported in China. This finding raises concern that this species could serve as a vector for spread of the virus, and could thus create a need to bird-proof poultry barns [102]. Experimental studies have demonstrated that infected sparrows can transmit virus to domestic chickens [235]. However, more importantly, the domestic duck population has become a major reservoir of H5 Gs/GD-lineage HPAIV in parts of Asia and also in Egypt [87, 175].

Historically, some domestic waterfowl production systems have had high infection rates for AIV resulting from close interactions and cohabitation with free-living aquatic birds. For example, southern China has many duck ponds, and ducks are reared on lakes with intermingling of large numbers of domestic ducks and wild aquatic birds [24, 170]. This environment favors fecal–oral transmission through ingestion of virus-contaminated water by outdoor-reared poultry in the region, especially domestic waterfowl [169]. In the late 1970s and early 1980s, AIVs were detected in the markets of Hong Kong, primarily in domestic ducks (6.5% of samples were AIV positive), and also in domestic geese (1.1% positive), but to a lesser extent in chickens (0.4% positive) and other gallinaceous birds [168, 169]. None of these viruses were HPAIVs. Similarly, in the USA, outdoor duck production systems on Long Island, New York during 1979–1980 had a 23% positive rate of AIV isolation in 2- to 5-week-old ducklings, but showed no signs of disease [156].

Transmission of AIVs within and between different enterprises or premises

When H5 Gs/GD-lineage HPAIVs emerged in multiple countries in Asia during 2003–2004, questions were raised about the relative importance of village poultry and commercial farms in the transmission and maintenance of these viruses. It was concluded that the highest risks arose from poultry production and marketing practices in which biosecurity measures were not commensurate with the threat of virus incursion [176]. Although in numerical terms more cases were detected in village poultry, on a "per-flock" basis there were fewer cases than in the commercial sector (even allowing for reporting bias). Many pathways exist for transmission in both directions. Of particular importance are links to poultry traders and LPMs, both of which are recognized as high-risk enterprises. LPMs have played an important role in dissemination of AIVs. In one example from the USA, the H7N2 LPAIVs that were endemic in LPMs of north-east USA from 1994 to 2006 were introduced into the index commercial farm and then disseminated to cause cases

in commercial poultry as follows: layers in Pennsylvania during 1996–1998, broiler breeders in Pennsylvania during 2001–2002, commercial poultry in Virginia, West Virginia, and North Carolina during 2002, a large layer company in Connecticut during 2003, a small layer farm in Rhode Island during 2003, and three broiler farms in Delmarva during 2004 [1, 44, 49, 165, 182, 193, 202, 238]. LPMs have also been implicated in infection of commercial poultry in Hong Kong SAR in 2002 [105]. The level of risk posed by LPMs depends on the management measures in place to prevent poultry in the market from becoming and remaining infected. In Hong Kong SAR, a series of measures have been introduced to LPMs, and these have markedly reduced the risk that these markets pose to poultry farms. However, these measures require very strict adherence to biosecurity and management protocols. If poultry in small-scale commercial and village flocks are infected, they can play a role in transmission to large-scale commercial farms, but this will depend on the direct and indirect links between the two types of production. Transmission of virus can also occur in the opposite direction, especially if poultry are sold through poorly managed LPMs or there are high concentrations of poultry rearing of different types in the same area, and infection gains entry to one or more farms.

The main high-risk activity for introduction of AIV to commercial poultry is direct contact with infected birds or indirect contact with contaminated fomites, such as transport cages, and shoes and clothing of employees. For example, the first case of H7N2 LPAI in Pennsylvania (in December 1996) occurred on the premises of an LPM dealer who had 50 birds in the facility at any time, and the dealer had made 405 pick-ups from neighboring farms during the previous 3 months [75]. The second case was in a commercial layer flock located within 1.5 miles of the first premises, and the third case was in a small layer farm. Four LPM dealers from Pennsylvania and New York had made multiple load-outs from each flock. Such risks can be mitigated by education of employees on the risks of AI introduction and prohibition of bringing other birds or equipment onto the farm, and of raising or having contact with village or LPM birds by employees. If commercial poultry are to be supplied to an LPM, extra biosecurity precautions must be taken in order to avoid introduction of pathogens into source flocks by only allowing clean and disinfected cages and vehicles on the farm, and requiring personnel to wear disposable outer clothing and clean boots. Other measures can be used to further reduce the risk, such as vaccination of all poultry destined for LPMs against the strain of virus of concern (e.g. H5N1 HPAIV in Hong Kong SAR), and very tight controls on sources of poultry.

Transport of AIVs via air or wind over short distances can occur between enterprises, especially wind dispersion of virus during depopulation activities such as dust generation during removal of non-composted contaminated litter or grinding of carcasses before composting [18, 130]. Water can also be a pathway for introduction of virus if it is contaminated by fecal waste from infected poultry, in particular by infected domestic ducks, or from infected wild birds. Fecal material and litter from farms and markets spread on fields can also play a role in virus transmission between the different types of production.

Practicing high-level biosecurity on farms will mitigate the risk of farm-to-farm spread, as was evident in the Shenandoah Valley of Virginia during 2002, where over 78% of poultry farms (667 farms) remained free of H7N2 LPAI [164, 166]. However, in areas of high farm density, local transmission can occur even if seemingly appropriate measures are taken. In several H5 HPAI outbreaks, virus was isolated from blow flies, house flies, black garbage flies, and small dung flies, which suggests that these insects could serve as mechanical vectors of AIV spread between farms [21, 157]. Mechanical transmission of AIVs can occur between farms by any animal that can walk, crawl, or fly, including insects, humans and other mammals, and wild birds, but confirmation of the exact routes and attribution are often difficult [21].

The frequency of AI infections within different man-made systems varies according to the structure of the poultry sector, including the density of poultry production in the area, the specific production sectors, the quality of biosecurity practices used, marketing and transport systems, and the infection status of the country. Among the 25–30 billion chickens that are raised annually within the integrated commercial poultry systems in developed countries, HPAI has been rare and LPAI has been sporadic. This demonstrates that the risk of virus incursion and infection can be managed. In

the USA, AIV infections have been more frequently reported in poultry within LPM systems [205, 216]. In the industrial sector of developed countries, LPAI outbreaks have been reported most frequently in turkeys, slightly less frequently in laying chickens, and even less frequently in other domesticated poultry [21, 107]. By contrast, various HPAIV and LPAIV infections have been commonly reported in both village poultry and commercial poultry in many developing countries. For example, H9N2 LPAIV became endemic in commercial chickens in countries of Asia and the Middle East during the mid to late 1990s [33, 34, 109, 111, 123, 128, 142]. Similarly, since 2003, H5 Gs/GD-lineage HPAI has become endemic in a number of Asian countries, being maintained in LPMs, and probably in some areas with high concentrations of poultry that are reared and sold using inappropriate biosecurity measures, and also in domestic ducks. When AIV infections have occurred in the industrial sector or live market system, they have spread rapidly throughout these systems from farm to farm when biosecurity practices have been inadequate, resulting in epidemics of HPAI or LPAI.

Outdoor rearing

The rearing of poultry outdoors or on range in areas with access to AIV-infected free-living birds is a risk factor for transmission from wild birds to agricultural systems. For example, in Minnesota between the 1970s and the 1990s, significant commercial turkey production was raised on range each year to provide extra birds for the Thanksgiving and Christmas holidays [205]. Such range rearing in the early autumn, during staging of wild ducks in lakes in Minnesota before migration south for the winter, exposed turkeys to migrating free-living waterfowl infected with LPAIVs, which resulted in infections [68]. The number of flocks with infected turkeys varied from year to year, with a minimum of two affected flocks (in 1983), and peaks of 141 (in 1978), 258 (in 1988), and 178 (in 1995) affected flocks [71]. In 1998, the Minnesota commercial turkey industry eliminated range rearing of turkeys, and as a result only 33 flocks were infected between 1996 and 2000, and most of these were infected with H1N1 swine influenza [72]. This outdoor access is especially prominent in production systems that supply the LPM systems. Typically, such poultry suppliers include small operations where birds are raised in backyards and in other outdoor settings, especially for domestic waterfowl [161, 212].

Prior to the development of the modern vertically integrated commercial poultry production and temperature-controlled supply chains for perishable products in the 1950s, most meat and egg type stock were raised outdoors and locally in backyard and hobby flocks or on small commercial farms with immediate slaughter and consumption [64]. Such small local outdoor production and slaughter operations still exist today as the LPM system in developed countries, and remain a major production system for poultry in much of the developing world. Outdoor-raised village and rural poultry and LPM systems have higher AIV infection rates than industrial poultry production systems in the developed world [171].

Species susceptibility

The LPAIVs from free-living aquatic birds are most closely host-adapted to domestic waterfowl, making them more likely hosts. In addition, some studies suggest that turkeys, pheasants, and Japanese quail are more susceptible to AIVs from free-living aquatic birds than are chickens [89, 141, 204]. The co-mingling of different species in the LPM systems, especially with domestic waterfowl, favors transmission of AIVs between species and initiation of infections [212]. Although chickens are generally resistant to direct transfer of AIVs from free-living waterfowl, after passage through an intermediate host (such as turkeys, Japanese quail, or pheasants) and subsequent adaptation to the chickens, AIVs can more readily infect chickens and be transmitted efficiently. Nevertheless, there are reports of wild-bird-derived AIV infecting chickens directly, including cases in Australia associated with H10-subtype viruses [222]. There has been one published field report of some strains of native chickens being resistant to H5N1 HPAIV based on the presence of the B21 haplotype [16]. However, experimental studies utilizing B21-haplotype white leghorn chickens did not prevent illness or death upon challenge with a low intranasal dose of Indonesian H5N1 HPAIV [90]. One experimental study has reported more severe disease in commercial white leghorn chickens than in broiler chickens following inoculation with H4N8 LPAIV [197].

Lack of movement controls and biosecurity

Historically, the lack of movement controls, including quarantines, and poor biosecurity were associated with the spread of HPAI in Europe between 1900 and 1930, and were responsible for the establishment of sustained endemic infections [191]. Similar conditions exist today in places where H5 Gs/GD-lineage viruses have become endemic [54]. Since the 1980s, surveys of poultry in LPMs in Hong Kong, New York, and some other large cities have identified infections by HPAIVs and LPAIVs [162, 169, 171, 212, 227]. In some cities these infections have been prevented and controlled by a combination of approaches, including appropriate biosecurity and hygiene measures, the implementation of movement management (including strict control on sources of poultry), species segregation, cleaning and disinfection protocols, market rest days, compulsory vaccination of all poultry destined for LPMs, and bans on overnight keeping of poultry. These measures have been applied for prevention of H5N1 HPAI in Hong Kong markets [173], and similar measures have eliminated the H7N2 LPAIV from LPMs of the north-eastern USA [213, 214]. In many countries, the sale of live poultry through LPMs is banned. Temporary and permanent market closures have been successful in controlling zoonotic infections with H7N9 LPAIV in China, at least temporarily. Introduction of measures such as market rest days and other hygiene measures, or shifts to centralized slaughtering, require cooperation from farmers and traders who in many cases do not see strong reasons for making changes to their existing practices.

Prevention of spread on commercial farms requires the implementation of high levels of biosecurity. High-risk activities must be identified, assessed, and mitigated or eliminated as necessary. For example, a high-risk activity that led to transmission of H7N2 LPAIV in Virginia in 2002 was the movement of dead infected birds from farms through a shared rendering system, with removal from the farm for burial without adequate sealing and decontamination of transport vehicles [18, 166]. Risks of this type should be controlled by proper biosecurity procedures and practices. In addition, to minimize the risk of introduction and dissemination of AIVs, 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 level of biosecurity. A number of case–control studies on H5N1 Gs/GD-lineage HPAI have also demonstrated the importance of well-managed biosecurity measures [105, 133]. High-level biosecurity measures only reduce the risks. Some farms that appeared to have appropriate measures in place have still become infected with influenza (and other) viruses. These include some outbreaks of H5 GS/GD-lineage HPAI in intensively reared poultry (especially turkeys) in a number of developed countries, including the USA in 2014–2015. Clearly, for virus to enter a farm, there has to be a breach of existing biosecurity measures, but even after thorough investigations the mode of entry into some farms could not be determined.

Modelling ecological factors

A number of research groups have developed models to identify ecological factors associated with outbreaks of H5N1 HPAI. These models have emphasized the importance of a number of known risk factors, including the role of free-ranging domestic ducks, links to LPMs, and the importance of enhanced farm biosecurity for disease prevention [43]. Some models have used molecular epidemiology techniques and demonstrated correlation and clustering of virus strains, indicating crossover of infection between wild and domestic poultry [61].

Epidemiological models seek to condense complex interactions into a more or less simplistic model. These models are further hampered by imperfect data with often limited and biased surveillance and disease investigation information, particularly in developing countries where disease reporting is incomplete. Models can quickly become dated as new measures such as changes to movement controls, market management, and farm biosecurity measures are implemented to prevent infection, thus requiring changes to the structure and parameters used to build the models. Models can be of value in refining disease control strategies [190], provided that they are verified under field conditions to ensure that their outputs match field experiences.

Other studies have used social network analysis to identify risk factors in market chains [59, 118]. In some cases these studies identified markets that

had already been identified by other means as being high risk for H5N1 HPAI persistence and transmission, and interventions were already being introduced to reduce the risks [177].

Epidemiological investigations

Whenever an outbreak of AI in poultry is suspected, it is essential to undertake a thorough investigation in order to obtain a specific diagnosis. It is also necessary to determine the likely source of the infection and possible onward spread by identifying locations likely to be at risk through tracing. Standard epidemiological data should be recorded, covering the location of the premises, the location of different age groups or batches, the size of the population at risk in each house, and the numbers and type of dead and sick birds. Timelines should be constructed highlighting key events on the farm prior to and during the outbreak. The exact location of outbreaks within houses should be recorded, including the point in the house where the outbreak started. In investigations of HPAI outbreaks (other than H5 Gs/GD-lineage viruses), serological investigation of other flocks on the same farm should be conducted to assess whether an LPAIV had infected other flocks on the premises before converting to an HPAIV.

In locations where multiple cases of AI have occurred, case–control and spatio-temporal studies have been conducted to determine the most likely risk factors for entry of the agent, and factors associated with transmission of the disease [74, 105, 120]. These studies are at times hampered by lack of information (e.g. reliance on the presence or absence of clinical signs, rather than tests to detect evidence of infection in vaccinated flocks). If genetic information about viruses is available using deep sequencing it may be possible to establish links between farms, as has been done with a major outbreak in the Netherlands [9, 237]. In many cases the exact route of entry of virus and/or the initial source of the virus cannot be determined despite detailed investigations. Molecular data can assist in this process, but this approach depends on detecting a representative number of infected premises. Ultimately a thorough examination of practices on the farm and related premises should yield equivalent information on routes of infection.

Conclusions

Over thousands of years, human activity has changed the natural ecosystems of birds through captivity, domestication, agriculture, and commerce, and these changes are continuing today. The man-made systems are very diverse, and include hobby, village, and rural poultry, fighting cocks, captive wild birds, outdoor-reared non-commercial and commercial poultry, and industrial indoor-reared poultry. The size of commercial farms has changed dramatically since the late 1800s, and the development of indoor commercial production has accelerated since the 1950s.

Similarly, LPAIVs have changed from being a diverse group of viruses circulating asymptomatically in certain free-living aquatic birds to also include a less diverse group of IAVs, which have arisen from reassortment or adaptation of whole viruses, causing endemic respiratory disease in horses, pigs, humans, and domestic poultry. In addition, the diverse LPAIVs of free-living aquatic birds have caused sporadic infections in a variety of wild and domestic mammals and poultry. HPAIVs are not maintained in a wild bird reservoir like LPAIVs, at least not for an extended period of time. HPAIVs have arisen by mutation of H5 and H7 LPAIVs, usually following uncontrolled circulation of the viruses in susceptible gallinaceous poultry. Historically, HPAIVs have not been very infectious for domestic or free-living waterfowl (geese and ducks), but over the past two decades, the H5 Gs/GD-lineage HPAIV that originated in southern China has adapted to both wild and domestic waterfowl, causing infection, morbidity, and death.

Transfer of LPAIVs from free-living aquatic birds may require a complex, multi-step process that includes exposure and adaptation of the viruses to a new host species. The emergence of novel strains of AIV, such as H10N8 viruses that were first detected in Jiangxi Province of China, provides an example of how this process can occur [115]. Such transmissibility involves exposure, host adaptation, and efficient virus replication in the host species, with easy transmission between individuals. In other cases, transfer of LPAIV from wild birds to domestic poultry occurs readily, as has been seen with multiple outbreaks in turkeys in the USA.

At high risk for introduction of AIVs from free-living aquatic birds are outdoor-reared domestic

poultry, especially domestic waterfowl (ducks and geese). Additional risk factors include intermixing of poultry species on the same premises, and lack of biosecurity and movement controls. There are six main pathways (one direct and five indirect) for introducing an AIV onto a premises:

1 direct contact with infected wild or domestic birds
2 exposure to contaminated agricultural equipment, vehicles, or materials
3 through human movement, with AIV present on shoes, clothing, hair, hands, or skin
4 contaminated water or feed
5 airborne contaminated dust or water droplets
6 (potentially) insects or rodents.

The importance of each of these pathways depends on the individual farm circumstances. An understanding of which of these pathways are involved in disease outbreaks, gained through well-structured epidemiological investigations, is crucial for developing appropriate disease control and prevention programs.

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13 Pathobiology of avian influenza in domestic ducks

Mary J. Pantin-Jackwood

Introduction

Domestic ducks are an important source of food and income in many parts of the world. The susceptibility of domestic ducks to avian influenza (AI) virus (AIV) infection and the presentation of disease can vary depending on many factors, including the species and age of the ducks, the virus strain, and management practices. Although wild waterfowl are the principal natural reservoirs of AIVs, domestic ducks also have an important role in AI epidemiology, serving as intermediaries in the transmission of viruses between wild waterfowl and other poultry species [46, 49, 67, 123]. Ducks that are naturally or experimentally infected with most AIVs, including highly pathogenic (HP) strains, only develop subclinical to mild disease. However, many Asian goose/Guangdong (Gs/GD)-lineage H5N1 HPAIVs have been shown to replicate systemically and produce severe disease and mortality in ducks (reviewed elsewhere [104]). More recent H5 HPAIVs, related to H5N1 Gs/GD-lineage HPAIVs, can also cause disease and in some cases mortality in ducks [63, 69].

Ducks are raised for meat, eggs, and down feathers, and are able to subsist and grow to maturity on relatively simple diets based on locally available feedstuffs [40]. Domestic ducks are members of the order Anseriformes, family Anatidae, and include two different species and many breeds, which fall within two major genetic classifications – common ducks and Muscovy ducks. Most domestic ducks are common ducks (*Anas platyrhynchos* var. *domestica*), and are believed to have originated from the mallard (*Anas platyrhynchos*). The mallard duck was domesticated in two independent events, first in South-East Asia and again in Europe during the Middle Ages [137]. Some of the better-known breeds of common ducks include the Pekin, Aylesbury, Rouen, Call, Indian Runner, Khaki Campbell, Cayuga, Alabio, Maya, and Tsaiya. Different breeds and varieties of common ducks can interbreed and produce fertile offspring [40]. Domestic ducks are the predominant poultry species in much of South Asia, where they are reared for both meat and eggs. Most production is of a semi-commercial type, but industrial production does exist as specialty meats, mostly in Europe and North America [137]. For centuries, ducks in the rice-producing areas of Asia have been managed under the traditional herding system, whereby native ducks are selected for generations for their ability to glean most of their food from harvested rice fields, levees, swamps, and waterways. Examples of breeds of ducks selected for herding are the Alabio and Bali of Indonesia and the native Maya in China. In addition to the Maya, there are a number of distinct lines or breeds in China, such as the Shao, Gaoyou, Jinding, Baisha, Yellow Colophony, and Pekin [40].

The Muscovy duck (*Cairina moschata*) is distinctly different genetically from the common ducks, and originated in South America [36]. From the Americas it was taken to Europe (primarily France) and to Africa by the Spanish and Portuguese explorers, but was also transported to Asia, where it adapted well to the hot climates [36]. Although Muscovy ducks can be crossed with common ducks, their offspring are sterile. These sterile hybrids are called mule (Muscovy male × common female) or hinny (common male × Muscovy female) ducks. In some

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cases, commercial breeders assign special names to hybrids. For example, one hybrid produced by crossing Muscovy males with Pekin females is called "Moulard." In Taiwan, the hybrid produced by crossing a White Muscovy male with a Kaiya (Pekin × Tsaiya) female is referred to simply as the "Mule Duck" [40]. The Muscovy duck has remained a minor poultry species, with the greatest commercial production located in France, Eastern European countries, and South-East Asia as a meat source. Production in other parts of the world is as village poultry and in sustenance farming for eggs and meat [137].

In most cases, domestic ducks infected with AIVs do not show clinical signs. However, many Gs/GD-lineage H5N1 HPAIVs, and recently H5 viruses derived from the H5N1, including the H5N8 HPAIV, have caused disease in ducks, consequently affecting duck production. Domestic ducks play an important role in the epidemiology of H5N1 HPAIVs. These viruses are highly lethal to chickens. However, in domestic ducks they can produce a range of clinical outcomes, ranging from asymptomatic infections to severe disease with mortality [12, 63, 80, 94, 103, 104, 109, 135, 145]. Both sick and asymptomatic infected ducks can shed large quantities of virus into the environment, resulting in an increased risk of transmission and potential outbreaks in commercial chickens. Infected migratory waterfowl contributed to the spread of H5N1 and H5N8 HPAIVs from Asia to other parts of the world [25, 66, 67]. However, the circulation of H5N1 HPAIVs in domestic duck populations is considered to be one of the major sources of infection, thus perpetuating the enzootic cycle of H5N1 HPAI in several countries in free-range farmed, as well as backyard or village-reared, poultry [46, 49, 67, 123]. The farming of domestic ducks in open fields, flooded rice paddies, or on ponds or other bodies of water allows direct exposure to wild waterfowl and to domestic ducks of many duck farmers, providing multiple mechanisms for the introduction or spread of virus between farms [10]. In addition to direct contact with infected birds, contamination of the environment with viruses shed by infected ducks plays an important role in the indirect transmission of AIVs to susceptible birds [77, 129, 130].

In order to better control AI, it is important to understand the pathobiology of AIVs in domestic ducks. Several reviews on the pathobiology of AIVs in poultry have been published [104, 138, 140, 142]. The following sections of this chapter will summarize the experimental studies that have examined AIV infection in ducks.

General concepts in avian influenza pathobiology

AIVs are influenza A viruses that infect a wide variety of domestic poultry, captive birds, and free-ranging wild bird species under natural and experimental conditions [142]. Wild aquatic birds, especially those belonging to the orders Anseriformes (ducks, geese, and swans) and Charadriiformes (shorebirds, gulls, terns, and auks), are the natural reservoirs of AIVs [128, 138, 156]. These AIVs are highly host-adapted, typically replicating in the epithelial cells of the gastrointestinal tract and producing subclinical infections in these species. Periodically, these AIVs are transmitted from wild aquatic to domestic birds (chickens and turkeys), producing subclinical infections, or occasionally respiratory disease and drops in egg production. This phenotype of virus is typically termed low-pathogenicity or low pathogenic AIV (LPAIV), and can be any combination of the 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes [141]. However, a few H5 and H7 LPAIVs, after circulating in domestic poultry, have mutated to produce high pathogenicity or highly pathogenic AIVs (HPAIVs). These HPAIVs cause severe systemic disease with lesions of necrosis and inflammation in the skin, viscera, and brain of gallinaceous poultry [142]. Historically, HPAIVs have not infected wild birds, with the exception of the die-off among common terns (*Sterna hirundo*) in South Africa in 1961 [11], but since 2002 the Gs/GD-lineage H5N1 HPAIVs have caused infections, illness, and death in a variety of captive, zoo, and wild birds, including waterfowl.

Several factors affect the complex biology of AIVs, including infectivity, host adaptation, virulence, and tissue tropism [104]. Although LP and HP pathotypes of AIVs are defined in the laboratory, natural infection by AIVs results in a wide range of clinical outcomes which are dependent on many factors, including virus strain, host species, host age, host gender, concurrent infections, acquired immunity, and environmental

factors [138]. Four potential clinical outcomes can follow AIV exposure – no infection, asymptomatic infection, mild disease, or severe disease with death [104]. Exposure, or access to the virus, is critical to beginning the process. However, with some AIVs and some hosts, exposure to virus may not result in infection, especially if the route of exposure is inappropriate, the viral dose is below the threshold required to initiate infection, immunity is present against the virus strain, or the virus strain is not adapted to the specific host species [104]. The mean infectious dose for selected AIVs determined in domestic poultry under experimental conditions has been shown to be both host and virus strain dependent, and could be considered one measure for assessing the infectivity and adaptability of the virus to a specific host (Table 13.1) [139].

Table 13.1 Variability in intranasal infectivity of several LPAIVs and HPAIVs for domestic ducks as mean bird infectious dose (BID_{50}) and lethal infectious dose (LID_{50}).

Virus strains	Subtype	BID_{50}[a]	LID_{50}	Reference
LPAIV				
A/chicken/ Alabama/1975	H4N8	3.3		[139]
A/mallard/ Ohio/338/1986	H4N8	3.1		[139]
A/mallard/ Ohio/184/1986	H5N1	1.9		[139]
A/mute swan/MI/06	H5N1	4.2		[110]
A/parrot/CA/04	H5N2	3.7		[110]
A/mallard/MN/00	H5N5	2.3		[110]
HPAIV				
A/mallard/ Korea/W452/14	H5N8		≥6	[69]
A/environmental/ Korea/W149/06	H5N1		2.5	[69]
A/mallard/ Korea/W401/11	H5N1		5.3	[69]
A/environmental/ Korea/W149/06	H5N1		<2.0	[74]
A/mallard/Korea/ W401/11	H5N1	<1	5.3	[74]
A/turkey/ Turkey/1/05	H5N1	≤4.2	3	[4]
A/ostrich/Italy/984/00	H7N1		>6	[4]

[a]Expressed in $\log_{10}$ mean chicken embryo infectious doses (EID_{50}).

The pathogenesis of AIV infections in ducks is dependent on the virus strain. The natural occurring LP endemic viruses are typically enterotropic, with shedding primarily through feces [45]. However, when waterfowl viruses jump to and become adapted in gallinaceous species, the virus typically changes to become more respiratory-tropic, with much smaller amounts of detectable virus in feces. When these "chicken"-adapted viruses infect ducks, the virus typically retains the respiratory-tropic replication pattern [100, 125].

Genetic determinants of virulence

Virulence or pathogenicity is the ability of a virus to cause disease, and is determined by the capacity of the virus to grow, be invasive, infect susceptible cells, evade the immune system, and cause cellular damage [152]. These capacities are encoded in the viral genome by individual virulence genes, and the specific property associated with virulence is termed virulence determinant. The pathogenicity of AIVs is a polygenic trait that affects host and tissue tropism, replication efficacy, and immune evasion mechanisms, among others. The HA is the major determinant of virulence, but maximum expression of virulence requires an optimal combination of internal genes [14]. In order to initiate the infection process in birds, the HA must first bind to α2,3-galactose-linked cell receptors to initiate receptor-mediated endocytosis. This is a poorly understood phenomenon, but it has an impact on both host specificity and cell or tissue tropism [138]. In addition, fusion of the viral envelope with the endosome wall requires the cleavage of the HA protein into the HA1 and HA2 proteins, which is essential for the virus to be infectious and produce multiple replication cycles. LPAIVs are released from the host cell with an uncleaved HA protein, and are not infectious. The protein can be cleaved by trypsin-like proteases found in restricted anatomical sites, such as respiratory and intestinal epithelial cells, which accounts for the restricted replication and lower virulence. The difference between the cleavage site of LPAIVs and HPAIVs is the number of basic amino acids in the HA1 near the cleavage site, or an insertion of amino acids near the cleavage site that determines whether trypsin-like proteases or furin-like proteases can cleave the protein. The LPAIVs 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 HPAIVs have either multiple basic amino acids or an insertion of amino acids at the carboxy-terminal of the HA1 protein, which allows proteolytic cleavage by ubiquitous furin proteases that are present in many cells throughout the body [133]. This increases the cell tropism of the virus, leading to virus replication in numerous visceral organs, the nervous system, and the cardiovascular system, resulting in systemic disease with high mortality. An additional factor, namely the presence or absence of a glycosylation site at the amino-terminal end of the HA1 protein, has been shown to influence HA cleavage. Changes in glycosylation sites of the NA also play a role in the pathogenicity of HPAIVs in chickens [55], as do deletions in the NA stalk region, which are considered to be a characteristic of wild-bird AIV adaptation to chickens [8, 23, 92].

Reverse genetic techniques and classical reassortment studies have shown that other genes and specific mutations are also important contributors to the pathogenicity of AIVs. Changes in the polymerase PB2 and PB1-F2, the nucleoprotein (NP), and the non-structural protein (NS) can experimentally alter the pathogenicity of HPAIVs in chickens [2, 26, 81, 143, 144, 171]. In addition to mutations in these genes, different combinations of the ribonucleoprotein (RNP) components (NP and polymerases PB1, PB2, and PA), which function as a unit, can effectively attenuate or increase virus virulence in chickens [55, 116, 144, 154, 155].

Molecular determinants of AIV pathogenesis in ducks are not well understood, and it is still not clear why HPAIVs do not induce the same disease in ducks as in chickens. The pathogenicity of the Gs/GD-lineage H5N1 HPAIVs is unique in that it can cause mild to severe disease in ducks. As in chickens, reverse genetics and studies with reassortant viruses have helped to identify the genetic factors that change a non-virulent H5N1 virus into a virulent one. This increase in virulence of H5N1 HPAIVs in ducks appears to be multigenic, and has been associated with changes in the HA, NS, NP, PA, PB1, and PB2 genes [51, 53, 61, 62, 88, 118, 122, 145]. For example, certain amino acid substitutions (Q/L at position 9) and deletions (at position 2) within the HA cleavage site appear to be important for H5N1 pathogenicity in ducks [145], as is a K deletion at position 2 [62]. However, the HA clade to which the virus belongs does not correlate with the virulence exhibited by the viruses in ducks, as representatives of virulent and avirulent H5N1 viruses can be found in most HA clades (Table 13.2) [99, 153]. Mutations in the PA (T515A) and PB1 (Y436H) genes abolish the pathogenicity of virulent viruses in ducks, but changes in the HA do not have an effect [53]. Other sites in the PA gene, such as PA 224P and PA 383D, are also associated with the high virulence of H5N1 HPAIVs in ducks [122], and three amino acid changes in PB1-F2 (T51M, V56A, and E87G) decrease the pathogenicity of an H5N1 HPAIV [88]. Moreover, the same gene also contributes to the efficient systemic replication of another H5N1 virus [118]. Multiple genes, including the PB2, PA, NP, HA, and NS genes, have been shown to be responsible for the efficient replication and full virulence of an H5N1 HPAIV in 2-week-old domestic ducks [61]. However, exchanging the NS gene from H5N1 HPAIVs of different pathogenicity did not alter the virulence of the viruses [117]. In a study in which the PA and HA genes were associated with efficient replication of a virulent H5N1 HPAIV in ducks by comparing the host immune response to different recombinant H5N1 viruses, it was found that the increase in virulence correlated with sustained and strong innate immune responses in ducks, especially in the brain [51]. In summary, the increase in virulence observed with some Gs/GD-lineage H5N1 HPAIVs appears to occur by many different mechanisms involving many different genes.

Immunology and immunopathology

The pathogenicity of AIVs is also affected by the host immune responses to the virus, which can alter the course of infection and the outcome of the disease. AIV infection in naive hosts triggers a cascade of host defenses that are responsible for control and clearance of the virus, and include innate and subsequent adaptive immune responses. Comprehensive reviews on duck immunology have been published [87, 119], although the role of host immune elements in the control of AIVs in ducks is still not well understood. Some studies have addressed innate immune gene expression in

Table 13.2 Mortality, mean death time (MDT), and viral replication titers from oropharyngeal and cloacal swabs of 2-week-old ducks inoculated intranasally with 10^6 EID_{50} of Gs/GD-lineage H5N1 HPAIVs. Data from previously published and unpublished experiments [78, 94, 103, 106, 107, 147, 153].

Virus[a]	Mortality[b]	MDT[c]	Virus isolation[d]	
			Oropharyngeal titers 3 DPI	Cloacaltiters 3 DPI
A/chicken/Egypt/3/2013	8/8	4.0	5.0	3.1
A/chicken/Egypt/102d/2010	4/8	6.0	4.0	1.9
A/chicken/Egypt/1063/2010	0/8	–	2.4	0.9
A/chicken/Egypt/08124S-NLQP/2008	8/8	4.1	5.5	1.5
A/chicken/Egypt/9402-CLEVB213/2007	3/8	7.0	4.0	1.5
A/duck/Vietnam/218/2005 (2.3.4)	8/8	2.7	6.5	3.3
A/duck/Vietnam/203/2005 (2.3.2)	8/8	3.4	4.8	1.5
A/Vietnam/1203/2004 (1)	7/8	4.2	4.9	2.0
A/goose/Vietnam/113/2001 (1)	0/8	–	1.8	<1.6
A/whooper swan/Mongolia/244/2005	7/8	4.3	NA[e]	NA
A/Vietnam/1203/2004	7/8	4.2	4.9	2.0
A/Thailand PB/6231/2004	3/8	6.3	3.7	1.3
A/crow/Thailand/2004	8/8	4.4	4.4	1.9
A/egret/HK/757.2/2002	7/8	4.1	5.8	2.3
A/chicken/Indonesia/7/2003	4/8	7.0	2.5	NA
A/chicken/Korea/ES/2003	2/8	4.0	1.62	1.55
A/goose/Hong Kong/739.2/2002	7/8	4.0	3.8	NA
A/Hong Kong/213/2003	0/8	–	2.7	NA
A/goose/Vietnam/113/2001	0/8	–	1.8	<1.6
A/chicken/Hong Kong/317.5/2001	0/8	–	1.92	2.45
A/duck/Anyang/ALV-1/2001	0/8	–	2.5	1.3
A/environment/HK/437-6/1999	0/8	–	2.05	2.57
A/chicken/Hong Kong/220/1997	0/8	–	1.97	1.22

[a]Ducks were inoculated intranasally with 10^6 EID_{50} of the viruses.
[b]Number of dead ducks/number of inoculated ducks.
[c]Mean death time in days.
[d]Mean $\log_{10}$ titers expressed as EID_{50}/mL from oropharyngeal and cloacal swabs were sampled from 3–8 individual ducks on the days indicated. The limit of detection was $10^{0.9}$ EID_{50}/mL.
[e]NA, not available.

duck-origin cells infected with AIVs [1, 9, 34, 37, 60, 71, 72, 82, 89, 124], and others have studied host gene expression in AIV-infected ducks [9, 21, 22, 33, 34, 43, 90, 101, 117, 150]. Few studies have specifically addressed humoral and cellular immunity against AIV in ducks, with most of the information obtained from pathogenesis and vaccination studies when looking for the production of antibodies against AIV after vaccination or virus challenge.

Comparative studies indicate that ducks and chickens respond differently to LPAIV and HPAIV infections [33, 34, 92]. When a virus enters the host, the innate immune system is activated, resulting in the induction of pro-inflammatory cytokines. The activation of the host's innate immune system by a virus is mediated by, among other factors, pathogen recognition receptors (PRRs), including Toll-like receptors (TLRs) and retinoic acid-inducible gene-I (RIG-I)-like receptors [30, 65]. Antigen-presenting cells produce high levels of interferon (IFN)-α and IFN-β in response to virus infection, due to activation of the PRRs such as TLR3, TLR7, RIG-I, and melanoma differentiation-associated gene 5 (MDA-5). Chickens, unlike ducks, lack RIG-I, but they do express MDA-5, which functionally compensates for the absence of RIG-I. Duck RIG-I is

the cytosolic recognition element for AIV recognition, whereas chicken cells sense AIV through MDA-5 [30].

In addition to differences in expression of PRRs between ducks and chickens, the cytokine and IFN responses upon activation of PRRs might differ, and could play a role in the outcome of infection with AIVs. A study of the expression of pro-inflammatory cytokines and PRR mRNA in duck and chicken tissues after infection with an LPAIV found a difference in correlation between MDA-5, TLR7, and IFN-α between chickens and ducks, which could determine the differences between the two species in the regulation of the IFN-α pathway in AIV infections [33]. Immunological responses were also examined in the lung, brain, and spleen of ducks and chickens after infection with an HPAIV [34]. This virus caused significant morbidity and mortality in chickens, whereas in ducks the infection was asymptomatic. The HPAI viral mRNA load was higher in the chicken tissues compared with the duck tissues. Excessive delayed cytokine inflammatory responses but inadequate cellular immune responses appeared to contribute to the pathogenesis in chickens, whereas ducks initiated a fast lower cytokine response followed by the activation of major PPR and a persistent cellular response [34]. The immunological process in which the HPAIV infection is cleared from the organs of the duck is complex, but it seems that ducks immediately initiate an IL-1β, Il-6, iNOS mRNA response, with a quickly induced apoptosis that could prevent the early replication of the virus, whereas later in the infection a robust cellular immune response helps to clear the infection [34, 71]. The inducible form of nitric oxide synthase (iNOS) expression levels observed in ducks during H5N1 HPAIV infection may also be important in the inflammatory response that contributes to the pathology [19].

The elevated pro-inflammatory response in chickens that appears to be a major pathogenicity factor in H5N1 HPAIV infection is mediated in part by the inhibition of STAT-3 [72]. H5N1 HPAIV-challenge studies in chickens and ducks found that STAT-3 expression was down-regulated in chickens but was up-regulated or unaffected in ducks *in vitro* and *in vivo* following H5N1 virus infection. In another study, H5N1 viruses caused fatal infections in chickens, as well as high viral loads and increased production of pro-inflammatory molecules, when compared with ducks [20]. Cytokines, including IL-6 and the acute phase protein serum amyloid A (SAA), were rapidly induced at 24 hours post infection with H5N1 in chickens. In contrast, low induction of these cytokines was observed in ducks, and only at later times during the infection period. These observations support the view that hypercytokinemia may contribute to pathogenesis in chickens, whereas the lower cytokine response in ducks may be a contributory factor in their apparent resistance to disease and their decreased mortality. Chickens are overwhelmed by intense viral replication and an associated cytokine influx following H5N1 HPAIV infection. Conversely, ducks survive and their cytokine response is relatively low, paralleling that of chickens infected with LPAIVs [20].

Other studies have compared the host immune responses of ducks infected with H5N1 HPAIVs of different pathogenicity [101]. Muscovy ducks died earlier than Pekin ducks, and presented with more severe neurological signs. However, Pekin ducks had significantly higher body temperatures and higher levels of nitric oxide in the blood at 2 days post inoculation than did Muscovy ducks, indicating possible differences in their innate immune responses [21, 22]. Comparison of the expression of innate immune-related genes in the spleens of the non-vaccinated infected ducks showed differences, including significantly higher levels of expression of RIG-I in Pekin ducks and of IL-6 in Muscovy ducks [21]. In another study it was found that the levels of expression of IL-6 and IL-8 in the brain of ducks following infection with a more virulent H5N1 virus were higher than those in ducks infected with the less virulent H5N1[159].

In order to characterize the duck immune response to LPAIVs and HPAIVs, innate immune genes expressed early in an infection were identified [150]. Major histocompatibility complex class I (MHC I), interferon-induced protein with tetratricopeptide repeats 5 (IFIT5), and 2′,5′-oligoadenylate synthetase-like gene (OASL) were all increased by more than 1000-fold in relative transcript abundance in duck lung with an H5N1 HPAIV. These genes were induced much less in lung or intestine following LPAIV infection. The expression of these genes after infection suggests that ducks initiate an immediate and robust

response to potentially lethal influenza strains, and a minimal response to a low pathogenic strain [150]. Several immune pathways were shown to be activated in response to LPAIV infection of ducks. Ducks elicited a unique innate immune response to different species-of-origin H7 LPAIV isolates. However, 12 identifiable genes and their associated cell signaling pathways (RIG-I, nucleotide oligomerization domain-NOD, TLR) were differentially expressed regardless of isolate origin, indicating that this core set of genes is critical for the duck immune response to AIV [90]. Genes that are responsive to influenza A viruses were also identified using the lung transcriptomes of control ducks and ducks that were infected with either an H5N1 HPAIV or an LPAIV. The duck's defense mechanisms against AIV infection have been optimized through the diversification of its β-defensin and butyrophilin-like repertoires [52].

There is less information on the acquired immune responses to AIV infection (cellular and humoral) in ducks. The T-cell response was stronger in ducks than in chickens following an H9N2 LPAIV infection, and distinctive kinetics of transcriptional levels of interleukins and interferons were found, with ducks showing more active and robust cellular immune responses than chickens [52]. Differences in the humoral immune response of ducks and chickens to infection with AIVs have also been reported [28]. Following homologous LPAIV reinfection, ducks were only partially protected against viral shedding in the lower intestine in conjunction with a moderate antibody response, whereas chickens were completely protected against viral shedding in the upper respiratory airways and developed a stronger antibody response. In contrast, heterologous reinfection was not followed by reduced viral excretion in the upper airways of chickens, whereas ducks were still partially protected from intestinal excretion of the virus, with no correlation with the antibody response [28]. Duck immunoglobulins have been shown to have a smaller version of IgY, lacking the Fc portion, which would affect immunoglobulin functions such as complement fixation, opsonization, and Fc-mediated macrophage clearance of viruses [87, 119]. It is unclear precisely how this affects the humoral immune response to AIV infection.

Influenza A in domestic ducks

Low-pathogenicity avian influenza (LPAI)

Ducks that have been experimentally infected with LPAIVs of different subtypes rarely show clinical signs [5–7, 15, 28, 32, 35, 39, 44, 45, 64, 92, 95, 96, 100, 110, 111, 125, 157, 160, 164]. When present, clinical signs have been found to consist of mild respiratory signs [95] or a decrease in body weight gain [38, 39]. The number of ducks shedding virus, the route of virus shedding (oropharyngeal versus cloacal), and the amount of virus shed vary depending on the strain and the origin of the virus (gallinaceous origin versus wild bird origin). In field conditions, co-infections of LPAIVs with bacteria are common, and are usually associated with production of more severe clinical disease, especially with lesions of airsacculitis, pneumonia, and sinusitis [7, 142].

Tracheitis, laryngitis, and bronchitis were the most common microscopic lesions observed in ducks experimentally infected with LPAIVs [45, 92]. Lesions in the trachea and bronchus were mild to severe, and consisted of congestion and edema, sloughing of epithelial cells, and increased amounts of mucin in the tracheal lumen. Also common were deciliation, hyperplasia, and metaplasia of the epithelium, epithelial degeneration, and lymphoplasmacytic infiltration. Lesions in the lungs, when present, were characterized by a lymphocytic, histiocytic, and heterophilic interstitial pneumonia [32, 39, 92]. No lesions were associated with infection of the intestinal tract and cloacal bursa in LPAIV-infected ducks, despite the relative abundance of infected cells, particularly in the intestinal villous epithelium. No significant histopathological lesions were reported in other tissues [32]. Cells positive for AIV antigen were confined to the trachea, lungs, air sacs, intestinal tract, and cloacal bursa of infected birds [32, 39, 45, 110, 111, 121].

In ducks inoculated by the intranasal route, virus can be detected in oropharyngeal (OP) and cloacal swabs from 1 day post inoculation (DPI). Virus titers peak at 2–3 DPI, and are low by 10 DPI, although virus can be detected in some ducks until 14 DPI [38]. Higher amounts of LPAIV are usually detected in cloacal swabs compared with OP swabs [15, 38, 39, 44, 45, 126, 127]. LPAIV has been isolated from

the trachea and intestines, but not from the brain, kidney, liver, or spleen [164].

High-pathogenicity avian influenza (HPAI)

Mortality caused by HPAIVs in domestic ducks had been infrequently reported before the H5N1 HPAI outbreaks in Asia. Death in ducks occurred as a result of experimental infection with an H7N1 HPAIV [5], and neurological signs and deaths were reported in Muscovy ducks following infection with another H7N1 HPAIV in Italy [24]. In most experimental studies, ducks that have been intranasally inoculated with HPAIVs have not shown clinical signs [6, 120, 160, 164, 165]. However, virus has been isolated from tracheal and cloacal swabs [160], and has been recovered from trachea, gut, liver, brain, and spleen [164]. With regard to microscopic lesions in tissues, mild pneumonia characterized by infiltrates of lymphocytes and macrophages has been reported, as well as air sacs with edema and similar cellular infiltrations. Intermittent antigen staining was found in epithelial cells lining the airways, but there was no staining in the lung [32].

Pathobiology of the Gs/GD-lineage H5N1 HPAIVs

The Gs/GD-lineage H5N1 HPAIVs developed the unique capacity among HPAIVs to infect and cause disease in domestic ducks and wild birds, producing a range of syndromes including asymptomatic respiratory and digestive tract infections, systemic disease (limited to just a few critical organs, usually the brain, heart, adrenal glands, and pancreas), and severe systemic infection and death (as seen in gallinaceous poultry) [104]. The H5N1 HPAIVs continue to evolve, and antigenic and genetic divergent strains have emerged, many of them expressing distinct pathobiological features and increased virulence for ducks (Tables 13.2 and 13.3). The early Hong Kong isolates (1997–2000) replicated only in the respiratory tract of domestic ducks, and produced associated mild respiratory lesions and no morbidity or mortality [106]. Similarly, intranasal inoculation of domestic ducks with H5N1 HPAI strains isolated from ducks in China (1999–2002) produced respiratory and alimentary tract infection, but no illness or death [29]. However, in 2001, an H5N1 HPAIV isolated from frozen duck meat that had been imported from China into South Korea replicated and spread systemically in ducks, and was isolated and visualized in muscle, heart, and brain, but did not produce clinical signs or death [147]. Strains isolated from captive waterfowl in Hong Kong during 2002 produced high mortality in experimentally inoculated young domestic ducks, with systemic infection and high virus titers within the respiratory tract, heart, and brain, whereas other strains from South-East Asia produced low mortality with neurological lesions and disease [54, 78, 103, 135, 136]. Domestic ducks experimentally inoculated with three H5N1 HPAIV isolates from Thailand from 2004 induced 50–75% mortality, with neurological symptoms observed in most of the domestic ducks, but less severe disease observed in the cross-bred ducks [115]. Feather lesions have been reported in domestic ducks infected with an H5N1 HPAIV [166]. Many other H5N1 HPAIVs isolated in different countries have shown increased virulence in ducks, but many are still of low virulence, producing no clinical disease in ducks (Tables 13.2 and 13.3).

In addition to the virus strain, the susceptibility of domestic ducks to H5N1 HPAIV infections and the presentation of disease can vary depending on other factors, including the age and species of the ducks, and management practices [101, 103, 123]. Although some H5N1 viruses can cause severe disease and death similar to that observed in chicken infections, the underlying pathophysiological mechanisms are different, with the viruses primarily causing severe vascular damage in chickens, resulting in severe pulmonary edema, congestion, hemorrhage, and microthrombosis in capillaries [105], whereas in domestic ducks the virus replicates and causes damage to multiple organs and tissues, including the respiratory tract, pancreas, central nervous system, adrenal glands, and myocardium [98, 142]. The pathogenicity of H5N1 virus in ducks is directly correlated with the efficiency of virus replication [51, 103, 153]. Ducks infected with the more virulent H5N1 HPAIVs have higher viral loads in the brain, which cause severe neurological symptoms, including head twitching, ataxia, tremors, and torticollis, followed by death. Viral dissemination to the brain, leading to severe neurological dysfunction, may be a cause of the high virulence of H5N1 virus in ducks [12, 54, 61, 86], but lesions in other important organs,

Table 13.3 Morbidity, mortality, mean death time (MDT), and viral shedding in ducks inoculated with Gs/GD-lineage H5N1 HPAIVs.

Species/breed	Virus	Sick/dead/total	MDT	Virus detection (EID_{50}/ml)		Remarks and references
				Oropharyngeal	Cloacal	
A. platyrhynchos/ mallard	A/goose/Hong Kong/739.3/02	3/2/3		3/3 (5.7)	3/3 (2.2)	4 weeks old; $2 \times 10^{7.5}$ EID_{50} IT, IN, IO, cloaca [135]
A. platyrhynchos/ Pekin	A/chicken/Yamaguchi/7/04	0/0/3	–	5.5	NA[a]	5 weeks old; 10^8 EID_{50} IN; average titers from trachea (EID_{50}/g) [70]
	A/duck/Yokohama/03	2/0/3	–	4.7		
	HK/483/97	0/0/3	–	4.5		
	Tn/SA/6	0/0/3	–	–		
A. platyrhynchos/ Call ducks	A/chicken/Yamaguchi/7/04	4/0/9	–	NA	NA	2 weeks old; 10^7 EID_{50} IN [166]
A. platyrhynchos/ Pekin ducks	A/chicken/Suphanburi/1/04	8/5/8	6.0	2.2/3.5	3.2/3.6	4 weeks old; 10^6 EID_{50} IN; average titers at 4 DPI [115]
	A/quail/Angthong/71/04	8/5/8	6.0			
	A/duck/Angthong/72/04	4/4/8	6.0			
Cross-bred ducks	A/chicken/Suphanburi/1/04	0/4/8	6.3	NA	NA	4 weeks old; 10^6 EID_{50} IN [115]
	A/quail/Angthong/71/04	0/4/8	5.3			
	A/duck/Angthong/72/04	0/6/8	4.8			
A. platyrhynchos/ Pekin	A/Thailand /PB/6231/04	0/8	5.5	2.4	0.8	5 weeks old; 10^6 EID_{50} IN [99, 103]
	A/egret/HK/757.2/02	2/8		4.9	4.1	
A. platyrhynchos/ Pekin	A/duck/Nigeria/1071-28/07	2/2/16	6	NA	NA	4 weeks old; 10^7 EID_{50} oronasal [3]
A. platyrhynchos/ Pekin	A/duck/Nigeria/1071-28/07	1/1/16	6	NA	NA	24 weeks old; 10^7 EID_{50} oronasal [3]
Domestic ducks	A/environment./Korea/W149/06 (H5N1) (2.2)	100%	7	7/7 (5.3±0.3)	7/7 (2.3±0.3)	3 weeks old; $10^{4,6}$ EID_{50} oronasal; titers at 3 DPI [74]
	A/mallard/Korea/W401/11 (H5N1) (2.3.2)	60%		7/7 (3.3±0.5)	7/7 (1.3±0.3)	
A. platyrhynchos/ Pekin	A/duck/Sleman/BBVW-1003-34368/07 (2.1.1)	1/1/14	5	7/14	0/13/14	7 and 9 weeks old; $10^{6.7,7.7}$ EID_{50} IO, IN and oral; virus shedding at 2 DPI [162]
	A/ducks/Sleman/BBVW-598-32226-07 (2.1.3)	0/0/13		3/13		
A. platyrhynchos/ Beijing	A/goose/Guangdong/1/96 (0)	0/0/3	–	3/6 (2±0.6)	0/6	3 weeks old; 10^6 EID_{50} IN; virus titers at 3 DPI [169]
	A/duck/Guangdong/E35/12 (2.3.2.1)	1/0/3	–	2/6 (1.8±0.8)	0/6	
	A/chicken/Henan/B30/12 (7.2)	0/0/3	–	0/6	0/6	

(*continued*)

Table 13.3 (*Continued*)

Species/breed	Virus	Sick/dead/total	MDT	Virus detection (EID_{50}/ml)		Remarks and references
				Oropharyngeal	Cloacal	
A. platyrhynchos/ Pekin	A/duck/BBvW-1003-34368/07 (2.1.1)	1/0/10	–	2/10	0/10	4 weeks old; $10^{8.4}$ EID_{50} IN, IO, oral; positive at 2 DPI by VI [161]
A. platyrhynchos/ Pekin	A/chicken/Kr/Gimje/08 (2.3.2.1)	8/8/8	3	8/8 (5±0.1)	2.9±1.4	2 weeks old; $10^{6.5}$ EID_{50}; titers at 3 DPI $TCID_{50}$ [62]
	A/whooper swan/Mongolia1/09	8/8/8	5.3	8/8 (4±1.4)	1.7±1.2	
	A/whooper swan/Mongolia/7/10 (2.3.2.1)	8/4/8	5.3	8/8 (3.3±0.9)	1.2±1.9	
A. platyrhynchos/ Pekin	A/turkey/Turkey/1/05 (2.2)	4/4/5	4.5	4/5	–	3 weeks old; 10^{6} EID_{50} IO, IN; positive at 3 DPI [4]
C. moschata/ Muscovy	A/mute swan/France/070203/07 (2.2)	11/11/15	7 ap.	15/15	15/15	5.5–6.5 weeks old; 10^{6} EID_{50} oculonasal; number of birds shedding at 3.5 DPI [47]
	A/mute swan/France/006299/06/07 (2.2.1)	15/15/15	5 ap.			
A. platyrhynchos/ Pekin	A/turkey/Turkey/1/05 (2.2)	18/18/18	<7	18/18	–	8 weeks old; 10^{6} EID_{50} oculonasal [85]
A. platyrhynchos/ Pekin	A/turkey/Turkey/1/05 (2.2)	18/0/18	–	18/18	–	12 weeks old; 10^{6} EID_{50} oculonasal; number of birds shedding at 2 DPI [85]
A. platyrhynchos/ Pekin	A/turkey/Turkey/1/05 (2.2)	5/5/5	<5	5/5/5	–	4 weeks old; $10^{6.7}$ EID_{50} oculonasal [86]
A. platyrhynchos/ Pekin	A/chicken/Korea/IS/06	?/0/20	–	?/20 (2±0.7)	0/20	2 weeks old; $10^{6.5}$ EID_{50} IN; titers $TCID_{50}$ at 3 DPI [58]
A. platyrhynchos/ mallard	A/mallard/Huadong/Y/03	0/0/14	–	14/14 (1.9±0.2)	14/14(2.1±0.1)	6 weeks old; 10^{6} EID_{50} IN, IT, IO; titers at 2 DPI [145]
	A/mallard/Huadong/S/05	14/14/14	3 ap.	14/14 (3.6±0.7)	14/14(3.7±1.1)	
A. platyrhynchos/ Khaki Campbell	A/duck/Thailand/144/05	3/5/10	4 ap.	10/10		4 weeks old; 10^{6} EID_{50} IN; number of ducks shedding at 2 DPI [123]
	A/quail/Thailand/551/05	2/1/10	5 ap.	8/18		
	A/Thailand/MK2/04	2/2/10	5 ap.	9/10		
	A/duck/Thailand/71.1/04	10/10/10	2.1	10/10		
A. platyrhynchos/ Pekin	A/muscovy ducks/Vietnam/453/04 (1)	8/1/5/1/5	NA	NA		5 weeks old; $10^{7.2}$ EID_{50} oral-nasal-ocular [20]
	A/ducks/Indramayu/BBVW/109/06 (2.1.3)	0/0/15				
A. platyrhynchos/ Pekin	Chicken/Hong Kong/220/97	0/0/10	–	2/3(1.5)	0/3	4 weeks old; 10^{5} EID_{50} IN [101]
	A/egret/HK/757.2/02	10/6/10	6.5	3/3(3.2)	3/3(2.3)	
	Duck/Vietnam/218/05	10/10/10	4.4	3/3(3.4)	3/3(2.3)	

[a]NA, not available; IN, intranasal; IT, intratracheal; IO, intraocular.

including the heart, could lead to multi-organ failure and death [99]. However, differences in virus replication alone may not fully account for the differences in pathogenicity seen with H5N1 HPAIVs, and host innate immune gene expression probably plays a role [101].

Continued circulation of H5N1 HPAIVs in countries where the virus is endemic has driven changes in H5N1 HPAIVs that have translated into more pathogenic viruses. For example, in Egypt the HA clade 2.2 H5N1 HPAIVs have evolved such that some have become virulent to ducks [153]. This has also been observed with viruses from Vietnam [27, 107], but not with the HA clade 2.1 viruses circulating in Indonesia for many years, which remained of low virulence for ducks [162].

A comparison of studies that have examined the pathogenesis of H5N1 HPAIVs in domestic ducks can be found in Tables 13.2 and 13.3. Table 13.2 compares the pathogenicity of H5N1 HPAIVs in 2-week-old Pekin ducks under identical experimental conditions. Table 13.3 compares studies conducted by several other research groups. Challenge of ducks with these and other H5N1 viruses has also occurred in vaccination studies [10, 13, 21, 22, 31, 41, 68, 83, 91, 108, 112, 114, 131, 132, 146, 148, 149, 158, 168], which have been reviewed by Pantin-Jackwood and Suarez [102].

Clinical signs and gross lesions

The presentation of disease and associated lesions varies depending on the virulence of the H5N1 HPAI strain. With the less virulent strains, clinical signs are mild or absent. With the more virulent strains, commonly observed clinical signs include listlessness, anorexia, watery greenish diarrhea, conjunctivitis, corneal opacity, weight loss, increase in body temperature, and neurological signs (including whole-body tremors, uncontrollable shaking, marked loss of balance, tilted head, seizures, loss of vision, and paralysis) (Figures 13.1–13.3) [21, 22, 85, 86, 97, 103, 145, 153, 167]. Gross lesions in severely affected domestic ducks include dehydration, empty intestines, splenomegaly, thymus atrophy, dilated and flaccid heart with increased pericardial fluid, pinpoint necrosis of the pancreas, airsacculitis, and congested malacic brain. Decreased egg production has been reported in commercial ducks [113].

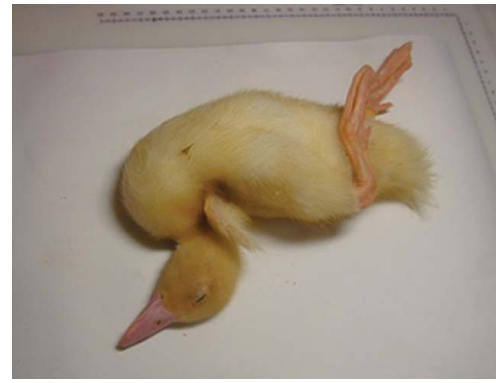

Figure 13.1 Two-week-old Pekin ducks showing severe neurological signs at 3 days after IN inoculation with A/egret/HK/757.2/02 H5N1 HPAIV. Reprinted with permission from *Avian Diseases*. Copyright held by the American Association of Avian Pathologists, Athens, Georgia, USA. Source: USDA – M. Pantin-Jackwood [103]. *See Plate section for color representation of this figure*.

Figure 13.2 Two-week-old Pekin ducks showing severe neurological signs at 3 days after IN inoculation with A/egret/HK/757.2/02 H5N1 HPAIV. Reprinted with permission from *Avian Diseases*. Copyright held by the American Association of Avian Pathologists, Athens, Georgia, USA. Source: USDA – M. Pantin-Jackwood [103]. *See Plate section for color representation of this figure*.

Microscopic lesions

Microscopic lesions commonly observed in ducks infected with virulent H5N1 HPAIVs include the following [58, 85, 86, 97, 101, 103, 123, 153, 162, 163]:

1 mild to moderate localized mononuclear cell submucosal inflammation of the respiratory tract, including turbinates, infraorbital sinuses, trachea, bronchi, air sacs, and lungs

Figure 13.3 Bile-stained loose droppings from a 2-week-old Pekin ducks at 3 days after IN inoculation with A/egret/HK/757.2/02. Reprinted with permission from *Avian Diseases*. Copyright held by the American Association of Avian Pathologists, Athens, Georgia, USA. Source: USDA – M. Pantin-Jackwood [103]. *See Plate section for color representation of this figure*.

2 randomly scattered foci of malacia with gliosis, mild lymphoplasmacytic perivascular cuffs, and mild perivascular edema in the brain
3 severe multifocal cellular swelling and necrosis of the pancreatic acinar epithelium
4 multifocal to confluent areas of vacuolar degeneration to necrosis of the adrenal corticotrophin and chromaffin cells and epithelia of the Harderian glands
5 degeneration to necrosis of individual myofibers in skeletal muscle and heart
6 moderate to severe proventriculitis with diffuse lymphoid infiltration
7 moderate to severe lymphoid depletion in the cloacal bursa and thymus
8 mild inflammatory changes in the lamina propria of the intestinal mucosa.

Viral antigen staining has been found to be present in multiple tissues of ducks infected with H5N1 HPAIVs, indicating systemic infection (Figures 13.4 to 13.15). Viral antigen has been observed in epithelial cells of the respiratory system (Figures 13.4 and 13.5), in the air sacs and infraorbital sinuses, with occasional small foci in the air capillary wall of lung (Figure 13.10), in the pancreatic acinar epithelium (Figure 13.13), in neurons and glial cells of the brain (Figure 13.7), in tracheal epithelium, air capillary epithelium, fragmented cardiac (Figure 13.6) and skeletal (Figure 13.9) myofibers, adrenal corticotrophic cells (Figure 13.8), Harderian gland epithelia (Figure 13.11), and tongue epithelia. In lymphoid organs, viral antigen has been identified in resident

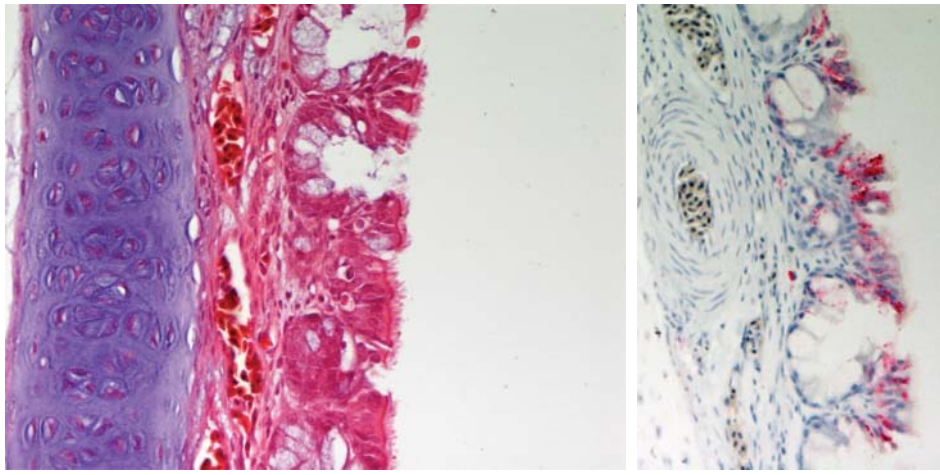

Figure 13.4 Moderate necrotizing rhinitis, with submucosal congestion and edema, and glandular hyperplasia of the nasal epithelium of a 2-week-old duck that died 3 days after IN inoculation with A/crow/Thailand/04 H5N1 HPAIV. HE. Inset. Demonstration of viral antigen in the epithelial cells (shown in red). Reprinted with permission from *Avian Diseases*. Copyright held by the American Association of Avian Pathologists, Athens, Georgia, USA. Source: USDA – M. Pantin-Jackwood [103]. *See Plate section for color representation of this figure*.

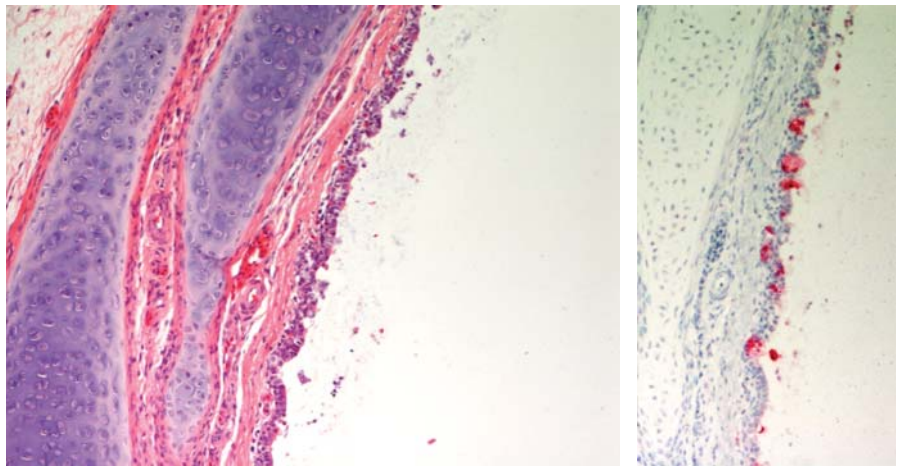

Figure 13.5 Degeneration and necrosis of the tracheal epithelium with mucocellular exudate containing sloughed epithelial cells of the trachea of a 2-week-old duck IN inoculated with A/crow/Thailand/04 and found dead at 4 days after inoculation. HE. Inset. AI viral antigen staining (shown in red) present in the epithelial cells. Reprinted with permission from *Avian Diseases*. Copyright held by the American Association of Avian Pathologists, Athens, Georgia, USA. Source: USDA – M. Pantin-Jackwood [103]. *See Plate section for color representation of this figure*.

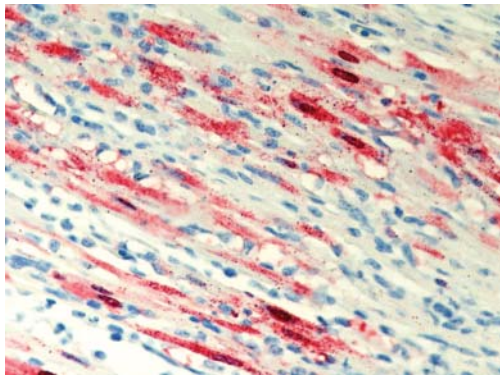

Figure 13.6 Extensive intranuclear and intracytoplasmic AI viral antigen (shown in red) in degenerated and necrotic myocytes of the heart of a 2-week-old duck IN inoculated with A/Thailand PB/6231/04 H5N1 HPAIV and found dead at 5 days after inoculation. Reprinted with permission from *Avian Diseases*. Copyright held by the American Association of Avian Pathologists, Athens, Georgia, USA. Source: USDA – M. Pantin-Jackwood [103]. *See Plate section for color representation of this figure*.

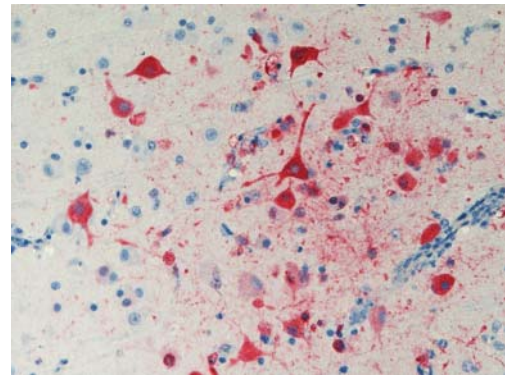

Figure 13.7 Strongly positive AI viral staining (shown in red) present in neurons of the cerebrum of a 2-week-old duck IN inoculated with A/Vietnam/1203/04 H5N1 HPAIV and found dead at 4 days after inoculation. Reprinted with permission from *Avian Diseases*. Copyright held by the American Association of Avian Pathologists, Athens, Georgia, USA. Source: USDA – M. Pantin-Jackwood [103]. *See Plate section for color representation of this figure*.

and infiltrating phagocytes (Figure 13.14), but not in apoptotic lymphocytes. Viral antigen has also been identified in the glandular epithelium of the proventriculus, in hepatocytes and Kupffer cells in the liver (Figure 13.12), in smooth muscle of the ventriculus, periosteum, bone marrow, and endosteum of the skull, autonomic ganglia of the enteric tract, corneal endothelial cells, and feather epidermal cells (Figure 13.15) [58, 85, 86, 97, 101, 103, 153, 162, 163, 167]. An important difference in pathogenesis compared with that in chickens is the lack of virus replication in endothelial cells and the absence of associated vascular damage [73, 101].

H5N1 HPAIV shedding occurs primarily by the oropharyngeal route [48, 54, 58, 99, 104, 135, 136]. The amount of virus shed and isolation of the virus

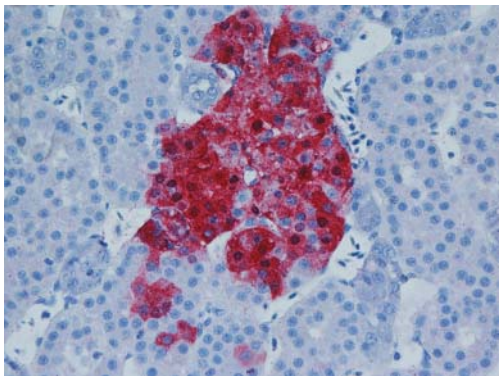

Figure 13.8 AI viral staining (shown in red) of the corticotrophic cells of the adrenal gland of a 2-week-old duck IN inoculated with A/Vietnam/218/05, 2 days after inoculation. Reprinted with permission from *Avian Diseases*. Copyright held by the American Association of Avian Pathologists, Athens, Georgia, USA. Source: USDA – M. Pantin-Jackwood [103]. *See Plate section for color representation of this figure*.

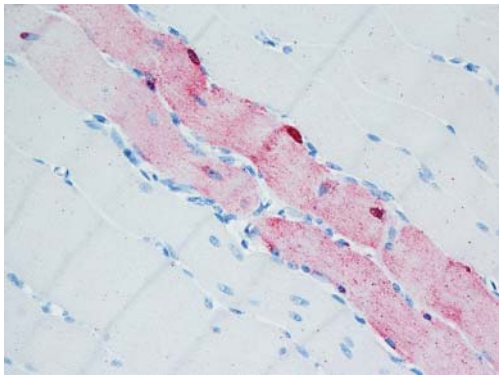

Figure 13.9 AI viral staining (shown in red) of the myocytes of skeletal muscle of a 2-week-old duck IN inoculated with A/crow/Thailand/04 and euthanized at 4 days after inoculation. Reprinted with permission from *Avian Diseases*. Copyright held by the American Association of Avian Pathologists, Athens, Georgia, USA. Source: USDA – M. Pantin-Jackwood [103]. *See Plate section for color representation of this figure*.

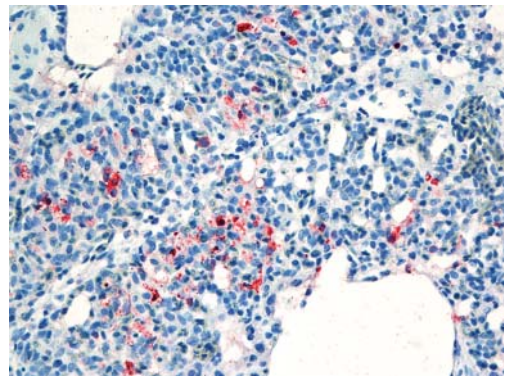

Figure 13.10 AI viral staining (shown in red) of phagocytic cells and air capillary epithelium of the lung of a 2-week-old duck IN inoculated with A/chicken/Egypt/08124S-NLQP/2008. *See Plate section for color representation of this figure*.

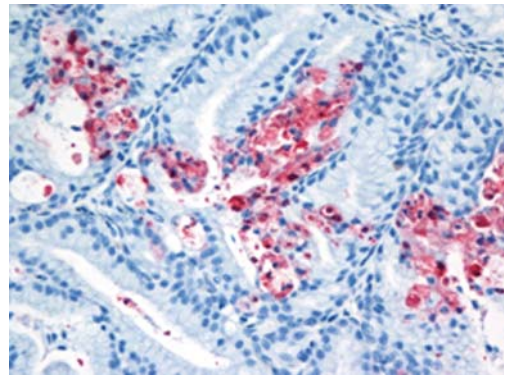

Figure 13.11 Vacuolar degeneration and AI viral staining (shown in red) of the Harderian gland epithelia of a 2-week-old duck IN infected with A/chicken/Egypt/08124S-NLQP/2008. *See Plate section for color representation of this figure*.

from internal tissues will vary depending on the virulence of the H5N1 HPAIV.

Effect of species on H5N1 HPAIV pathogenicity in ducks

Both in domestic ducks and in wild ducks, the species has been shown to affect the outcome of H5N1 HPAIV infection, with some duck species being more likely to show clinical signs and higher mortality [16, 21, 22, 42, 47, 66, 76, 86, 97, 109, 131, 132]. A comparison of three separate studies using either Pekin, Muscovy, or mallard ducks, all involving infection with the same H5N1 HPAIV, dose, and mode of inoculation, revealed differences in the initial appearance of clinical signs and time elapsed to reach 100% mortality [47, 66, 86]. Mallard and Muscovy ducks infected with different H5N1 HPAIVs showed clear differences in response to infection, with the Muscovy ducks exhibiting high mortality regardless of the virus administered, in contrast to the mortality in mallards, which ranged from 0 to 100%, suggesting that Muscovy ducks are more susceptible to H5N1

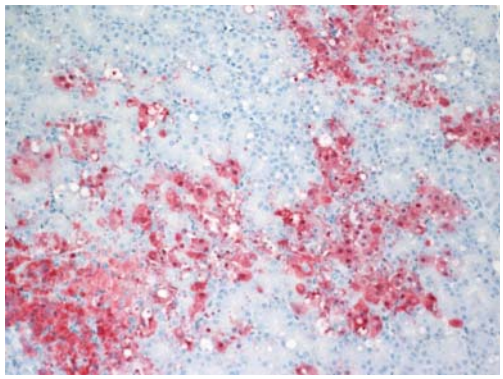

Figure 13.12 Severe multifocal cellular swelling and necrosis of the pancreatic acinar epithelium with viral staining (shown in red) of a 2-week-old duck IN infected with A/chicken/Egypt/08124S-NLQP/2008. *See Plate section for color representation of this figure.*

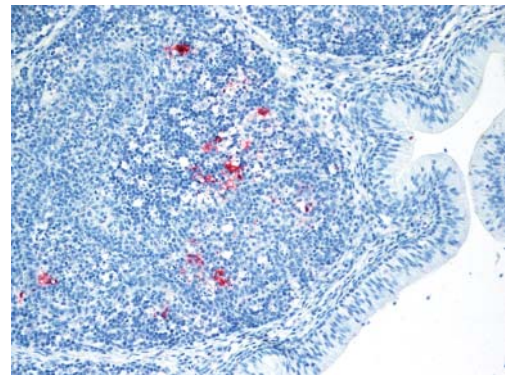

Figure 13.14 AI viral antigen (shown in red) in resident and infiltrating phagocytes in a bursa follicle of a 2-week-old duck IN infected with A/chicken/Egypt/08124S-NLQP/2008. *See Plate section for color representation of this figure.*

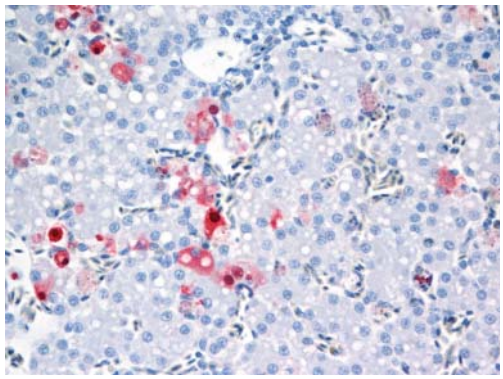

Figure 13.13 Viral staining (shown in red) in hepatocytes and Kupffer cells in the liver of a 2-week-old duck IN infected with A/chicken/Egypt/08124S-NLQP/2008. *See Plate section for color representation of this figure.*

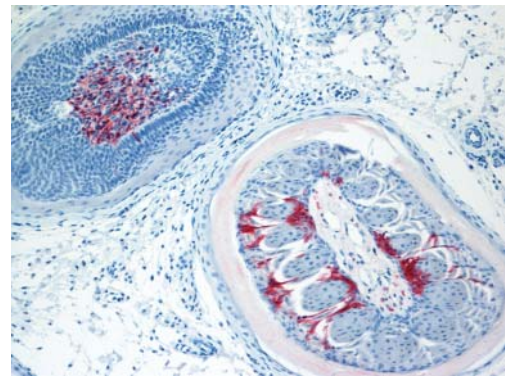

Figure 13.15 AI viral antigen (shown in red) in the epithelium and pulp of feathers of a 2-week-old duck IN infected with A/duck/Vietnam/218/2005. *See Plate section for color representation of this figure.*

HPAIV infection [109]. Similarly, differences in pathogenicity were found between Pekin and Muscovy ducks infected with the same clade H5N1 HPAIV, with only 20% of Pekin ducks exhibiting clinical signs, compared with 100% of the Muscovy ducks [131, 132].

In addition, clear differences in responses to vaccination were observed between Muscovy and Pekin ducks [21, 22]. Muscovy ducks developed lower viral antibody titers induced by the same vaccination as Pekin ducks, and presented with higher morbidity and mortality after challenge with an H5N1 HPAIV [21]. When comparing the response to infection in non-vaccinated ducks, differences were also observed, with infected Muscovy ducks showing a shorter mean time to death and more severe neurological signs than Pekin ducks [22]. However, Pekin ducks had significantly higher body temperatures and higher blood levels of nitric oxide at 2 DPI than Muscovy ducks, indicating possible differences in innate immune responses [21, 22].

In another study examining the pathogenicity of H5N1 HPAIVs in different species and breeds of domestic ducks with regard to the outcome of infection, it was shown that the pathogenicity of H5N1 HPAIVs varies between the two common farmed duck species, with Muscovy ducks (*Cairina moschata*) presenting with more severe

disease than various breeds of *Anas platyrhynchos* var. *domestica*, including Pekin, mallard-type, Black Runners, Rouen, and Khaki Campbell ducks [97]. The observed differences in pathogenicity between the two species can be explained in part by differences in the immune response between ducks. *Anas platyrhynchos* var. *domestica* ducks might be more efficient in controlling virus replication and spread after infection than Muscovy ducks, and consequently able to clear the virus and survive the infection, or at least to survive for longer [22].

Effect of age on H5N1 HPAIV pathogenicity in ducks

The age of the duck also affects the outcome of HPAIV infection and viral replication in tissues [3, 85, 99, 101, 103]. In 2- and 5-week-old Pekin ducks infected with three different H5N1 HPAIVs, viral-induced pathology ranged from no clinical signs to severe disease and mortality, with the 2-week-old ducks being more severely affected by the more virulent viruses [101]. These viruses induced higher body temperatures in the 5-week-old ducks than in the 2-week-old ducks, indicating possible differences in innate immune responses. IFN-α, RIG-I, IL-6, and IL-2 RNA levels were increased in spleens regardless of the virus given and the age of the ducks. However, differences in the levels of up-regulation were observed between the 2- and the 5-week-old ducks [101].

When young (meat-type) and older (breeder) ducks naturally infected with H5N1 HPAIV were compared, it was found that the meat-type ducks had a high mortality rate (30%) and exhibited severe neurological signs [113]. In contrast, HPAIV-infected breeder ducks showed minimal clinical signs but had a decreased egg production rate. Younger ducks had high viral titers in organs, high levels of viral shedding, and a high mortality rate after experimental HPAIV infection. Compared with the breeder ducks, the meat-type ducks were raised in smaller farms that had poor quarantine and breeding facilities. It is therefore possible that better biosecurity in the breeder farms could have reduced the infection dose and subsequently the severity of the disease. Thus management may be a factor contributing to HPAI susceptibility in ducks [113].

Effect of route of virus infection

Domestic ducks might become infected by different routes with H5N1 HPAIVs. In contrast to LPAIVs, H5N1 HPAIVs replicate preferentially in the respiratory tract of ducks, although they still replicate in the intestinal tract, and virus is excreted in both feces and respiratory or oral secretions [104]. Most studies that have examined the pathogenicity of H5N1 HPAIVs in ducks have used the intranasal (IN) route of inoculation [17, 21, 70, 76, 99, 101, 106, 107, 109, 115, 166]. However, other routes of exposure have been used to experimentally infect ducks. In an attempt to emulate natural exposure, ducks were infected by inoculating virus simultaneously via the cloaca, trachea, throat, nares, and eyes [54, 135, 136]. Simultaneous inoculation by the IN and intraocular (IO) routes, or by the IN, IO, and oral routes, has also been used to infect ducks with H5N1 HPAIV [12, 85, 86]. Infection with an H5N1 HPAIV caused morbidity and mortality in domestic ducks after ingestion of infected meat and inoculation by the intragastric and IN routes [75]. Ducks also became infected after ingestion of feathers with H5N1 HPAIV [166].

Mallard ducks were infected with LPAIVs by various routes of inoculation with very similar pattern of viral shedding [44]. Muscovy and Pekin ducks also became infected with two H5N1 HPAIVs of different virulence when given by any one of three routes – IN, intracloacal (IC), or IO [97]. Regardless of the route of inoculation, the outcome of infection was similar for each species, and depended on the virulence of the virus used. Infection with either virus was lethal for all Muscovy ducks, but only one of the viruses caused high mortality in Pekin ducks, again highlighting the clear differences in pathogenicity of H5N1 HPAIVs in these two duck species. Irrespective of the initial site of replication, the virus rapidly becomes systemic, and produces similar lesions and grows to similar high titers in tissues [97].

LPAIV transmission in aquatic bird populations is thought to occur through an indirect fecal–oral route involving contaminated water [50, 156, 157]. In experimental trials, it has been demonstrated that unlike that of wild-type LPAIVs, replication of the H5N1 HPAIVs is primarily associated with the respiratory tract. In ducks infected with H5N1 viruses, viral shedding by the oral route is more pronounced than that by the cloacal route, with

peak virus shedding occurring between 2 and 4 days after infection [99]. Ducks infected with non-lethal H5N1 HPAIVs can shed infectious virus for up to 10 days after infection, and in some cases for longer [161].

Infected ducks can contaminate the ponds, fields, or wetlands that they inhabit with H5N1 HPAIVs, which can survive in these environments for variable lengths of time [18, 93, 123]. Most domestic ducks are "dabblers", tending to feed superficially (by skimming the surface of water for feed), but they can also feed on and filter mud in shallow waterways [134]. Ducks in water also allegedly practice "cloacal sipping" (in which water is sucked into the cloaca), which could potentially enhance the spread of infection if the water is contaminated with virus [134]. The fate of respiratory-borne virus from ducks in water is not known. Since ducks are gregarious animals, the shift towards increased excretion of H5N1 HPAIV via the respiratory route could potentially facilitate duck-to-duck transmission when birds are in close contact [134]. However, studies of the rates of transmission between ducks of viruses that are excreted predominantly via the cloacal or oropharyngeal route have yet to be conducted. Improved knowledge of virus exposure would provide a better understanding of the pathogenesis of influenza viruses and thus enable optimization of poultry husbandry to prevent disease outbreaks.

New H5 reassortant viruses

Despite great efforts to control the spread of H5N1 HPAIVs, these viruses continue to survive and evolve in Asia, and this has led to the emergence of multiple genotypes or sub-lineages. The endemicity of H5N1 HPAIVs in Asia has also led to the generation of reassortant H5 strains with novel gene constellations. Recently, new subtypes of H5 HPAIVs (H5N8, H5N5, and H5N2) with the genetic backbone of clade 2.3.4.4 viruses have been detected in wild birds, ducks, geese, quail, and chickens [57, 84, 170]. Among the reassortant viruses, an H5N8 HPAIV was isolated from ducks in China during 2010, but it was not reported in other countries until January 2014 [170], when two types of H5N8 HPAIVs were isolated from a poultry farm in South Korea [80]. Clinical signs of HPAI, such as decreased egg production (60%) and slightly increased mortality rates, were detected in ducks on a breeder duck farm followed by reports of clinical signs of HPAI in broiler ducks [80]. In November and December 2014, HPAIVs of the H5 subtype originating from China were detected in poultry and wild birds in various countries of Asia and Europe, and for the first time in North America [56, 59, 79, 151]. From December 2012, H5N8, H5N2, and H5N1 viruses were detected in wild birds, backyard poultry, and commercial poultry operations in western North America. The H5N2 HPAIV continued to spread east, affecting commercial poultry operations in many states in the USA.

Experimental studies of the H5N8 virus showed that it replicated systemically and was lethal in chickens, but appeared to be attenuated, although efficiently transmitted, in ducks. Mild to severe clinical signs were observed in ducks that had been intranasally inoculated with H5N8 viruses, and mortality rates were in the range 0–20% [63, 69, 170]. Viral shedding and replication rates in tissues were high, and the duration of viral shedding was long [63]. The ability of these novel reassortant H5N8 viruses to replicate efficiently in the respiratory and intestinal tracts without killing the infected ducks enables them to circulate within the duck population, and increases the risk of transmission on poultry farms.

Conclusions

The increase in pathogenicity observed in ducks with certain H5N1 HPAIVs and the more recent H5 reassortants has implications for the control of AI. Ducks infected with more virulent strains shed more virus, thus perpetuating the virus in the environment and increasing the risk of transmission to susceptible birds and other mammalian species, including humans. On the other hand, ducks infected with less virulent HPAIVs do not show clinical signs, but can still spread virus. The cause of the increased pathogenicity of H5N1 HPAIVs in ducks is still unknown. Because of the many genetic differences observed between viruses it has been difficult to specifically determine the causative changes for the observed differences in pathogenicity. In addition, differences in response to infection

and vaccination observed between the two domestic duck species should be taken into account when developing effective vaccination programs for controlling HPAI in different species of ducks.

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14 Avian influenza control strategies

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Introduction

There is no single control or preventive strategy for avian influenza (AI) to fit every country, virus, and avian species [19, 70]. The strategies that have been developed and used have depended on a variety of factors, including the presence or absence of AI virus (AIV) in the country, the pathogenicity of the virus (low pathogenicity [LP] versus high pathogenicity [HP]), the hemagglutinin (HA) subtype of the AIV (H5 and H7 HA subtypes versus the other 14 HA subtypes found in birds), the species of birds at risk or infected (wild birds, captive birds, or domesticated species), the type of ecosystem (natural, zoological exhibits/nature preserves, household pets, or agricultural systems), the demonstration of regionalization or compartmentalization, the financial resources available (government versus private sectors), the veterinary medical infrastructure, political will and authority, the extent of infection, the importance of export markets for poultry products, and the desired goal or outcome. Strategies have been modified over time, building on experiences from earlier outbreaks [19].

In most developed countries over the last 125 years, HPAI has been handled by variations of stamping-out programs, using concepts originating with Giovanni Mario Lancisi in Italy during the early eighteenth century to control exotic livestock diseases such as Rinderpest in cattle. However, in the last 20 years, especially with H5 A/goose/Guangdong/1/1996 (Gs/GD-lineage) HPAI in a number of developing countries, the poultry industry and governments have not been able to achieve eradication through traditional stamping-out programs, but have instead managed HPAI by using combinations of stamping out, changes to farming and marketing practices, and measures that reduce shedding of virus, thereby reducing environmental contamination and spread. Stamping out is not the only method available for controlling AI, but in small outbreaks it remains the method of choice. It is the first line of attack in most national contingency plans [69]. There are some situations where the overall cost of alternative approaches is lower and, in some places, vaccination should be considered as an alternative or complementary approach to stamping out.

Until around 50 years ago, LPAI was not considered a significant health issue for poultry. This view changed in the 1960s, when clinical syndromes of respiratory disease and drops in egg production emerged, primarily in turkeys, but also in pheasants, quails, and partridges [16]. Moderate to severe disease associated with H9N2 LPAI in Asia and the Middle East from the 1990s also resulted in the implementation of control and preventive measures for this virus [55]. Globally, control programs for LPAI have been more diverse than those for HPAI, and have ranged from no action to vaccination to stamping-out strategies, usually coupled with improvements in farm biosecurity [67]. The emergence of zoonotic H7N9 LPAI viruses (LPAIVs) in China in 2013 has increased the pressure on veterinary authorities to control this and other potentially zoonotic LPAIVs.

Low-pathogenicity avian influenza (LPAI)

The existence of LPAIVs in a variety of wild aquatic birds, causing mostly asymptomatic infections and

Animal Influenza, Second Edition. Edited by David E. Swayne.

as part of ecosystems on all seven continents, suggests that the presence of LPAIVs is natural, has existed for eons, and is of minimal consequence in its natural setting. Humans have had and will continue to have minimal impact on control of LPAIVs in wild bird populations. Given the ubiquity of LPAIVs in wild birds, the primary focus for control of LPAI in poultry and other captive birds should be on preventing the introduction of wild-bird-origin AI viruses (AIVs) into these populations. In the USA, Canada, the European Union, Australia, and other developed countries, serological and virological active and passive surveillance programs in commercial poultry have demonstrated that most commercial farms are free of AI infections, presumably as a result of biosecurity measures introduced to prevent infection [21]. Nevertheless, occasional viral incursions to commercial farms do occur. The village/rural poultry sectors, and outdoor-reared specialty poultry, such as organically grown birds, are potentially at higher risk for introduction of LPAIVs from wild bird reservoirs because of the difficulties in preventing all contact with wild birds. Once poultry are infected, secondary spread and transmission can occur to other poultry, including the outdoor and indoor commercial systems and live poultry markets (LPMs). The LPAIVs are not maintained in wild gallinaceous species of birds [46, 61].

In the USA, control of LPAI is the responsibility of state governments and/or private industry, except for H5 and H7 LPAI, which is handled jointly by federal and state government authorities. Notably successful state LPAI control programs have been developed in Minnesota [22, 47], Pennsylvania [8], and the Delmarva region [15] through partnerships between the poultry industries and state governments. These programs have been used to eradicate a variety of LPAIVs on multiple occasions over the past 30 years [12, 23, 24, 53, 67]. The Minnesota plan, initially developed in the early 1980s, has been the model for many other state and national LPAI control plans, and has five specific components: education, preventing exposure, monitoring, reporting, and a "responsible response" [47]. Since 2000, Italy has developed and implemented successful control programs for H5 and H7 LPAI, including the use of emergency or prophylactic vaccination with inactivated AI vaccines containing a neuraminidase (NA) subtype different from the field virus (i.e. heterologous NA), monitoring for infection in vaccinated poultry by a range of methods that include NA differentiating serological tests (differentiating infected from vaccinated animals, DIVA) and use of sentinel birds, elimination of infected flocks by stamping out or controlled marketing, and strict restriction measures [9, 10, 41, 42]. Although these programs have been successful in containing the threat, the multiple incursions of both LPAIVs and HPAI viruses (HPAIVs) into commercial poultry farms demonstrate that biosecurity measures, as implemented, only reduce risks, especially in areas frequented by migratory birds.

In the past two decades, some H5 and H7 LPAIVs have mutated and become HPAIVs, whereas others have circulated in poultry as LPAIVs for a number of years (e.g. H7N9 in China since 2013). Predicting which LPAIVs will or are likely to mutate to HPAIVs has proved difficult even using laboratory models [64]. In the mid-1990s, molecular criteria based on the presence of multiple basic amino acids of the H5 and H7 HA proteolytic cleavage site were added to the OIE definition of HPAIVs irrespective of *in-vivo* lethality for chickens [43, 73]. However, because some H5 and H7 LPAIVs without these molecular criteria have mutated to HPAIVs, national and international guidelines for AI control have had to be updated to include not only HPAIVs, but also all H5 and H7 LPAIVs. The World Organisation for Animal Health (OIE) Code now includes all infections with H5 and H7 AIVs in its definition, for trade purposes, of AI [44]. This change has increased the use of stamping-out programs to deal with these two AI subtypes, even when only LPAIVs are present, as a means of preventing the emergence of HPAIVs. A side effect of these changes is that non-tariff trade restrictions have been placed on countries or parts of countries when only a single H5 or H7 LPAI outbreak or infected flock is detected.

High-pathogenicity avian influenza (HPAI)

HPAIVs are not believed to have arisen in wild birds, but there is evidence of at least short-term maintenance (up to 3 years) of Gs/GD-lineage H5 HPAIVs in wild birds (see Chapter 9). This is best illustrated by the role of wild birds in the

transmission of Gs/GD-lineage H5 viruses belonging to HA clade 2.3.4.4 to and within Europe and North America during 2014–2015 [26, 35]. These viruses almost certainly emerged initially in poultry in Eastern Asia, but once established in wild birds have persisted in this population over winter and into spring in 2014–2015. Persistence of virus in wild birds was seen between 2005 and 2008 with clade 2.2 H5N1 viruses that were first detected in Qinghai Province in western China and subsequently in places with no poultry, such as lakes in remote parts of Mongolia and Russia (see Chapter 9). Given the close interactions between poultry and wild birds in many parts of Asia, it has not been possible to establish in all cases whether virus has persisted in wild birds or if virus has been reintroduced from infected poultry. Nevertheless, it is now evident that wild birds play a significant role in long-distance transmission of Gs/GD-lineage H5 HPAIVs, providing further reasons for implementing appropriate measures to reduce the likelihood of direct and indirect contact between poultry and wild birds. In many production systems in Asia, such as free-grazing ducks and scavenging poultry, this is not possible without changes to production methods [49, 58]. Until the emergence of Gs/GD-lineage H5 HPAIVs, the accepted pathway consisted of H5 and H7 LPAIVs being introduced from wild aquatic birds into poultry flocks, and HPAIV strains arising through specific mutations in the HA gene following circulation for a period ranging from weeks to years [46].

Until the emergence of Gs/GD-lineage H5 viruses, in most HPAI epizootics, wild aquatic birds had not been infected by HPAIVs and thus were not biological vectors of HPAIVs. In some of these outbreaks, wild birds were believed to have been mechanical vectors, spreading the virus by carrying it on feet and feathers, but this has been dependent on the quality of biosecurity measures implemented to prevent wild bird access to premises. However, the inclusion of wild birds in stamping-out programs is not condoned, for ecological reasons, and such measures may in any case be counterproductive as they encourage wild birds to disperse, thus moving the virus to new premises and geographic regions.

Because some wild aquatic bird populations are reservoirs of LPAIVs, some individuals have the misconception that all species of wild birds are infected and are spreading HPAIVs, especially Gs/GD-lineage H5 HPAIVs. In areas where these viruses are present, the movement of infected poultry, contaminated products such as manure, and contaminated equipment and clothing and shoes remain the most likely means of viral spread between farms and between neighboring countries. Such movement of infected poultry or derived materials may be legal or illegal, including movement across porous borders between countries. In some outbreaks, aerogenous spread during depopulation or cleaning has been implicated in farm-to-farm transmission. Wild birds should only be implicated when all other potential pathways of introduction have been fully explored and eliminated (or considered unlikely), and there is reasonable evidence to support their involvement. Sometimes the introduction of a virus to an area, and the subsequent movement of that virus to farms, occur by different means. The former may be related to movement of wild birds, whereas the latter may be due to fomite or possibly aerogenous transfer. Outbreaks of H5N2 HPAI in North America in 2014–2015 have raised many questions about the mode(s) of introduction of virus to large-scale poultry farms with sophisticated biosecurity measures in place, and it is likely that multiple mechanisms are involved. The recommendations and responsibilities for containing HPAI outbreaks have been described previously [20], but have been modified over time to take into account experiences in places where virus remains entrenched, and developments in the use of vaccines [10].

From 1959 to 1992, most developed countries eradicated HPAI epizootics or outbreaks within a few weeks to a year by traditional stamping-out programs. However, since 1992, some developing countries have been unable to achieve immediate eradication through traditional stamping-out programs. The major reasons for this include the difficulties in detecting and eliminating all infected poultry, especially in countries with a large duck population and large volumes of sale of poultry through LPMs. In both of these populations, infection can go undetected unless intense active surveillance is undertaken, beyond the capacity of veterinary services [58]. Difficulties in elimination were compounded by the late detection of virus introduction in some countries, which allowed the

virus to become widespread before concerted action was taken. However, although the initial incursions of Gs/GD-lineage H5 HPAIVs were not detected in Thailand (in 2003) and Nigeria (in 2006), allowing the virus to become widespread, control programs were eventually successful, which suggests that other factors are also important for persistence of virus. The main factor appears to be the structure of the poultry sector. Locations where Gs/GD-lineage H5 viruses remain entrenched share a number of features, including high concentrations of poultry reared under different (and often inappropriate) biosecurity systems, sales of poultry through poorly regulated LPMs, and in some cases the presence of a large population of ducks reared outdoors. This is compounded by limited financial resources, one consequence of which is that veterinary services are still undergoing development. This creates difficulties in developing and enforcing movement controls and identifying all foci of infection [19]. In such outbreaks, management of the disease to a low infection rate has been the only realistic short- to medium-term option. The long-term goal is still virus elimination, but this will not be feasible until appropriate systems are in place to achieve this goal, and the required changes to marketing and production systems have been or can be implemented. For the five countries/regions where Gs/GD-lineage H5 HPAIV remains endemic (China, Vietnam, Indonesia, Egypt, and the area around Bangladesh), the prospects of virus elimination by 2025 remain extremely remote.

Goals of avian influenza control, and components of a control strategy

There are three different goals or outcomes in the control of AI, namely prevention, management, and elimination or eradication [66, 67]. The goal that is to be pursued will depend on the infection status of the country, zone, or compartment (CZC). If the CZC is free from AI, the goal should be to prevent introduction either from wild birds or from infected poultry (LPAIV or HPAIV) within a neighboring affected CZC. If the CZC has infected flocks, the goal could be to manage the disease to reduce viral loads or economic losses, followed by eventual eradication or immediate eradication through stamping out if this is feasible. The main goal of programs designed to manage the disease is to decrease the quantity of circulating virus. This will usually reduce the clinical manifestations of the disease and the negative economic consequences of infection by allowing production to continue. At the same time it reduces the zoonotic risk and the potential for emergence of a human pandemic strain of virus.

The dilemma that arises when managing HPAI is that the standard method of dealing with known infected flocks is depopulation, but in places where the vast majority of infected flocks go undetected (due either to non-reporting or to subclinical infection, as can occur in domestic ducks), the end result of responding only to cases that are identified is "disease harvesting" [58]. This approach has little effect on long-term control of the disease, especially if the conditions that allow infection to occur are not modified. In a number of developing countries where Gs/GD-lineage H5 HPAIVs are entrenched, surveillance programs have been conducted to determine whether and where virus is circulating. These programs result in testing of only a small subset of all poultry farms. However, if one of the farms that is sampled returns a positive test result, that farm will probably be depopulated and quarantined. This response acts as a disincentive for involvement in surveillance programs, and also inhibits efforts to understand the epidemiology of the disease.

Virus elimination is usually the ultimate goal of any AI control plan, but this may not result in eradication of that particular strain of virus, especially if it is present in multiple locations or countries. In addition, virus elimination may not be feasible, either due to the costs involved or because the risk of reinfection is high. Decisions taken by managers of individual farms or compartments may also be different to those taken by countries. For example, well-managed commercial farms or compartments may choose to eliminate a particular AIV and maintain freedom from infection, whereas the country may choose not to embark on country-wide elimination because of the costs involved and the limited likelihood of success across the entire country. This is the case with H9N2 LPAI and Gs/GD-lineage H5 HPAI in parts of Asia.

These goals or outcomes are achieved based on comprehensive strategies developed using

a combination of five specific components or features [66, 67]:

1. education, communication, public awareness, and behavioral change (i.e. improving knowledge of how AI is transmitted, making individuals aware of their role in prevention, management, or eradication, and promoting appropriate behavioral change)
2. changes to production and marketing systems that result in enhancement of biosecurity (i.e. facilities, management practices, and procedures that prevent virus from being introduced or, if virus is already present, from leaving a premises)
3. diagnostics and surveillance (i.e. the ability to detect the virus or evidence of infections in bird populations or their environment, or a means of verifying "freedom" from such infections)
4. elimination of infected poultry (i.e. removal of the infection source or susceptible sources in order to prevent continued environmental contamination and dissemination)
5. decreasing host susceptibility and reducing viral shedding (i.e. increasing host resistance, usually via vaccination, so that if exposure occurs, infection is prevented or the negative consequences of infection are minimal).

The overall effectiveness of the comprehensive strategy in controlling AI will depend upon how well the appropriate components are used and how thoroughly they are practiced in the field. The goals for individual LPAI and HPAI control strategies may differ depending on the country, the subtype of the virus, the economic situation, and the risk to public health. The following sections will focus on components of control programs relating to agricultural and allied industry production systems.

Education, communication, public awareness, and behavioral change

One critical aspect of control is the education of all personnel working in the poultry and allied industries, government personnel, and others involved in the control process with regard to how AIVs are introduced, how they are spread, and how such events can be prevented. The control of risky behaviors and actions greatly reduces the spread of AIVs by controlling fomite and aerosol dispersion of virus, thus preventing AIV movement onto the farm and between farms. This also applies to village-level producers, transporters, and market stall managers. The general public should be included in the education process by communicating information on risks and dispelling incorrect information and rumors, especially concerning the safety of properly prepared poultry products. Of particular importance is the training of producers and on-farm personnel in biosecurity measures to prevent introduction of AIV to a premises, and how to prevent spread once AIV has been introduced.

One important conclusion from 10 years of intervention on HPAI in locations where Gs/GD-lineage H5 viruses remain entrenched is that, using standard communication and extension methods, it is relatively easy to improve knowledge about AI, but much harder to change long-standing behaviors [45]. Behavioral change requires a thorough understanding of the drivers of existing practices, as well as consideration of the costs of and constraints on implementing new measures. One example was the recommendation that village-level households should confine their poultry to help to control Gs/GD-lineage H5 HPAI. Uptake of this measure was very low, because the shift from scavenging to confinement requires confined poultry to be fed. This in turn usually requires the purchase of feed, which cash-poor families could not afford. In villages in Cambodia, alternative strategies based on housing of young chicks in cages for the first month of life, which increased chick survival, proved to be more successful because the costs were low and the return on the investment was high (through increased survival of chicks). This program was coupled with temporary confinement of poultry in any village once one or more households had identified an increase in mortality in their birds. This was achieved using upturned cane baskets and a small supply of feed, sufficient for 1–2 weeks, until it was evident that the disease was not spreading and appropriate measures were applied in the affected household(s). Poultry were then allowed to return to scavenging. Through extension programs, villagers learned to stop allowing traders to enter their premises. Instead birds for sale were brought to the trader at the household entrance [75].

Poverty is a major driver of behavior, as are past experiences, such as preparation of sick or dead chickens with no apparent adverse consequences, which was common in a number of

Asian countries. Changing these behaviors through increasing awareness of the risks was not always successful, but was possible when poultry survival was increased, thereby removing the need to prepare sick and dead birds for food. Novel programs for providing information about AI, such as school-based activities, have proved to be valuable in Vietnam [76].

Changes to production and marketing systems resulting in improved biosecurity

Measures adopted in order to reduce the likelihood of introduction of pathogens to farms or markets and their onward transmission fall under the broad term "biosecurity." Biosecurity is the first line of defense against both LPAI and HPAI, and involves the use of appropriate facilities and management practices to prevent or reduce AIV spread by preventing contamination, controlling the movement of birds or their products, people, and equipment, or reducing the amount of virus (e.g. by cleaning and disinfection) [22, 70]. Conceptually, biosecurity falls into two broad categories [66, 67]. Inclusion biosecurity, or biocontainment, uses measures such as quarantine that are designed to keep the AIV on an affected premises or in an affected CZC. Exclusion biosecurity, or bioexclusion, is practiced to keep the AIV out of an AI-free premises or CZC. Many guides on poultry farm and market biosecurity have been produced [13, 57]. The highest-risk source of AIV for naive birds is direct exposure to infected birds, which shed high levels of virus from the respiratory and/or alimentary systems into their immediate environments. Typically, transmission occurs either when naive birds come into close direct contact with infected birds, or indirectly when they are exposed to contaminated materials from the environment of infected birds, especially poultry manure or equipment contaminated by poultry manure. Exposure and infection usually result from inhalation, contact with mucous membranes, or ingestion of AIV-contaminated dust, water droplets, or other forms of contaminated materials [70]. Cleaning and disinfection of equipment, and of clothing and footwear of personnel, are critically important for prevention of the introduction and farm-to-farm spread of AIV. Adoption of appropriate biosecurity measures by farmers, workers, and market stallholders depends on appropriate awareness campaigns and training as well as an understanding of behaviour. Effective management of movement of poultry is also part of this process.

In order to prevent primary introduction of AIVs from wild aquatic birds, poultry should be raised in confinement or, if raised with outdoor access, confined or separated during specific periods that correspond to the migration periods of potentially infected wild aquatic birds. Guidance has been prepared for outdoor flock owners, providing advice on avoiding stresses associated with confinement for free-ranging poultry [13]. From the late 1970s to the mid-1990s, some commercial turkeys in Minnesota were reared outdoors on range and experienced outbreaks of LPAI following exposure to infected wild ducks, but moving commercial turkey production indoors in late 1990s has almost eliminated LPAIV infections in the Minnesota turkey industry [25]. This change was not sufficient to prevent the incursion of H5N2 HPAIV onto a significant percentage of farms in this area in 2015, requiring reassessment of ways to minimize the risk of future outbreaks through improvements in biosecurity practices. In some countries, production and marketing systems with low-level biosecurity, including free-grazing ducks, selling poultry through traders, and associated LPM systems, have become an important entry point for AIVs into agricultural systems, and have served as the major reservoir for AIVs in the agricultural systems of many developed countries [34, 60, 62]. If biosecurity is lax, if farms are interconnected or located close together, and if the poultry density is high, AI viruses can spread through the commercial industry and rapidly move within the integrated commercial system, resulting in epidemics of HPAI or LPAI.

In many developing countries the only way to improve biosecurity is to modify the way that poultry are reared, transported, and sold. This is particularly applicable where poultry is sold through poorly managed LPMs, or where traders keep birds temporarily from different sources. Mixing of species can also occur in these places, and this too can result in virus transmission. Unless these practices are eliminated by a move towards centralized slaughtering, or modified so that there

are strict controls on sources of poultry and limits on the duration of stay for poultry in markets, there is a high likelihood of persistent AIV infection of poultry in these markets.

The previous edition of this book contains more detailed information about biosecurity [11], farm biosecurity audits [54], and methods of inactivation of AIV [5].

Diagnostics and surveillance

Early and successful control of AI requires an accurate and rapid diagnosis [70]. In new outbreaks the speed with which AI is controlled and the cost of such control are largely dependent on how quickly the first case or cases are diagnosed, the level of biosecurity practiced in the area, and how quickly control strategies can be implemented, especially if eradication is the goal.

Passive surveillance or diagnostic work-ups are critical for identifying LPAIV as the etiology of respiratory disease or drops in egg production, or HPAIV as the cause of high-mortality events. More broadly, active surveillance through planned statistical or targeted sampling is critical for identifying where the AIV or AIV infection is located within a CZC, or for certifying a CZC as AI-free. Such testing is typically accomplished through serological detection of AIV-specific antibodies (which is of limited use for HPAIV in gallinaceous poultry if the virus kills a very high percentage of infected poultry) and/or detection of AI virus by real-time reverse transcriptase–polymerase chain reaction (rRT-PCR) or antigen-capture ELISA tests. The tests used for diagnosis and surveillance of AI are described in Chapter 2. This chapter will provide a synopsis of the classical methods used to diagnose AI, including virus isolation in cell cultures and embryonating chicken eggs, immunological methods for identifying and subtyping AIVs, and newer biotechnology methods for virus detection, including antigen and nucleic acid detection methods.

Finally, surveillance and monitoring are critical both for evaluating the success of control and preventive strategies, and for use in decision making as a prelude to improving control strategies. Special virological and serological strategies to identify infected birds within vaccinated populations (i.e. DIVA), will be discussed in Chapter 15. Nevertheless, it is worth pointing out that the best method for detecting AIV infection in vaccinated flocks is routine testing of dead birds for AIV.

Elimination of infected poultry

Elimination of the source of AIV in a CZC is critical for stopping an outbreak and eradicating the disease. Once an affected flock has been identified, the high-risk materials should be eliminated, including infected birds, eggs, and manure [70]. However, the safest and most economical method of elimination varies according to the virus strain, local conditions, biosecurity level practiced on the farm and in the area, and available financial and personnel resources. For HPAI, elimination has typically meant humane depopulation, and disposal of carcasses, eggs, and manure using an environmentally friendly method such as composting, incineration, rendering, or landfill burial. The reader is referred to the previous edition of this book for information concerning euthanasia methods [32] and carcass disposal [7].

However, for LPAI, traditional stamping-out and disposal methods have been used less commonly, and instead alternative control methods, including controlled marketing of birds 2–3 weeks after recovery from infection, and washing of eggs before they are marketed, have been used successfully [70]. This practice has also been combined with vaccination. The alternatives are plausible because most AIV shedding occurs during the first 2 weeks after initiation of infection, and usually virus cannot be detected by 4 weeks. Therefore antibody-positive flocks have a low risk of transmission if they are maintained under strict biosecurity. Antibody-positive flocks should be tested for viral shedding before movement is allowed, with testing of a sufficient number of birds to allow reasonable confidence that the virus is no longer circulating. Because the economic losses due to AI may be severe, any AI control program should not unnecessarily penalize the growers, especially the small producers and farmers who cannot withstand the economic losses without financial compensation or the ability to market recovered poultry. Stamping-out and disposal programs will only be successful if indemnities are paid by federal or state governments in a timely manner. Success in using stamping out in areas with high poultry density depends on finding all infected flocks early. An

inability to do this has been one of the reasons why Gs/GD-lineage H5 viruses have been able to persist for 18 years in Asia (as of 2015). In some places, stamping out was carried out in a ring of 1–5 km around infected premises. However, most developing countries have abandoned this practice because it was highly disruptive and was not successful in eliminating virus. In some developing countries, stamping out is also undertaken several weeks after an outbreak has commenced (due to delays in reporting and centralized testing), by which time some birds in the affected area have been moved to other locations or sold. As there is no long-term carrier state for HPAIV in individual birds, there is little justification for this approach. However, this requires flexibility in the approach, which can be difficult to achieve in highly centralized, rule-bound systems.

Decreasing host susceptibility

If poultry are at risk of exposure to infected birds or a contaminated environment, decreasing the susceptibility of the birds to infection may be necessary to break the infection cycle [70]. Such decreased susceptibility can be achieved either by using a host strain or breed that is genetically resistant to AIV infections or, more commonly, by producing active or passive immunity against the AIV in a susceptible host species, breed, or strain.

At the present time, human anti-influenza drugs are not recommended for treatment of food-producing animals. Use of such medications has been shown to favor rapid development of AIV strains that are resistant to the antiviral products and compromise the effectiveness of the specific antiviral therapies for humans infected with AIVs [2, 3, 14, 17, 33, 74]. Unsanctioned use of antiviral products in poultry has occurred, and should be actively discouraged. For LPAIV infections, supportive care and antibiotic treatment have been used to reduce the negative effects of concurrent bacterial infections.

Genetic resistance

Very little research has been undertaken to identify the natural occurrence or for classic selection of resistance to AIVs in poultry. Some commercial chicken strains show resistance to renal pathology following intravenous challenge with LPAIVs [63]. A population survey of Leung-Hahng-Kow and Pradoo-Hahng-Dam native chicken breeds in Thailand identified the A9, B14, and B21 major histocompatibility class (MHC) I and II haplotypes as being more frequently present in survivors after H5N1 HPAI village outbreaks, whereas A1, B12, B13, and B19 haplotypes were predominant in the chickens that died, and B2, B4, and B5 haplotypes were present equally in survivors and fatalities [6]. However, experimental trials using congenic chicken lines that differed at MHC haplotypes B2, B12, B13, B19, and B21 did not show significant differences in mortality rates following a low challenge dose of H5N2 HPAIV, thus refuting the hypothesis that MHC haplotype determines resistance to HPAIV infection and lethality [27].

Studies in mice have identified a functional *Mx1+* gene as conferring some resistance to laboratory-adapted human influenza A viruses [28]. An Mx homolog has been demonstrated in ducks and chickens, and has shown variable *in-vitro* antiviral properties against influenza A viruses and other viruses [1, 4, 31, 36], but it is not known whether it can confer resistance to AIV infection in the bird. Finally, a technology that inserts small interfering RNAs (siRNA) has silenced expression of some AI viral genes in avian and mammalian cells, thus conferring resistance to AIV replication [37]. Use of siRNA in the respiratory tract produced protection in a mouse model system following lethal influenza A virus challenge [72]. Combining siRNA with transgenic technology holds the potential for development of AIV-resistant birds. One study was conducted in which transgenic chickens were developed expressing a short-hairpin RNA designed to function as a decoy that inhibits and blocks influenza virus polymerase. Transgenic chickens infected experimentally with HPAIV did not prevent infection or mortality, but there was reduced contact transmission of challenge virus to transgenic or non-transgenic chickens [40].

Immunity

The established and practiced method of producing resistance to AIVs is through active or passive immunity, principally against the AIV HA, and to a lesser extent the NA, but such protection was subtype specific and in some cases strain specific. In practice, immunity has been achieved mainly through vaccination and to a lesser extent through

maternal antibodies passed to progeny via the egg yolk. Maternal antibodies only provided protection for the first 1–3 weeks after hatching, whereas active immunity was effective for longer periods of time. Avian influenza vaccines have been used in a number of different poultry species, including chickens, turkeys, and ducks.

A variety of vaccine technologies have been developed and shown to be efficacious against LPAIVs and HPAIVs in the laboratory setting [66]. Field usage has been dependent upon licensing by national veterinary authorities following demonstration of purity, safety, efficacy, and potency [50], and a demonstrated need for them in the control of AI. The majority of vaccine used in the field has been inactivated whole-AIV vaccines, typically made using LPAI field outbreak strains and more recently reverse genetic generated AI vaccine strains, followed by chemical inactivation and oil emulsification [68]. Use of HPAIVs as inactivated AI vaccine strains has occurred, but requires manufacturing in special high biocontainment facilities to prevent accidental escape and infection of susceptible poultry in the community. HPAIVs are not recommended for vaccines [43]. Since the late 1990s, live fowl poxvirus and avian paramyxovirus type 1 (lentogenic Newcastle disease viruses) vectored vaccines with gene inserts of AI HA have been licensed and used in some countries. Other viral vector vaccines that have been used for control and prevention of Gs/GD-lineage H5 virus include vaccines based on herpesvirus of turkeys [29, 59], and duck viral enteritis vaccine [38]. The former have been applied with some success as a hatchery-level vaccine in some countries. The latter has been used successfully in field trials in ducks, and may also provide protection in broiler chickens [39].

Experimental studies have demonstrated that high-quality, properly used AI vaccines can provide protection against mortality, morbidity, and declines in egg production [70]. Furthermore, vaccines increase resistance to AIV infection, reduce the number of birds shedding virus, greatly reduce the titer of challenge virus shed, prevent contact transmission, and reduce environmental contamination. Therefore vaccines can be a useful tool in a comprehensive AI control program when used in combination with other disease control components. The 2015 North American H5N2 HPAI outbreak has resulted in the culling of millions of healthy poultry in unaffected houses on farms with one affected house. It suggests that approaches based on emergency vaccination, especially for long-lived poultry, may be possible if appropriate vaccines are available and trade issues relating to vaccine use can be resolved. This approach was used successfully in outbreaks in Hong Kong SAR [18].

The topic of vaccines and their use will be covered in more detail in Chapter 15.

Economic costs

The economic costs of AI can result from direct and indirect losses due to morbidity and mortality in affected flocks, loss of consumer confidence in poultry products from non-affected CZCs, and downtime in farming operations, as well as the costs of preventing, managing, or eradicating the disease or infection. The costs are variable, and are dependent upon the virus strain, host species, type of agricultural system affected, number of premises involved, control strategies used, and the speed with which the control program is implemented [70]. In most developed countries, neither HPAI nor LPAI have been an endemic disease in the commercial poultry industries, but LPAI has been identified as causing sporadic to endemic infections in some backyard premises and in LPM systems that serve ethnic populations of large metropolitan areas. Most outbreaks, and the resulting economic losses, have been from HPAI epidemics in commercial as well as non-commercial chickens and turkeys. By contrast, in many developing countries, LPAIVs have become endemic in commercial and non-commercial poultry, especially viruses of the H9N2 subtype since the 1990s, and have caused ongoing increased costs for poultry production. Since 1996, the Gs/GD-lineage H5 HPAIV has caused epidemics in various Asian, African, European, and North American countries. Beginning in 2003, H5N1 HPAI became endemic in poultry production systems, especially in domestic ducks and LPMs, in some Asian countries and in Egypt.

Infections by LPAIVs in poultry have caused significant economic losses due to the illness and mortality in infected birds, especially when accompanied by secondary bacterial or viral pathogens

[70]. However, accurate figures for economic losses are generally either not documented or unavailable. In general, the losses from LPAI outbreaks have been less than those from HPAI outbreaks, because of the lower morbidity and mortality rates associated with LPAI. In some situations, recovered LPAI-infected flocks have been eliminated through a controlled marketing program which provides some financial recuperation for farmers. Federal or state eradication costs have been less commonly incurred with LPAI, and typically LPAI has caused minimal disruption to national and international trade in poultry and poultry products. Endemic H9N2 LPAI infections of poultry in Asia and the Middle East, and H5N2 LPAI infections of poultry in Mexico and Central America, have placed a significant financial burden on poultry production, and are now controlled by routine vaccination and management programs to control secondary bacterial and viral pathogens. However, in some developed countries, H5 and H7 LPAIVs have been managed by traditional stamping-out programs at a higher financial cost. For example, the stamping-out program that was undertaken in Virginia for H7N2 LPAI during 2002 had a Federal eradication cost of US$461 000 per farm, which was slightly more than the figure of US$275 000 per farm for H5N2 HPAI eradication in the USA when adjusted for inflation to 2006 funds [70]. Typically, the economic losses have been greater for HPAI than for LPAI, with costs being proportional to the number of birds that died plus those that were pre-emptively culled. However, the projected cost of not implementing an HPAI eradication program would be even higher in terms of animal health losses and loss of export markets [70]. A more detailed discussion of the costs of AI, especially the H5N1 HPAI epidemic, can be found in Chapter 3 [70].

Public health aspects

In general, influenza A viruses express host adaptation with transmission and infection occurring most frequently and with ease between individuals of the same or closely related species (e.g. chicken to turkey), occasionally causing infection in unrelated species but within the same class (e.g. pig to human, or wild duck to turkey), and, rarely, interspecies and interclass infections (e.g. chicken to human) [65]. Infections by AIVs have occurred in humans, but have been uncommon considering the number of exposures to H5N1 HPAIV and H9N2 LPAIV that have occurred in Asia and Africa over the past 10 years, and compared with the number of human infections with endemic H1N1 and H3N2 human influenza A viruses that occur each year around the globe. It is likely that there is a higher rate of infection than that detected by clinical disease, especially for H9N2 and H7N9 viruses. Although rare, AIVs have caused individual sporadic infections, or AIV genes have appeared in influenza A viruses that infected humans (i.e. there has been reassortment of gene segments).

Between 1959 and 1997, only six incidences involving 15 non-fatal cases were reported. However, between 1997 and 2014, three AIV strains were responsible for 356 cases, with severe consequences, namely the 2003 Netherlands H7N7, the Guangdong-lineage H5 viruses from 1997 onwards, and the H7N9 LPAIVs that emerged in 2013 in Eastern China (reviewed by Swayne *et al.* [70]). In these incidents, multiple deaths occurred in association with H5N1 HPAIVs, and one death was associated with H7N7 HPAIV. In most human cases, the H5 HPAIV-infected individual had close contact with live or dead infected poultry, including preparation of poultry for consumption at the village level, or exposure to infected poultry in the LPM system, while H7N7 HPAIV infections were in farmers, poultry veterinarians, and depopulation crews in the commercial production sector. Exposure to live or dead infected birds was determined as the primary risk factor [46, 56]. This highlights the need for precautions when working with birds infected by some strains of HPAIVs. The H7N7 HPAIV was eradicated. At the time of writing, the H7N9 LPAIVs and H5 HPAIVs that still persist have not become readily transmissible between humans. However, the potential for this to occur, resulting in a human influenza pandemic, has led to considerable investment of both public and private funds in control measures for these viruses, including considerable donor support to affected and at-risk countries.

AIVs have contributed genes to previous human pandemic influenza viruses through reassortment. The 1957 (H2N2) and 1968 (H3N2) human pandemic influenza viruses arose following reassortment of three (HA, NA, and PB1) and two (HA

and PB1) AI viral genes with five and six human influenza internal viral genes, respectively [30, 48, 51, 52]. It is unclear whether this reassortment occurred in humans, or in a "mixing-vessel" species such as swine, but with the discovery of multiple human infections by AIVs in the past 20 years, it would seem that a mixing vessel may not be necessary to produce human pandemic influenza A viruses. Some recent evidence suggests that the 1918 pandemic virus may have been entirely avian, and may have arisen by direct adaptation to humans [71]. It has also been proposed that reassortment may have occurred between an avian-origin H1 virus that circulated in humans for a number of years and another avian virus of the N1 subtype just prior to the pandemic [77].

The zoonotic aspects of AIVs and the infections that they have caused are discussed in more detail in Chapter 5.

The role of international animal health organizations

Guidelines on trade in poultry and poultry products are provided by the World Organization for Animal Health (Office International des Epizooties, OIE) *Terrestrial Animal Health Code*. This document provides technical guidance on the measures to be taken to minimize the likelihood of importing AI through trade. The information in the *Terrestrial Animal Health Code* is updated as new assessments become available on pathogenicity, pathobiology, epidemiology, and molecular features of AIVs. As an exotic disease, AI has been used as a legitimate trade barrier to protect countries and regions from the introduction of this devastating animal health disease. Since 2004, OIE has codified animal health measures for AI, as defined by OIE. Chapter 4 discusses in more detail the measures adopted to reduce risk in trade of birds and their products (topics covered include the risk of transmission of AIVs through meat or other products to poultry and humans, the distribution of LPAIV and HPAIV in poultry meat and tissues of infected birds, and methods used to inactivate AIV in foods).

While OIE has a major role in setting international animal health standards for trade in animals and animal products, the Food and Agriculture Organization (FAO) of the United Nations plays a major role with regard to livestock production and animal health programs in the developing world. The FAO facilitates countries in developing programs that enable the production of clean and safe animal products for consumers, particularly in helping to build and improve opportunities for smallholder livestock farmers in developing countries. The FAO has been very active in helping countries to tackle the control and prevention of avian influenza. For Gs/GD-lineage H5 viruses there are three distinct country categories, and the approach in each country will differ. For countries where the virus is not present in poultry, the main objective is to maintain this status. Countries that have detected virus recently will usually attempt to contain and preferably eliminate the virus, and a decision needs to be taken once the situation is clear as to whether virus elimination is likely to be possible. If it is, the country will then work towards virus elimination. If it is not, the country will be considered a place where virus remains endemic, and measures will be taken to contain the virus in preparation for eventual elimination, although experience suggests that for a number of countries where the virus remains entrenched, this will be a long-term process that will not occur until there is major restructuring of the poultry sector and changes to existing production and marketing practices.

Conclusions

There is no single control strategy that fits all types of AI in every country and in all avian species. Each control strategy must be tailored to the specific AIV and to the local situation and needs. In most developed countries, HPAI has been handled by variations of stamping-out programs, but with the emergence of GS/GD-lineage H5 HPAIVs in Asia 18 years ago, the strategies for controlling HPAI as well as LPAI have broadened, especially for countries where the prospects of virus elimination are poor, and these have adopted a variety of options.

There are three different goals or outcomes in the control of AI, namely prevention, management, and elimination or eradication. The achievement of these goals is based on comprehensive strategies

developed using a combination of five specific components or features:

1 education, communication, public awareness, and behavioral change (i.e. improving knowledge of how AI is transmitted, making individuals aware of their role in prevention, management, or eradication, and promoting appropriate behavioral change
2 changes to production and marketing systems resulting in enhancement of biosecurity (i.e. facilities, management practices, and procedures to prevent virus introduction to a premises or, if virus is already present, from leaving a premises)
3 diagnostics and surveillance (i.e. the ability to detect the virus or evidence of infections in bird populations or their environment, or a means of verifying "freedom" from such infections)
4 elimination of infected poultry (i.e. removal of the infection source or susceptible sources in order to prevent continued environmental contamination and dissemination)
5 decreasing host susceptibility and reducing viral shedding (i.e. increasing host resistance, usually by vaccination, so that if exposure occurs, infection is prevented or the negative consequences of infection are minimal).

The effectiveness of the comprehensive strategy in controlling AI is dependent upon how many of these five components are used and how thoroughly they are practiced in the field. The immediate goals for individual LPAI and HPAI control strategies may be different, depending on the country, the subtype of the virus, the economic situation, and the risk to public health, but the long-term goal should be the elimination of all AIVs of the H5 and H7 subtype from domestic poultry.

The economic costs associated with AI can result from direct and indirect losses due to illness and mortality in affected flocks, loss of consumer confidence in poultry products from non-affected CZCs, and downtime in farming operations, as well as the costs of preventing, managing, or eradicating the disease or infection. The costs are variable, and are dependent upon the virus strain, the host species, the type of agricultural system affected, the number of premises involved, the control strategies used, and the speed with which the control program is implemented.

Infections by AIVs have occurred in humans, but such infections have been uncommon considering the number of exposures to H5 HPAI and H9N2 LPAI viruses that have occurred in Asia and Africa over the past 18 years, especially when compared with the annual number of human infections that occur globally with endemic H1N1 and H3N2 human influenza A viruses.

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15 Vaccines and vaccination for avian influenza in poultry

David E. Swayne and Darrell R. Kapczynski

Introduction

Avian influenza (AI) vaccines and their application in the field can be an effective tool within a comprehensive control program, which should include the following additional components:

1. biosecurity (bioexclusion and biocontainment, including quarantine, limiting human access to affected premises, cleaning and disinfection, and movement controls for poultry and equipment)
2. education on how to prevent AI, including risk communication
3. diagnostics and surveillance for accurate and rapid detection of AI virus (AIV), disease or infection
4. elimination of AIV-infected poultry through humane euthanasia and environmentally sound disposal of carcasses or, when appropriate, controlled marketing [330, 331].

Usage of these components in various combinations within a control strategy can prevent, manage, or eradicate AI. However, the use of AI vaccine alone can severely limit the effectiveness of any control strategy, especially to achieve eradication, unless used properly. Use of AI vaccine can manage the disease, but addition of the other four components of a comprehensive control program is needed to prevent or eradicate the disease and/or the infection.

History of avian influenza vaccines

In the early part of the 1900s, a few chickens infected with fowl plague virus (i.e. H7 high pathogenicity avian influenza [HPAI] virus [HPAIV]) were observed to recover from the disease and were refractory to fowl plague upon re-exposure (two reviews provide further information [24, 358]). The blood of those chickens contained virus-neutralizing substance (i.e. neutralizing antibodies). Initial attempts to produce vaccines were unsuccessful, or the vaccines were inconsistent in producing immunity [254, 358]. The first vaccines were derived by drying spinal cord from fowl plague cases (an attempt to use the technology responsible for the successful rabies vaccine of Pasteur), or they were produced by using heat, light, and various chemicals (e.g. formalin, phenol glycerine, tricresol glycerine, etc.) to inactivate the virus in blood or liver of chickens that had died from fowl plague. Vaccine failures usually resulted from incomplete inactivation of the HPAIV such that vaccine administration produced fowl plague, or the vaccine did not provide adequate protection because there was an insufficient amount of inactivated virus to produce a protective immune response, such that vaccinated birds succumbed following challenge with fowl plague virus. However, when a successful vaccine was produced, it could maintain efficacy for at least 120 days if stored at –3°C. Early immunization and challenge studies indicated that the European fowl plague viruses were all cross-protective (i.e. they provided homosubtypic protection across all H7 HPAIVs). However, because of the success of stamping-out programs and inconsistency of vaccines, vaccines were not used in HPAI control programs until 1995, when they were specifically first used in Mexico and Pakistan.

Animal Influenza, Second Edition. Edited by David E. Swayne.

The development of vaccines to control low pathogenicity avian influenza (LPAI) arose after the mid-1960s, and was based on economic need. As one of the first observations on the potential for immunity to control LPAI, some flocks of turkey pullet breeders raised on range in California would develop LPAIV infections and mild clinical disease, but these recovered birds were protected from LPAIV-induced drops in egg production after being moved to the breeder production houses (R. McCapes, personal communication, 23 May 2007). The occurrence of severe losses in Minnesota breeder turkeys from LPAI during the autumn of 1978 resulted in special United States Department of Agriculture (USDA) approval of the first commercial inactivated AI vaccines through a new special conditional license. These first vaccines were produced in late 1978, and were initially used in 1979 to control LPAI in turkeys in Minnesota and California [223, 253]. Some AI vaccine was used in Minnesota meat turkeys, but in California the AI vaccines were only used in turkey breeders, each bird receiving two vaccine doses given 4–6 weeks apart. In 1980, a polyvalent H5N2, H6N2, and H10N2 inactivated AI vaccine along with Newcastle disease virus (NDV) was reported to have been used in Italy to control multiple subtypes of LPAIV plus Newcastle disease [402]. In the USA, conditional licensing was allowed for non-H5/H7 vaccines in 1985, but H5/H7 vaccines required federal government approval for distribution and use in order to prevent interference with eradication efforts [107].

Features of the ideal avian influenza vaccine for birds

The ideal AI vaccine is antigenically close to field virus for the best protection, usable in multiple avian species, compatible with single-dose protection, easily administered by mass application to large populations of poultry, compatible with methods for easy identification of infected birds within the vaccinated population (i.e. the so-called differentiating infected from vaccinated animals [DIVA] strategy), able to overcome maternal antibody block to produce an active immune response, possibly able to be administered in the hatchery at 1 day of age or *in ovo*, and inexpensive (Table 15.1) [116, 117, 342]. When developing new vaccines, these traits must be taken into

Table 15.1 Properties of ideal avian influenza vaccines and vaccination methods for poultry (modified from Swayne and Spackman [339]).

Desired property	Current situation
Inexpensive	Current cost for inactivated AI vaccine: US$0.05–0.10 per dose plus cost of administration (US$0.05–0.07 per dose for individual handling and injection) [342]
Use in multiple avian species	Most used in meat, layer, and breeder chickens, but large quantity also used in ducks; minor amounts in turkeys, geese, quail, etc. [336]
Single dose protection	Most situations require a minimum of two doses; prime-boost scenario is optimal, with additional boost in long-lived birds at 6- to 12-month intervals [338]
Mass application	95.5% is inactivated vaccine administered by handling and injecting individual birds, with 4.5% as vectored vaccine given by mass spray vaccination (rNDV vector) [299, 332]
Identify infected birds in vaccinated population	Serological differentiation tests are available, but have only minor use. Most vaccine is applied without using a DIVA strategy [338]
Overcome maternal antibody block	Maternal antibody to AIV hemagglutinin or virus vector inhibits primary immune response. Initial vaccination must be timed for declining maternal antibody titers to allow optimal primary immune response [332], and also a decline in active immunity is needed before giving booster vaccinations [213]
Given at 1 day of age in hatchery or *in ovo*	Inactivated vaccine provides poor protection if given at 1 day of age. Vectored vaccines can be given at 1 day of age, but generally require a field boost with inactivated vaccine 10 days or more later
Antigenically close to field virus	The majority of inactivated whole AI vaccine uses reverse-genetic-generated vaccine seed strains to antigenically match field viruses [324]

account, as a new vaccine is unlikely to be developed to market unless it contains additional "ideal" traits by comparison with the existing licensed poultry AI vaccines. This requires the scientist to have a practical knowledge of how poultry are produced in the vaccine-licensing country, and how poultry health problems are managed in the field in different countries or regions. In addition, the reader must accept that the ideal traits for human influenza vaccines will not be ideal for poultry AI vaccines. Furthermore, the novelty of the new vaccine technologies is insufficient by itself to be usable (i.e. the new technology must be practicable). None of the currently licensed AI vaccines meet all of the criteria for an ideal vaccine, so there is still much room for innovation and improvement.

Immunological basis for protection

AI vaccines provide protection to birds, principally through systemic humoral immunity against the hemagglutinin (HA) protein, and such protection is HA subtype specific. Similarly, infection with LPAIV produces protection against exposure or challenge by the same HA subtype of HPAIV, implicating mucosal and cellular immunity as contributors to protection [376]. Subtype-specific humoral antibodies against neuraminidase (NA) can provide partial to complete protection. Recent reports have demonstrated that cell-mediated immunity can contribute to protection against AIV [161, 284].

Theoretical attempts to develop universal vaccines for poultry are a long-term goal, with some small steps of success in reducing virus replication, mainly with LPAIV, but protection from lethality of HPAIV has not been achieved. For example, immunization of chickens with a prime-boost regime of recombinant Adenovirus-NP/M and recombinant Vaccinia-NP/M reduced cloacal shedding by A/Turkey/England/1977 (H7N7) LPAIV [161]. A prime-boost vaccination with M2 or M2e in chickens produced antibodies against their respective proteins, including neutralizing proteins, but failed to protect from lethality of HPAIV challenge [35].

Laboratory criteria for assessing vaccine protection

The goal of AI vaccination is the production of an immune response that is protective against the disease (morbidity and mortality), and ideally, the prevention of infection. Assessment of protection conferred by the vaccine is important to national regulatory authorities, which only license vaccines that are efficacious and potent, and for assessment of vaccines for practical use in the field. Protection can be directly measured by *in-vivo* laboratory studies using a variety of avian models and measurable criteria or metrics. Laboratory models can be useful for directly measuring protection in the target or surrogate species when variables such as vaccine seed strain, challenge virus strain, challenge virus dose, and vaccine antigen content are standardized. In addition, a variety of indirect measures can be used to assess protection when compared with the *in-vivo* protection data. These measures can include assays for immunological response, such as antibody titers or cell-mediated responses, or assays to quantify the amount of protein that will produce an immunologically protective response by the vaccine. In this section, the term "efficacy" will be used to indicate that the vaccine is protective in defined, standardized experimental studies, whereas the term "potency" indicates that the vaccine has passed quality control tests that ensure adequate antigenic mass of the protective immunogenic protein to produce a consistent immunological response that should be protective under experimental as well as a variety of field conditions.

Direct assessment of protection

The "gold standard" for assessing protective immunity of AI vaccines is the use of LPAIV or HPAIV challenge models in the target poultry or other surrogate avian species. Historically, most AI vaccine studies and subsequent field use of vaccine have focused on chickens, and to a lesser extent turkeys, because these have been the major poultry species reared in developed countries, and they have been affected by both LPAIV and HPAIV infections and disease. In addition, these species have experienced the highest death rates from

HPAIV exposure, and when infected, they have excreted high concentrations of virus into the environment, resulting in efficient viral transmission between individuals and viral spread between premises. With the changing epidemiology of the H5N1 A/goose/Guangdong/1996 (Gs/GD)-lineage HPAIV in Asia, domestic ducks and geese have emerged as very important contributors to the maintenance and spread of this HPAIV, and have resulted in increased economic losses, and thus need primary consideration for managing HPAI though vaccines and vaccination programs. Thus evaluation of protection in additional host species, such as ducks, geese, minor gallinaceous poultry species (e.g. Japanese quail, pheasants, partridges, guinea fowl, etc.), ostriches, and zoological birds, may be needed.

All *in-vivo* studies should include a group of birds vaccinated with a placebo control (sham) to ensure that proper challenge was accomplished. Studies should be properly designed and evaluated using statistical methods to establish unbiased treatment effects for the vaccine. Simple numerical differences in survival or other metrics for a small number of birds that are not statistically tested should not be interpreted as significant, and can lead to biased interpretations. Although use of an AIV challenge model is common in vaccine licensing for chickens, turkeys, and more recently domestic ducks, challenge models may not be economically viable in all countries for licensing AI vaccines for domestic geese, minor gallinaceous poultry species, ostriches, and diverse zoological birds. As an alternative, demonstration of HA-subtype-specific HI antibodies, especially after two vaccinations, has been associated with protection, and may be an adequate metric of protection in minor poultry and non-target non-poultry avian species for field usage [185, 345].

Criteria used to assess protection can vary depending on whether the challenge is a LPAIV or a HPAIV. For HPAIV challenge, prevention of respiratory and general clinical signs (morbidity) and death (mortality) are the criteria that have been most frequently used to assess protection (Table 15.2) [176]. Most experimental LPAIV challenge models typically do not produce clinical signs or death, rendering morbidity or mortality metrics unreliable for assessing LPAI vaccine protection. Recently, however, protection from conjunctivitis has been proposed as a metric in vaccine assessment for conjunctival sac exposure in an H9N2 LPAIV challenge model [41, 145, 304, 305, 394, 398]. In addition, both LPAIV and HPAIV can affect the reproductive health of laying chickens and turkeys, and prevention of drops in egg production or reduction in the number of virus-positive eggs (in the case of HPAIV only) can be quantifiable indicators of protection (Figure 15.1) [174].

For both LPAIV and HPAIV, the prevention of infection, or a qualitative and/or quantitative reduction in virus replication in the respiratory and digestive tracts, are protective criteria that indirectly assess the role of the vaccine in limiting environmental contamination and field virus spread (Table 15.3 and Figure 15.2) [2, 29, 41, 165, 249, 304, 341]. The reduction in challenge virus replication can be quantified using classical virus

Table 15.2 AI vaccine protection as measured by prevention of clinical signs (morbidity) and death (mortality) of vaccinated chickens following challenge with different doses of HPAIV (mean embryo infectious [EID_{50}] and mean chicken lethal [CLD_{50}] doses).

Vaccine	Challenge dose		Morbidity	Mortality
	EID_{50}	CLD_{50}		
rFPV-H5	0.5	0.003	0/10	0/10
	2.0	0.1	0/10	0/10
	3.5	3.2	0/10	0/10
	5.0	100	0/10	0/10
	6.5	3200	0/10	0/10
	8.0	100 000	2/10	2/10 (4.5)
Sham	0.5	0.003	0/10	0/10
	2.0	0.1	0/10	0/10
	3.5	3.2	8/10	8/10 (2.75)
	5.0	100	10/10	10/10 (2.4)
	6.5	3200	10/10	10/10 (2.0)
	8.0	100 000	10/10	10/10 (2.0)

Chickens were vaccinated subcutaneously at 1 day of age with recombinant fowl poxvirus containing H5 AIV gene insert of A/turkey/Ireland/1983 (rFPV-AIV-H5) and intranasally challenged at 3 weeks of age with various challenge doses of HPAIV ($10^{0.5–8.0}$ EID_{50}, A/chicken/South Korea/2003 [H5N1]). *Avian Diseases* 51(1):498–500, 2007 [338, 340]. Used with permission of the American Association of Avian Pathologists.

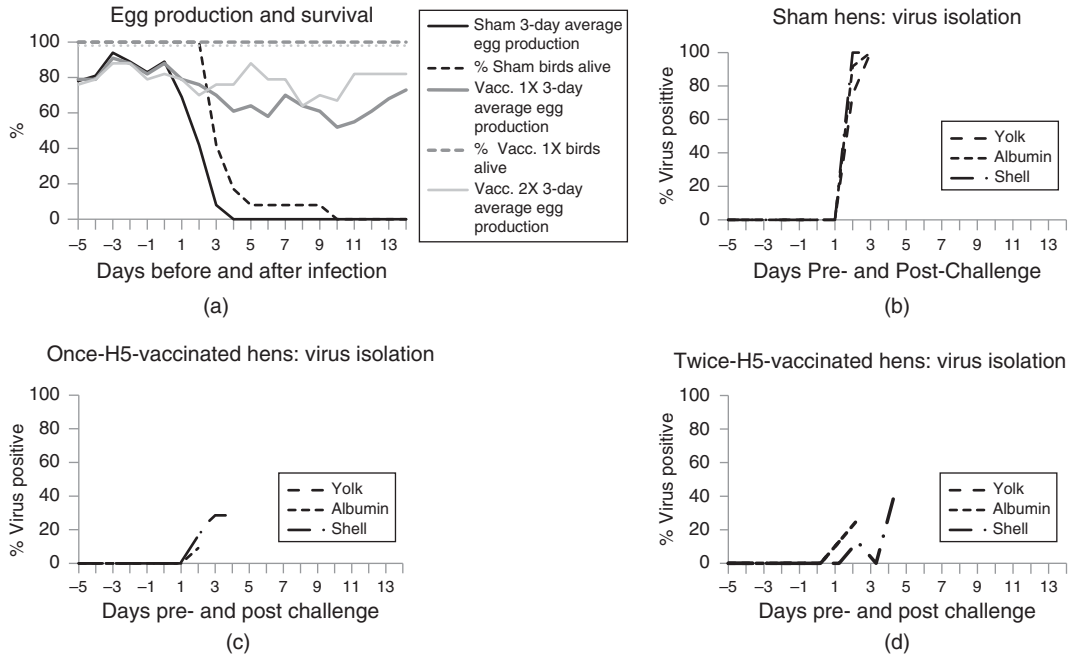

Figure 15.1 Example of protection metrics related to egg-laying poultry using egg production (3-day average of percentage of eggs/hen/day), hen mortality (percentage); and virus-positive eggshell surface, yolk, and albumin samples (percentage positive for virus) for sham-vaccinated and once or twice H5-vaccinated chickens challenged with A/chicken/Pennsylvania/1370/83 (H5N2) HPAIV. Swayne *et al.*, *Vaccine* 30(33):4964–4970, 2012 [11]. Used with permission of Elsevier.

Table 15.3 AI vaccine protection as measured by reduction in the number of vaccinated chickens shedding HPAIV from the oropharynx and cloaca.

Inactivated AI vaccine group	**Virus isolation, 2 days post challenge**	
	Oropharyngeal swab[a]	**Cloacal swab[a]**
Sham control	$10/10^{A}$	$10/10^{A}$
A/chicken/Mexico/232/94(H5N2)	$5/10^{B}$	$3/10^{B}$
A/duck/Potsdam/1402/86 (H5N2)	$6/10^{AB}$	$3/10^{B}$

[a]Number positive/total tested. Different upper-case superscript letters indicate significant differences in frequency of positives between different vaccine groups (Fisher's exact test, $P < 0.05$). Chickens were vaccinated subcutaneously at 3 weeks of age with inactivated whole AI vaccines and intranasally challenged at 6 weeks of age with a high dose ($10^{6.0}$ EID_{50}) of A/chicken/Indonesia/7/2003 (H5N1) HPAIV [45]. Chickens were swabbed 2 days post inoculation.

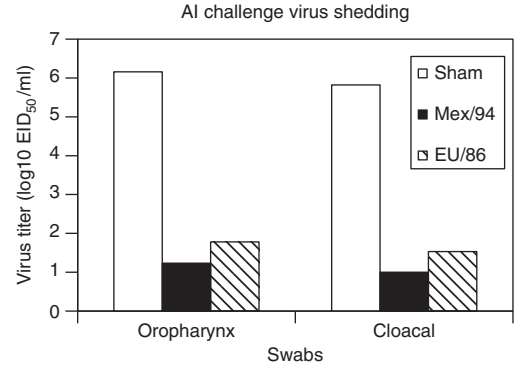

Figure 15.2 Reduction in titer of HPAIV shed from the oropharynx and cloaca of vaccinated chickens 2 days post challenge. See Table 15.3 for details. Minimum limit of detection 10_1 ELD_{50}/ml. Source: David E. Swayne.

Table 15.4 AI vaccine protection as measured by interruption of contact transmission.

IN challenge group	Contact group	Mortality in contact group	Virus shedding from contact groups (log10 EID_{50}/mL)	
			Oropharynx	Cloaca
WI/68 vaccine	WI/68 vaccine	0/10	0/10 (≤0.9)$^{A a}$	1/10 (≤0.9)^{A}
	Sham vaccine	10/10	2/10 (≤1.5)^{A}	1/10 (≤1.3)^{A}
Italy/98 vaccine	Italy/98 vaccine	0/10	0/10 (≤0.9)^{A}	0/10 (≤0.9)^{A}
	Sham vaccine	5/10	1/10 (≤1.0)^{A}	1/10 (≤1.0)^{A}
Sham vaccine	WI/68 vaccine	0/10	8/10 (≤2.0)^{B}	0/10 (≤0.9)^{A}
	Sham vaccine	10/10	10/10 (5.7)^{C}	10/10 (5.0)^{B}

[a]Number positive/total tested. Different upper-case superscript letters indicate significant differences in virus titers between different vaccine groups.

Chickens were vaccinated subcutaneously at 3 weeks of age with inactivated whole H5N9 AI vaccines (A/turkey/Wisconsin/1968 [H5N9], WI/68, and A/chicken/Italy/22A/1998 [H5N9], Italy/98) and intranasally challenged at 6 weeks of age with a high challenge dose (10^6 EID_{50}) of HPAIV A/chicken/Supranburi/2/2004 (H5N1) isolated in Thailand. Vaccinated (WI/68 and Italy/98) and non-vaccinated (sham) chickens were put in contact with intranasally challenged chickens 18 hours post challenge to determine the impact of vaccination in reducing AIV transmission [333].

isolation and titration methods in embryonating chicken eggs (ECE) or tissue culture systems [18, 63, 152, 322, 323, 328], or by quantitative or qualitative assaying for AIV-specific nucleic acids, such as with real-time reverse transcriptase–polymerase chain reaction (RRT-PCR) [304, 322], or by demonstration of AI viral proteins, such as in a ELISA test [152, 189]. Demonstration of a reduction in replication and shedding titers of virus from the respiratory and intestinal tracts should be at a minimum of 10^2 EID_{50} (100-fold) less virus in vaccinated compared with non-vaccinated birds [177], or the difference should be analyzed for statistical significance [314]. National veterinary biologics regulatory agencies may require demonstration that reduction in shedding is clinically relevant (i.e. that both shedding and contact transmission are reduced in experimental studies). In addition, immunized birds have a quantifiable resistance to induction of infection as measured by requiring a 10^2–10^5 EID_{50} greater challenge dose to produce infection in vaccinated compared with non-vaccinated birds, and such resistance could be used as a metric of protection, but such experiments are large and expensive to conduct [322]. Among the various protection metrics observed in experimental studies, AI vaccines most frequently demonstrate prevention of mortality, followed by prevention of morbidity, then prevention or reduction in replication and shedding from the alimentary tract, and most difficult to demonstrate is the prevention of challenge virus replication and shedding from the respiratory tract. Such reduction in shedding is best achieved if the hemagglutinin of the vaccine and challenge virus are genetically and antigenically closely related [45, 63]. Drops in egg productions are the most difficult to prevent of morbidity metrics. Recently, when assessing protection of chickens by inactivated vaccines after an LPAIV, a reduction in serum acute-phase proteins was associated with a reduction in challenge virus shedding from the oropharynx [152, 294, 295, 325].

The prevention of contact transmission is a more direct laboratory method of assessing the protective capacity of the vaccine to limit field spread (Table 15.4) [347]. Prevention of contact transmission has been used as an epidemiological evaluation tool to demonstrate that proper vaccination could stop HPAI epidemics by limiting bird-to-bird transmission and infection [322]. For example, one quantitative standardized model using caged chickens demonstrated that two different inactivated H7 AI vaccines completely blocked HPAIV transmission 2 weeks after vaccination, whereas 1 week after vaccination it was noted that transmission was only partially blocked [368]. Based on experimental studies, the presence of a high percentage of immunized birds within the population is critical for preventing contact transmission. To prevent major outbreaks, 60% of the birds in the population should be immunized, but using the upper

limits of the confidence interval, 90% (experimental data) and 80% (observational data) of chickens in the population should be vaccinated to prevent transmission, and reduction in contact transmission has been demonstrated as early as 1 week after vaccination [367, 368]. Although prevention of contact transmission is a desirable goal, routine laboratory assessment of contact transmission is typically not standardized, so comparisons across experiments and laboratories are not feasible. Most studies simply place non-challenged birds in cages containing challenged vaccinated birds, but the prevention of contact transmission is affected by multiple variables, including bird density, housing type, and sanitation and ventilation features [33, 369, 370]. For example, it may be more difficult to block transmission in birds that are housed on litter than in those housed in wire-floor cages.

In experimental studies, effective immunity based on prevention of mortality may be achieved in chickens and other gallinaceous poultry following a single vaccination, but single vaccination in commercial broilers was not effective in reducing contact transmission [362]. In some experimental studies in ducks and geese, more than one vaccination was needed to produce protective immunity [250]. Furthermore, in the field, because of immunosuppressive viruses, maternal antibodies, and other factors, a two-vaccination regime is typically needed to produce field protection, especially if the vaccine and field virus are antigenically divergent [102, 369]. In the field, protective immunity is more difficult to achieve than has been demonstrated in experimental studies with specific pathogen-free (SPF) poultry.

Multiple factors will affect the protection of AI vaccines in experimental studies, including the following:

1 Challenge virus dose. High-quality vaccines provide protection against high challenge exposure, whereas inferior-quality vaccines may only protect against low challenge doses, with the most consistent challenge dose being 10^6 mean embryo infectious doses (EID_{50}) or 10^3 mean chicken infectious doses (CID_{50}) [34, 74, 246, 343].
2 Quantity of hemagglutinin in the inactivated vaccine or titer of the live virus in the recombinant vectored vaccine. High hemagglutinin content (inactivated) or high titer (live) vaccines provide the best protection against challenged AIV replication in the respiratory and digestive tracts, whereas vaccines with a lower antigen or virus content may not protect at all, or may protect from morbidity and mortality but not reduce replication and shedding from the respiratory and digestive tracts [322].
3 Adjuvants. The use of proprietary oil adjuvants is common in inactivated poultry vaccines, including AI vaccines, and produces robust, broad, and longer-lasting protective immune responses than do non-adjuvanted vaccines [322, 323, 391].
4 Hemagglutinin match. The greater the genetic and antigenic similarity between the HA of vaccine and field viruses, the greater is the reduction in challenge virus replication and shedding from the respiratory tract. Prolonged usage of vaccines (for more than 3 years) was associated with the appearance of H5 antigenic variant field AIVs in China, Mexico, Hong Kong, Vietnam, Indonesia, and Egypt, against which registered vaccines did not provide protection [99, 309]. However, in Pakistan, antigenic variants did not appear that were not protected by available H7 vaccines [74, 77, 86, 172, 190, 325, 339, 344].
5 Length of protection. The best vaccines produce protection beginning 7–10 days after vaccination, with peak protection at 3–4 weeks, and the protection may last from 6 to 12 months, but the length of protection is directly associated with the quantity of protective antibodies produced (i.e. titers) following the immunization. However, in some species, long periods of protection may require multiple additional vaccinations [1].
6 Route of administration. Inactivated AIV, and recombinant fowl poxvirus (rFPV) and recombinant herpesvirus turkey (rHVT) with H5 AIV gene insert (rFPV-H5 and rHVT-H5, respectively) vaccines require parenteral administration, whereas some live vectored vaccines, such as recombinant Newcastle disease virus (rNDV) with H5 AIV gene insert (rNDV-H5), can be administered by mass topical routes such as spray or drinking water administration to achieve protection. The rFPV-H5, rHVT-H5, and other vaccines have the potential for *in-ovo* application [322].
7 Species of bird and number of vaccinations. Short-lived meat chickens may be protected for their entire production life following a single vaccination in experimental studies, but most field studies suggest that two vaccinations are

needed to provide optimal protection, because of maternal antibodies to the HA of the AIV or the vector. Some species of meat poultry (e.g. turkeys, ducks, and geese) and long-lived birds (e.g. layers and breeders) may require multiple additional vaccinations to achieve protection that will last through their production life cycle [19, 164, 244, 322, 327].

8 Age of vaccination. Optimal immune response with inactivated AI vaccines is achieved in most birds after 2 weeks of age and before puberty – that is, suboptimal protection may be seen in birds vaccinated before 2 weeks of age, and in adults during the stress of the laying cycle, but such suboptimal vaccination timing may be necessary as a priming or boost vaccination in a multi-dose vaccine program within some production systems.

9 Field versus laboratory protection. Field protection is less than achievable in laboratory efficacy studies because of maternal antibodies, immunosuppressive viruses, vaccine storage and transport problems, incomplete or missed vaccinations of poultry on a farm or within a region, and failure to follow the manufacturer's instructions, including use of a reduced vaccine dose [16, 34, 322]. The impact of maternal antibodies against AIV proteins or vaccine vector on protection can be assessed in experimental studies by using progeny from vaccinated breeders, or modeling via passive transfer of anti-AIV antibodies to day-old SPF birds [328, 330].

Prior to 2004, poultry AI vaccines had a longer life of field usage without changing vaccine strains as compared with human seasonal influenza vaccines, mainly because of more limited usage in poultry, reduced opportunity for antigenic drift in hemagglutinin, and the presence of antigenic similarity/broad cross-reactivity within a subtype. For example, the rFPV-H5 vaccine with a 1983 H5 AIV gene insert protected chickens against diverse H5 HPAI North American and Eurasian challenge viruses isolated between 1959 and 2004, before vaccine use was widely implemented as an HPAI or H5/H7 LPAI control tool [111]. Similarly, inactivated H5 AI vaccines based on classic H5 LPAI vaccine seed strains of A/turkey/Wisconsin/68 (H5N9), A/turkey/3689-1551/Minnesota/81 (H5N2), A/duck/Potsdam/1402/86 (H5N2), A/chicken/Mexico/232/1994 (H5N2), and A/duck/Singapore/F119/1997 provided protection against early H5N1 Gs/GD-lineage HPAIVs A/Hong Kong/156/1997 and A/chicken/Indonesia/7/2003 strains [43, 45, 325]. However, the loss of protection following drift of field viruses was identified for the 1994 H5N2 AI vaccine strain used in Mexico against the emerged 1998 Mexican and 2003 Guatemalan LPAI field strain [327, 333]. Furthermore, with the advent of large-scale vaccination campaigns against the H5 Gs/GD-lineage HPAIVs, field viruses that were resistant to licensed vaccines have been identified in China, Hong Kong SAR, Egypt, Indonesia, and Vietnam [190]. In response, China has changed H5 vaccine seed strains approximately every 4–5 years as vaccine-resistant field strains have emerged [2, 86, 344]. Similarly, in Korea and China the H9N2 field LPAIVs have drifted away from the commercial vaccine seed strains [339]. As a result, AI vaccines should be constantly evaluated for protection against emergent drift field strains. At the very minimum, *in-vivo* protection against current circulating field viruses should be assessed every 2 years, and a continual program for assessing serological cartography of new field viruses against HI antisera produced with vaccine strains should be a routine assessment tool.

Indirect assessment of protection

Direct assessment by an *in-vivo* challenge model for vaccine efficacy is time consuming and expensive, but is necessary for initial vaccine registration as a demonstration that the vaccine is protective against the specific field virus. However, indirect assessment can be a viable option in some situations to assess protection, especially when determining the consistency of vaccine batches as a means of ensuring a minimal protective level. Such indirect assessment of protection can be based on protective serological responses, such as neutralization or HI antibody titers in vaccinated birds, or on quantification of the HA (which elicits a protective immune response for inactivated AI vaccine) or the infectious titer of live vaccines [243, 319]. Quantification of HA in inactivated vaccines has been accomplished by the radial immunodiffusion assay [214], infectious titer prior to inactivation [394], hemagglutinating titer [323], receptor-binding surface plasmon resonance (SPR) assay [323], and immunological methods of quantifying HA protein, such as ELISA assays or other immuno-based assays [171]. Alternatively, the HA protein quantity can be calculated using a protein/nucleic acid formula and

nucleic acid content obtained from a quantitative RRT-PCR assay [109, 131, 197]. For recombinant vectored or live virus vaccines, virus titers in ECE or cell cultures are appropriate indirect measures. The lack of anamnestic HI response after challenge of vaccinated birds indicates a high level of protection, but such an observation is not feasible as an indirect measure of efficacy.

Potency

Potency measurements provide a quantitative assessment of protection that ensures adequate efficacy under a variety of field conditions, and not just to a minimal level of protection. Theoretically, potency testing ensures that there is sufficient and consistent antigen mass or virus titer to be efficacious under field usage. Potency for some poultry vaccines, including NDV and AI vaccines, has been quantified in a mean protective dose (PD_{50}) test in SPF chickens under laboratory conditions, with challenge occurring at 21 days after vaccination (i.e. the PD_{50} is the dose of vaccine that provides protection in 50% of the birds) [333]. In experimental studies, PD_{50} for AI vaccines has measured prevention of mortality in a series of reduced vaccine doses, such as 1×, 0.1×, 0.01×, and 0.001×, followed by intranasal challenge with a defined dose of HPAIV [323, 355]. Using these data, the PD_{50} becomes a simple mathematical calculation. Once the PD_{50} has been calculated, the minimum number of PD_{50} per dose for a potent vaccine must be decided. For NDV, potent vaccines should contain an average of 50 PD_{50} per dose with a minimum deviation to no less than 35 PD_{50} [84, 323]. However, potency could also be determined indirectly by serological response of the birds to different vaccine doses, or by quantification of the HA protein in the vaccine. A reduction of the vaccine dose below 50 PD_{50} per dose has had a negative effect on protection [355].

In previous experimental studies with inactivated AI vaccines in SPF chickens challenged with HPAIV, survival was associated with an HI antibody geometric mean titer (GMT) of ≥ 8 [128] or ≥10 [323], prevention of oropharyngeal shedding of the challenge virus in most vaccinated chickens was associated with a GMT of ≥ 40 [182], and complete prevention of such shedding in all vaccinated chickens was associated with a GMT of ≥ 128 in antigenically closely matched vaccines and challenge viruses [182]. Development and implementation of minimum serological potency standards would ensure that sufficient antigen is contained in commercial poultry inactivated AI vaccines that would produce a protective immune response in the field for vaccines with a close antigenic match to circulating field strains. Because of cell-mediated immunity induced by live recombinant vaccines, HI serological standards may not be directly applicable for potency determination on single vaccination, but use of recommended prime with live recombinant with boost using an inactivated or other HA protein-based vaccine vaccines may favor HI potency determination in the field.

Potency testing based on ≥ 90% survival in HPAIV challenge study at the recommended vaccine dose has been used, but is a much less stringent measure of potency than 50 PD_{50} standard [333]. Live NDV vaccine potency in the USA is based on virus titer per dose (9 CFR 113.329), which could potentially be used to determine the potency of live virus vectored AI vaccines.

Types of avian influenza vaccines

Categories of avian influenza vaccines

Over the past 50 years, the worldwide use of vaccines has profoundly reduced infectious diseases in poultry. High-quality vaccines can elicit immune protective elements such as circulating antibodies, specific cytokines, effector cells, and various antigen-specific memory lymphocytes. AI vaccines fall into four broad categories [355], namely inactivated whole AIV, *in-vivo* expressed HA protein (and potentially other AI viral proteins), *in-vitro* expressed HA protein (and potentially other AI viral proteins), and nucleic acids (Table 15.5). The vaccines in each of these categories have specific advantages as well as disadvantages.

Inactivated whole avian influenza virus vaccines

The majority of AI vaccines that are registered and used in the field are inactivated whole AI vaccines licensed for parenteral (subcutaneous or intramuscular) administration (Table 15.5) [330].

Table 15.5 Compiled information on AI experimental and field vaccine studies in poultry and other birds.

Vaccine category	Vaccine	Species	Challenge	Protection criteria			Comments	References
				Prevent morbidity/ mortality	Decreased shedding	Prevent contact transmission		
Inactivated AIV	Oil emulsion	Chicken: layer (SPF, C) and broiler (SPF, C)	Field, LPAIV, HPAIV	Yes	Yes	Yes	Experimental and commercial vaccines tested; SQ, IM and *in-ovo* routes protective; better protection produced if vaccinated at 4 weeks of age versus 1 day of age; DIVA strategies included non-vaccinated sentinels, and heterologous NA and NS1 serological tests; HA content of vaccine correlated with protection and gives broad subhomotypic protection; commercial vaccines varied in antigen content and quality; PD_{50} good measure of vaccine protection; transmission completely blocked at 2 weeks post vaccination but only partially blocked at 1 week; infectious clone vaccine strains available; protection to 43 weeks with 1 SQ vaccination; 9 doses given orally protected chickens from HPAIV challenge; can protect from egg production drops; metabolizable oils can be used in place of mineral oil as adjuvants; two doses of ISCOM used to achieve serological response and protection; the closer the relatedness of vaccine and challenge virus the better the protection from shedding; reduced levels of serum acute-phase proteins in vaccinated compared with shams after LPAIV challenge; multiple vaccinations will broaden the antigenic profile but will not protect against challenge by viruses that have broad antigenic differences; maternal antibody delays serological response by 14 days; reducing vaccine dose by half reduced HI response by 0.5log10; priming with gel-based H9N2 vaccine followed by boost with oil-based H9N2 vaccine had improved protection; H9+M1 VLP provided protection and DIVA compatible; bivalent inactivated NDV and rgH5(LPAIV) protected chickens from virulent NDV and H5N1 HPAIV challenge	[46]

(*continued*)

Table 15.5 (*Continued*)

Vaccine category	Vaccine	Species	Challenge	Protection criteria			Comments	References
				Prevent morbidity/ mortality	Decreased shedding	Prevent contact transmission		
		Turkey (C)	Field, LPAIV, HPAIV	Yes	Yes	Yes	Experimental and commercial vaccines tested; DIVA strategies included non-vaccinated sentinels, and heterologous NA and NS1 serological tests; increased reduction in challenge virus shedding with two or more vaccinations; vaccination increased resistance to infection by 10^2 virus; NP/HA ISCOM vaccine produced partial reduction in challenge virus shedding from respiratory tract; superior protection with positively liposomal-acridine adjuvant vaccine; multiple vaccinations can provide broader protection against antigenically divergent H1N1 LPAIV, protection from drops in egg production, improved egg quality, and decreased shedding from cloaca; demonstration field H5N1 HPAIV in Egypt not protected by some classical and heterologous H5 vaccines	[1, 2, 5, 8, 10, 11, 28, 33, 34, 41, 42, 46, 68, 83, 86, 89, 98, 105, 106, 122, 129, 163, 193–196, 205, 234, 264, 287, 294, 295, 302–305, 309, 323, 327, 333, 347, 353, 356, 364, 368, 392, 394, 398, 407]
		Duck (C)	HPAIV, LPAIV	Yes	Yes		Two vaccine doses produced best protection, with protection up to 52 weeks; bivalent AI vaccine did not interfere with protection; reverse genetic clone vaccine strains are available; two doses protected Muscovy ducks from HPAIV; two doses prevented viremia and visceral organ infection in Pekin ducks; heterologous H5 vaccines protected Pekin and Muscovy ducks from mortality, but suboptimal reduction in shedding; single vaccination in Pekin ducks protected better than in Muscovy ducks for heterologous GS/GD-lineage H5N1 HPAIV; two vaccination give best protection	[3, 63, 110, 115, 162, 165, 167, 173, 178, 223, 234, 363, 364, 384, 401]
		Geese (C)	HPAIV	Yes	Yes		Infectious clone vaccine strains are available, three doses required to provide 34 weeks' protection; one vaccination of geese inadequate for good protection from HPAIV challenge	[21, 32, 53, 54, 74, 102, 227, 241, 246, 251, 259, 298, 356, 392]
		Other poultry	No challenge				Houbara bustards produced HI antibodies; inactivated vaccine in Chinese painted quail	[102, 356]
		Zoo birds	No challenge				Serological response to H5 and H7 inactivated AI vaccines, two doses – 76%, 80.5%, and 81.5% had HI titers of ≥ 32	[273, 393]
In-vitro expressed HA	Baculovirus in insect cell culture	Chicken: layer (SPF); Muscovy duck (C)	HPAIV	Yes	Yes		HA content and virus strain influenced protection; NA insert only partially protective; HA/NA/M2 combined oil-emulsion vaccine was protective in Muscovy ducks against LPAIV; DIVA compatible; broad intraclade protection (2.2 Gs/GD lineage); can be used to produce protective VLP from H5N1 HPAIV and H5 LPAIV	[27, 247, 248, 279]

	Vaccinia in TK-143 or chicken embryo fibroblast cultures	Chicken: layer (SPF, C)	HPAIV	Yes		Good protection from intraperitoneal, IM, or subdermal routes; partial protection from scarified skin route; bursectomized chickens not protected; live virus non-infectious or poorly infectious to chickens; oil-emulsified vaccine protective; partial protection with NA-based vaccines	[22, 88, 120, 242, 251, 252, 327, 346]
	Non-replicating adenovirus in PER.C6 cells	Chicken: layer (SPF)	HPAIV	Yes	Yes	Complete protection when administered by SQ or *in-ovo* routes, but only partial protection by IN route; mucosal (ocular) prime and boost given provided protection against HPAIV	[75, 95, 388]
	Alphavirus-based virus-like replicon particles	Chicken: layer (SPF)	HPAIV	Yes	Yes	NA insert only partially protective after two to three vaccinations	[121, 226, 359, 360]
	Duckweed expressed HA	Chicken: layer (SPF)	HPAIV	Yes	Yes	Similar protection to that which HA produced in embryonating eggs	[30]
	Other systems					Polypeptide of HA produced in Rosetta 2 *E. coli* cells (DE3) produced HI antibodies; yeast (*Pichia pastoris* and *Schizosaccharomyces pombe*) expressed HA; *E. coli* expressed HA in expression vector pBCX	[282, 346]
In-vivo expressed HA	Live AIV	Chicken (C)	HPAIV	Yes		Homologous NA partial to occasionally complete protection from mortality when challenged with HPAIV; best HI response with spray or cloacal vaccination routes; wild-type LPAIV transmitted by contact and not recommended as AI field vaccine; AI-NDV chimera poor replication in post-hatch chickens, but *in-ovo* vaccination protects from HPAIV and velogenic NDV challenge; NS1 deleted mutant AIV given intranasally protected chickens from HPAIV challenge, and vaccine did not spread by contact; NS1 deleted mutant AIV work with NS DIVA test; passage in chickens resulted in NS1 deleted mutant reverting to wild-type virus phenotype; live attenuated H5, H7, and H9 administered *in ovo* protects; temperature-sensitive mutations in PB1 and PB2 generate a vaccine backbone for poultry H9 and H5 vaccines	[168, 387, 395, 397]
		Turkeys (SPF)	LPAIV		Yes	H3N2 strains with partial deletions in NS gene, reduced number of poultry and titer of challenge virus shed from oropharygnx	[4, 5, 13, 26, 38, 52, 55, 89, 225, 269, 289, 297, 382]

(*continued*)

Table 15.5 (*Continued*)

Vaccine category	Vaccine	Species	Challenge	Protection criteria			Comments	References
				Prevent morbidity/ mortality	Decreased shedding	Prevent contact transmission		
	Fowl poxvirus vectored	Chicken: layer (SPF, C) and broiler (SPF, C); Goose (C); Muscovy ducks (C)	HPAIV, LPAIV	Yes	Yes	Yes	H5 and H7 vaccines developed; broad protection by AI-H5 insert vaccine against 1959–2004 H5 HPAIV given in low to very high challenge doses in chickens; efficacious if given by SQ or WW routes but not by comb scarification, eyedrop, IN, or drinking water routes; duration of immunity over 20 weeks from single vaccination; pre-existing immunity to vector inhibits vaccine efficacy; greatest reduction in respiratory shedding of challenge virus if vaccine and challenge viruses were more genetically similar; protection 40–45% at 1 week, 95–100% after 2 weeks post vaccination; NP express vaccine not protective; maternal immunity against fowl poxvirus does not inhibit post-hatch immunization at 1 day; SQ immunization of geese at 21 days protected 80% from mortality, reduced viral shedding, and shortened infection; WW immunization only gave partial protection in turkeys; two doses provided protection in Muscovy ducks from HPAIV, but slightly less effective than inactivated vaccine; H7 insert 2002 Virginia H7N2 LPAIV protected chickens from H7N3 Mexican HPAIV; effective as a priming vaccine followed by inactivated boost for Pekin and Muscovy ducks and chickens; one study with raccoonpox vector with H5 HA insert elicited Hi antibodies in chickens	[384]
	Avian leukosis virus vectored	Chicken (SPF)	HPAIV	Yes			In chickens, vaccine with AI HA insert was protective, but with AI NP was not protective	[17, 19, 28, 37, 45, 47, 48, 56, 80, 124, 149, 156, 228, 251, 256, 257, 298, 322, 324, 325, 330, 352, 361, 389, 390]
	Paramyxovirus type 1 vectored (lentogenic Newcastle disease virus)	Chicken (SPF)	HPAIV	Yes	Yes		Mass immunization vector (eyedrop or spray); protects from both velogenic NDV and HA-subtype-specific HPAIV; prime with rNDV and killed boost better protection than 2 rNDV vaccinations; rNDV-H9, H7, H6 effective in SPF chickens against respective subtype challenge virus; chimeric rNDV with F and HN genes from APMV-8 was not inhibited by maternal antibodies against NDV	[39, 147]

	Gallid herpesvirus-1 (infectious laryngotracheitis virus [ILTV] vector)	Chicken: layer (SPF)	HPAIV	Yes	Yes	ILTV vector was attenuated by ΔUL0 or ΔUL50 insertions of AIV-H5 or H7 HA; mass immunization potential (eyedrop or IT routes)	[112, 125, 126, 244, 266, 272, 280, 281, 300, 329, 374]
	Herpesvirus turkey					rHVT with clade 2.2 Gs/GD-lineage H5 protected clade 2.1.3 HPAIV in maternal-antibody-positive broiler chickens, and prime-boost effect with inactivated vaccine	[212, 373]
	Duck viral enteritis herpesvirus	Domestic duck; chicken: layer (SPF)	HPAIV	Yes	Yes	Requires high vaccine doses to protect SPF chickens	[290]
	Vesicular stomatidis virus	Chicken: layer (SPF)	HPAIV	Yes	Yes	Recombinant vesicular stomatitis virus (VSV) vectors expressing H5 HA and deletion of G gene of VSV genome	[204, 381]
	Salmonella species vector	Chicken: layer (SPF)	LPAIV, HPAIV	Yes (LPAIV only)	Yes (LPAIV only)	*Salmonella enteritidis* Δ*aro*A-Δ*htr*A vector expressing M2e protein when given twice orally reduced shedding of LPAIV, but not from mortality of HPAIV; *Salmonella enteria* serovar Typhimurium vaccine strain with H5 (clade 2.2) insert after four oral immunizations (at 1-week intervals) reduced mortality by 50%	[136, 159]
	Lactococcus lactis	Chicken: layer (SPF)	HPAIV	No	No	Intranasal or subcutaneous M2e expressed in *Lactococcus lactis* prolonged time to death but did not prevent mortality	[185, 201]
DNA	Naked DNA	Chicken: broiler (SPF), layer (SPF); Muscovy ducks	HPAIV, LPAIV	Yes	Yes	Requires high doses of DNA and multiple immunization if given by IM, intraperitoneal, or IV routes to produce partial protection; response improved when using pCI-neo HA plasmid and lipofectin or lipotaxi adjuvants; gene-gun administration reduced quantity of DNA after two vaccinations to produce protection; NA only partially protective; NP gene not protective; DNA vaccine followed by HA protein prime boost in chickens gave the best protection from LPAIV challenge versus DNA/DNA or HA protein/HA protein primary and boost vaccinations; electroporation gave better serological response than intramuscular injection	[263]

Such non-replicating vaccines have been preferred because of their safety in conventional and immunocompromised hosts. However, in order to induce protective immunity, inactivated vaccines require injection of high antigen quantities, and the inclusion of adjuvants greatly enhances immunogenicity [338]. The route and timing of vaccination will affect the immunogenicity and efficacy of the immune response to the vaccine. Most often, AI vaccines are combined with other viral and bacterial vaccines, and administered concurrently at specific time periods in the bird's life, and such logistic issues must be considered when developing a program for AI control. Numerous experimental studies have described the efficacy of inactivated AI vaccines in avian species (Table 15.5).

Inactivated AI vaccines have primarily utilized seed stock of LPAIVs obtained from field outbreaks, and occasionally HPAIVs, the latter being used in high biocontainment manufacturing facilities [275–278]. Beginning in 2006, licensed vaccine strains have been developed and used in the field that utilized reverse genetic technologies incorporating the HA and NA of recent field AIVs and the remaining six gene segments obtained from a high growth influenza A vaccine virus such as PR8 [330, 332]. The reverse genetic (rg) generated seed strains usually have the HA proteolytic cleavage site altered from HPAIV to LPAIV. Of the 125 billion doses of H5 or H7 inactivated poultry AI vaccines that were used to manage HPAIVs between 2002 and 2010, 71.9 billion doses used rgLPAIV seed strains, and 53.1 billion doses were based on naturally occurring LPAIV or HPAIV seed strains [77, 152, 191, 205, 292, 344, 356, 392]. Regardless of the source, the seed viruses are grown in ECE, and the infective allantoic fluid is collected, chemically inactivated, and emulsified in a mineral oil adjuvant system, the latter being proprietary to each veterinary vaccine company. The degree of purification of the allantoic fluid can affect the overall response following vaccination, but in order to reduce cost, most AI vaccines in poultry use crude allantoic fluid without purification. Typically, inactivation of the virus is achieved with formalin, which cross-links the viral proteins such that viral replication cannot occur. Other chemicals, such as β-propiolactone or binary ethyleneimine, have been used as inactivants [78, 338].

Adjuvants

Inactivated whole AI virus or virosome vaccines are formulated with adjuvants prior to application. Vaccine adjuvants are chemicals, microbial components, or mammalian proteins that enhance immune responses to vaccine antigens [175]. Adjuvants are necessary to activate and direct the innate and adaptive immune responses to the rather poorly immunogenic inactivated vaccine antigens. In general, although it is dependent on purity and quantity, the antigen is a passive element, and the adjuvant represents the activating and modulating intermediate operating at the interface between the immune system of the host and the administered vaccine inoculum. A single adjuvant may have more than one mechanism of action. Interest in reducing vaccine-related side effects and inducing specific types of immunity has led to the development of numerous new adjuvants. Adjuvants in development or in experimental and commercial vaccines include aluminum salts, oil emulsions (including proprietary adjuvants such as Montanide, etc.), saponins, immune-stimulating complexes (ISCOMS), liposomes, microparticles, non-ionic block copolymers, polysaccharide derivatives (e.g. β-1,3/1,6 glucan [379]), CpG oligodeoxynucleotides [187], toll-like receptor (TLR) ligands [215, 216, 385, 396], small peptides (e.g. bursopentene [Cys-Lys-Asp-Val-Tyr] [200]), pathogen pattern recognition receptor agonists (CVCVA5) [198], cytokines [350], and a wide variety of bacterial derivatives [396, 399, 400]. Less purified inactivated vaccines sometimes contain bacterial or viral components that can serve as "built-in" adjuvants, whereas more purified antigens do not usually stimulate a strong and lasting immune response [9, 92, 110, 116, 130, 141, 146, 169, 267, 271]. Aluminum and calcium salts are relatively weak adjuvants that mainly induce type 2 T-helper lymphocyte (T_h2) responses and few, if any, antigen-specific cytotoxic T-lymphocytes (CTLs) [237].

Oil adjuvants

Oil emulsion adjuvants contain a mixture of oil and aqueous phase stabilized by a surfactant, and have been commonly used in experimental and licensed inactivated AI vaccines for poultry. These emulsions contain the antigen in either "oil-in-water" or "water-in-oil-in-water" formulations. Without

other components, oil-based adjuvants stimulate mainly antibody responses, although under some circumstances water-in-oil emulsions may be able to activate CTLs [237]. Most licensed and experimental poultry inactivated vaccines, including AI vaccines, use a mineral oil base, but metabolizable oils have been shown to be effective [9, 25, 114, 302, 304, 306–310, 398]. The advantages of using emulsions include enhanced antibody production, as well as extended release of the antigen, which results in an overall higher immune response of the birds to the vaccines.

Experimentally, nanoemulsion formulations made from soybean oil, tributyl phosphate and Triton X-100 provided early protection of mice against an intranasal lethal challenge with influenza virus [310]. The nanoemulsion was a mixture of non-ionic detergents, generally-recognized-as-safe (GRAS) list solvents, and soybean oil. The nanoemulsion had no toxicity in the upper respiratory tract in mice, and also protected them against challenge with influenza virus.

Liposomes

A variety of liposomes have been used as adjuvants in mammalian and avian experimental vaccine studies, including AI vaccines [100], but to date the technology has not been applied to licensed AI vaccines for use in poultry in the field. Liposomes are vesicles of cholesterol and phospholipids that resemble crude cell membranes. As adjuvants, liposomes can incorporate the desired antigens either within the center of the vesicle or within the cell membrane. They can induce humoral immunity and, in some cases, activate CTLs [110]. Nanoparticles and microparticles are tiny solid particles made from biodegradable polymers, certain cyanoacrylates and poly (lactide-co-glycolide) copolymers. Nanoparticles (10–1000 nm in diameter) differ from microparticles (1–100 μm) only in their size. Microparticles can induce cell-mediated immunity (CMI), including CTLs and humoral immunity [379]. Calcium phosphate nanoparticles induced mucosal immunity and protection against herpes simplex virus type 2 in mice [237]. Chitosan readily forms microparticles and nanoparticles that encapsulate large amounts of antigens such as ovalbumin, diphtheria toxoid, or tetanus toxoid. Chitosan particulate drug carrier systems are promising candidates for oral vaccination. After co-administering chitosan with antigens in nasal vaccination studies, a strong enhancement of both mucosal and systemic immune responses was observed in mice [141]. Saponins are complex chemical adjuvants extracted from plants, most often the tree *Quillaja saponaria*. Saponins are immunomodulators, and can induce strong type 1 T-helper lymphocyte (T_h1) and T_h2 responses as well as CTLs in animals. Saponins may stimulate CMI to an antigen that would normally induce only antibodies [150, 372]. ISCOMS are cage-like structures that contain saponins, cholesterol, and phospholipids. They can induce T_h1 reactions and CTLs as well as concurrent T_h2 responses under some circumstances [379]. Non-ionic block copolymers are synthetic adjuvants composed of hydrophobic polyoxypropylene flanked by blocks of polyoxyethylene. As adjuvants, these chemicals can enhance humoral immunity to many antigens, but most often they are used in an aqueous buffer, oil-in-water, or water-in-oil emulsions [236, 271, 275]. Muramyl dipeptide (MDP) is the active component of an immunomodulatory peptidoglycan from mycobacteria. The MDP induces mainly T_h1 and T_h2 responses. MDP derivatives are often incorporated into liposomes, water-in-oil, and oil-in-water emulsions [6]. Bacterial toxins, cholera toxin (CT), and *Escherichia coli* heat-labile exotoxin (LT) have been tested most extensively as mucosal adjuvants in animal models. They appear to induce strong humoral responses as well as CTLs [87, 237]. Cytokine protein and genes are themselves being considered as vaccine adjuvants [87, 114, 127, 260]. The specific effects vary with the cytokine – some enhance the activity of defined immune cells, while others act as general activators. Cytokines also induce other cytokines, and this property can make the effects of a specific cytokine difficult to predict [202].

In-vivo expressed hemagglutinin

With *in-vivo* expression systems, the immunogen is produced within the bird host by use of a live bacterial or viral vector such as rFPV, rHVT, recombinant duck virus enteritis (rDVE), some adenoviruses, replication-deficient Venezuelan equine encephalitis virus (rdVEE), recombinant avian leukosis virus (rALV), recombinant infectious laryngotracheitis virus (rILT), rNDV, AI-NDV chimera virus,

Salmonella species, or other organisms [143, 224] (Table 15.5). In theory the method has lower manufacturing costs because the process uses the bird's own cells to produce the immunogen, rather than expensive *in-vitro* expression systems. The advantage of this type of vaccine is that it can stimulate humoral and cellular immunity when given parenterally, and if it replicates at a mucosal site it can induce mucosal immunity. Live virus vaccines are usually superior to inactivated vaccines in inducing mucosal immunity and thus reducing shedding, but are susceptible to inhibition of vector replication if active or passive immunity is present against the vector and sometimes the insert AIV HA.

Live AIV vaccines

Live LPAI vaccines have been studied experimentally in poultry (Table 15.5). They offer the advantages of good protection against HPAIVs, can be mass applied by spray vaccination or in drinking water, are economical, and provide more rapid protection than inactivated vaccines [334]. However, live unaltered LPAIV strains are not recommended for use as poultry vaccines for a number of reasons [13, 14, 225]. First, they can produce economically important production losses associated with respiratory disease or drops in egg production. Second, they can easily spread from bird to bird and from farm to farm, potentially creating endemic infection and disease with the need for eradication of the vaccine strain. Third, some LPAIVs have the potential for mutation or reassortment, creating more pathogenic viruses, as has been reported, for example, in the case of some H5 and H7 LPAIVs becoming HPAIVs in the field. Currently, no live AI vaccines are registered and used in the field.

However, various genetically altered influenza A viruses that have been developed and investigated in mammals and birds, allowing regulated viral replication and the induction of immunity without negatively affecting growth or immuncompromising the individual. This strategy for developing a genetically altered live influenza A virus vaccine involved attenuating the virus to a lower pathogenicity level either through laboratory passage to generate cold-adapted, temperature-sensitive phenotypes, or through biotechnology to alter the viral genome directly. The majority of these types of vaccines have been developed and tested in mammalian models [14, 15, 18, 192]. Recently, through reverse genetic (rg) technology, temperature-sensitive mutations were introduced into the PB1 and PB2 genes of an H9N2 LPAIV to produce a live LPAI vaccine backbone [76, 97, 210, 318]. This cold-adapted H9N2 virus was used as a vaccine backbone with an rg system to replace the H9 and N2 genes with H5 and N1 genes, and produced a cold-adapted H5N1 LPAI vaccine strain that protected chickens against both H9N2 LPAIV (original H9N2 vaccine construct) and H5N1 HPAIV challenge (rgH5N1 construct) [291]. The vaccine elicited both cellular and humoral immunity. A theoretical concern is the potential of the live cold-adapted AI vaccine virus to revert or recombine with field viruses, resulting in a virus with enhanced pathogenicity or expanded host range. However, the reassortant, cold-adapted phenotype has been applied to influenza A vaccines for humans with no serious side effects and without evidence of reversion to virulent virus [233]. Live AI vaccines are not currently approved for use in commercial poultry.

In addition to temperature-sensitive mutant live influenza A virus, a laboratory-passaged, attenuated AIV has been described that is not based on cold adaptation. This AIV has a truncation of the NS1 gene in A/turkey/Oregon/1971 (H7N3) LPAIV, and resulted in decreased replication in chickens and attenuation of virulence [386], but this attenuation was insufficient to allow *in-ovo* vaccination, as hatchability was reduced [72, 192, 382]. This virus has the potential for use as a post-hatching vaccine, but its safety must be determined before licensure and use in the field. In one study of an NS-truncated mutant, five times passage of rgH5N3/NS1/144 live attenuated vaccine resulted in reversion to the wild-type LPAIV phenotype [383], indicating the need to adequately assess live AI vaccine for reversion to virulence before registration and application in the field.

The development of infectious clones through rg technologies for influenza A viruses has created a system that allows directed mutations in one or more influenza gene segments that could result in sufficient attenuation to allow usage as a live vaccine seed strain. Recently, an AI virus vaccine was developed that contains 8 gene segments of H5 HPAIV (HA cleavage site altered from HP to LP),

but includes the ectodomain of the HN gene from NDV instead of the NA gene of the AIV [38]. The resulting virus was attenuated as demonstrated by inconsistent replication in 2-week-old chickens, but virus administered *in ovo* produced a humoral antibody response, and vaccinated chickens were protected from both H5 HPAIV and virulent NDV challenges [244].

Live vectored vaccines (non-influenza A viruses and bacteria)

Other types of live virus vaccines have been developed for AI using alternative virus vectored constructs or bacterial vectors, and can provide some of the immunological advantages of a live vaccine, but without the reassortment risk of using a live, fully replication-competent AIV (Table 15.5) [297]. This category of vaccines provides broader protection across antigenically divergent AIVs within the same subtype [334]. These types of vaccines utilize recombinant DNA technologies to incorporate genetic material from the AIV genome, typically the HA gene, into a viral backbone for gene expression *in vivo*. Many examples of these types of vaccines have been documented in the literature, with varying levels of success, but the most frequently reported system has been the recombinant fowl poxvirus (rFPV), with H5 (rFPV-H5), H7 (rFPV-H7), or H9 (rFPV-H9) AIV HA gene inserts [164, 325]. An rFPV-H5 vaccine was licensed in 1998 in the USA for emergency use in HPAI outbreak situations, but has not been used to date in the field in the USA [17, 28, 36, 37, 43, 44, 79, 124, 155, 199, 255, 257, 258, 320, 321, 325, 352, 361, 389]. However, this vaccine has been used extensively in Mexico, El Salvador, and Guatemala against endemic H5N2 LPAIVs (1998–2015) [229]. Other recombinant live vectored vaccines have been engineered to express AIV genes and have demonstrated experimental protection in chickens. These include virus vectors (rILT virus [herpesvirus], rNDV [paramxyvirus type 1], rdVEE virus [alphavirus], rALV [retrovirus], rHVT [herpesvirus], rDVE [herpesvirus of ducks], recombinant adenoviruses) and bacteria (*Salmonella enteritidis, Salmonella typhimurium*, and *Lactobacillus lactis* vaccine vectors) [377, 378]. The rNDV, rILT, and some recombinant adenovirus vectors could be applied by mass administration via drinking water or sprays to reduce costs, because they replicate in the mucous membranes. However, some vectors, such as rdVEE virus [39, 75, 125, 185, 199, 204, 209, 212, 226, 235, 266, 282, 290, 329, 348, 349, 373, 374, 381], recombinant replication-incompetent adenoviral vector [282], rHVT [121, 301, 359], rDVE [290], rALV, and rFPV [204, 381], require injection in order to produce an effective immune response, but mucosal administration (via the conjunctival sac) of a recombinant replication-incompetent adenoviral vectored-H5 vaccine twice did induce protection against HPAIV [19].

Although protection derived from inactivated AI vaccines is largely based on the induction of neutralizing antibodies produced against the HA subtype, broadly cross-reactive cytotoxic T-lymphocytes (CTLs) have been reported to be critical for clearance of virus from infected cells. However, little is known about the induction of CMI against AIV in chickens. rHVT is a known inducer of cell-mediated immunity, and has been shown to induce specific cell-mediated immunity [142, 262]. Recently, cross-reactive CTL activity induced against the HA protein following vaccination with rHVT in chickens was demonstrated [161]. In addition, a T-cell epitope found within the H5 HA has been recognized by both $CD4^+$ and $CD8^+$ chicken T-cells [135]. The vaccine-induced CMI also recognized different subtypes of AIV, all of which contained this epitope with varying degrees of similarity. Thus recombinant live-vectored vaccines expressing the HA appear to induce CMI, which probably contributes to protection.

One additional hurdle to the application of virus-vectored vaccines in the field is the immune status of the birds against the vector. For example, most poultry raised in the developed world have been immunized against NDV, which will limit or restrict the immunogenicity of the rNDV vectored vaccines, unless NDV immunity is serologically monitored and the vectored vaccine is applied only when antibody levels are sufficiently low to produce an anamnestic response. Swayne and colleagues have described how pre-existing immunity, most likely cell-mediated immunity, against fowl poxvirus will interfere with the primary immune response of rFPV-H5 vaccine in chickens, preventing protection [226], but field data suggest that maternal antibody against H5 AIV or fowl poxvirus vector did not interfere with the priming immune response to rFPV-H5 vaccine given at 1 day of age

[324]. Furthermore, using a prime boost regime with rFPV-H5 and inactivated H5 AI vaccine in H5 maternal-antibody-positive chickens provided better protection from H5N1 HPAIV challenge than either vaccine alone [56, 124].

In order to make recombinant vaccines more effective, some modifications may be necessary to protect from multiple pathogens and improve replication in the host for immunity against individual pathogens. For example, in some developing countries with circulation of multiple AIVs plus NDV, the use of rFPV-H5, rFPV-H9, and rFPV-NDV in a trivalent vaccine can be effective against exposure to any of the respective challenge viruses [206]. With rNDV-H5, an improved vaccine was produced by modifying the open reading frame of the H5 gene so that the transmembrane and cytoplasmic domains of the H5 gene were replaced with those of the NDV F protein [207]. Recently, a chimeric NDV vector was developed utilizing the fusion (F) and hemagglutinin-neuraminidase (HN) proteins of avian paramyxovirus type 8 (APMV-8) as surface proteins, and not the F and HN genes of NDV. The resulting vector was not inhibited by maternal antibodies to NDV, and provided protection against inserted and expressed H5 gene [235]. APMV-8 has not been identified in poultry, and is thus unlikely to have naturally occurring maternal or actively acquired humoral antibodies.

Finally, the host range is important in terms of knowing which bird species can be immunized with each vectored vaccine. For example, rFPV and rILT have produced protective immunity and are only used in chickens because of the host restriction of the vector, but there is some evidence that rFPV-H5 can produce a protective immune response in cats and geese [300]. In addition, rFPV with NDV gene inserts is efficacious against virulent NDV in turkeys, so the rFPV with AIV HA genes would potentially be effective against AIV challenge in turkeys. The rFPV is best applied in the hatchery at 1 day of age. Thus, while it is clear that live vectored vaccines have advantages in terms of immunity, their use in the field may be limited by other factors. The recently developed rDVE-H5 vaccine provides good protection in domestic ducks, and when given at high doses as a vaccine it can protect chickens from H5N1 HPAIV challenge, and has no maternal antibodies to interfere with the vector, as occurs with rNDV-H5 [156, 166].

In-vitro expressed viral proteins

Various *in-vitro* expression systems have been used to produce experimental AI vaccines, but none of the technologies have been commercialized for poultry AI vaccines (Table 15.5). The specific expression methodology varies, but the total amount of viral proteins, principally the HA, to be administered is produced in eukaryotic cell cultures (e.g. plant and animal cells [204, 381]), plants (e.g. duckweed or tobacco [88, 183]), yeast [30, 160], or bacteria [221, 270], with virus vector systems being used for expression in some of the eukaryotic systems (e.g. vaccinia [170], some replication-deficient adenovirus and baculovirus [12, 75, 95, 387]). The most frequently used system involves insertion of the AIV HA into a baculovirus (insect virus) vector and infection of insect cell cultures, with expression and production of the HA [88, 301]. The HA is then recovered from the supernatant or cell lysates, inactivated with chemicals (if necessary), and emulsified in a similar manner to inactivated whole AI vaccines. Therefore the entire quantity of the immunogenic protein (i.e. HA) is produced external to the bird host.

DNA vaccines

Plasmid-based experimental DNA vaccines using the HA gene have elicited a protective immune response in chickens against a variety of H5 and H7 HPAIVs [22, 88, 327]. Such vaccines produce antigen *in situ*, inducing both adaptive humoral and cellular immune responses, similar to those produced by live virus infection or vaccination [118, 179, 180, 203, 265, 311]. Typically, DNA vaccines are naked nucleic acids containing AIV cDNA within various plasmids under control of a mammalian promoter gene [192]. Studies in mammals have shown that DNA vaccines expressing HA genes produced more effective protection against antigenic variant influenza A viruses than inactivated vaccines, and inclusion of the NP gene augmented the protection [118, 179, 311]. However, protection induced by DNA vaccine in an avian model (i.e. the chicken) has been less consistent, and requires more vaccinations than that produced by protein-based vaccines using inactivated whole AI vaccines or *in-vitro* or *in-vivo* expressed HA subunit vaccines [192, 311]. The main limitations of the use of DNA vaccines in

poultry are the requirement for large quantities of expensive nucleic acid per dose to produce a protective immune response in chickens, and the fact that protection is only achievable after three or more vaccinations. DNA vaccines will be economically prohibitive for use in the field until promotors are developed to reduce the number of immunizations and quantity of nucleic acids needed per bird, and in experimental studies, single DNA vaccination provides as effective protection as protein-based vaccines, which is the licensing standard. Recently, improvements in DNA vaccination have been demonstrated in some experimental studies. DNA priming followed by an AIV protein boost was more effective than a prime/boost strategy with DNA vaccine alone in chickens [180, 192, 261, 311], and the concurrent use of specific cytokines with DNA vaccines improved the immunological response compared with DNA vaccines alone [120].

Field use of vaccine and special issues

Avian influenza vaccination issues

In terms of AI vaccination to protect poultry, ideally four goals need to be met, namely induction of complete resistance to infection if birds are exposed, prevention of virus replication in and excretion by the vaccinated birds, prevention of clinical disease and death, and easy identification of infected animals within a vaccinated population (i.e. the DIVA principle) [203]. However, few if any commercially available or experimentally tested vaccines consistently fulfill all of these requirements [313]. In experimental studies, most AI vaccines provide consistent protection against clinical disease and death, but do not always provide absolute protection against mucosal infection or shedding of the virus from the oropharynx and cloaca, which is dependent on the virus challenge dose. The risk of infection of vaccinated birds and excretion of challenge or field virus is greatly reduced, and thus transmission and spread are reduced, but absolute prevention of infection is not feasible under most field conditions. The effectiveness of reduction of virus excretion is linked to both a reduction in titer of the virus excreted and the shortened duration of viral shedding. "Silent infections" have been proposed based on experimental studies where shedding is seen in chickens following high challenge doses (10^6 EID_{50}). However, such "silent infections" have not been as convincingly demonstrated in properly vaccinated poultry in the field [192, 313], and vaccination has been shown to dramatically reduce the risk of infection [288]. Furthermore, the risk of virus spread from potential "silent" infections in vaccinated flocks is much lower than that from infected non-vaccinated flocks, as evidenced by the finding that non-vaccinated chickens excreted 100- to 10 000-fold more virus when infected than did vaccinated chickens [49, 51], and the non-vaccinated chickens required 100 to more than 100 000 times less virus in order to produce infection compared with vaccinated chickens [139]. Thus even a less than ideal vaccine can be advantageous over no vaccine use. However, birds that receive vaccines of low quality (i.e. low quantities of HA antigen, poor adjuvant systems, or poorly antigenically matched vaccine seed) may be protected from clinical signs and death, but the challenge/field virus may replicate, with excretion of significant quantities of virus into the environment [45, 63].

Only AI vaccines that are licensed by a country's national veterinary authority should be considered for use in the field. In addition, such vaccines should meet the minimum requirements of the World Organisation for Animal Health (OIE) [122]. International standards are needed to ensure uniform potency and efficacy of AI vaccines [238]. At the very minimum, AI vaccines should meet the following criteria:

1. purity as determined by not being adulterated but containing only the desired immunogen(s) and other adjuvant or carrier compounds, and it must be consistent in composition
2. safety as demonstrated by the absence of adverse effects on the vaccinated host or the environment
3. efficacy to protect in specific quantifiable assays or tests against a specific challenge AIV
4. potency indicating that the vaccine has a sufficient HA antigen mass (inactivated vaccine) or dose (live vectored vaccine) to ensure protection under a variety of conditions.

Protection of vaccinated birds against AI is affected by the antigenic relatedness of vaccine seed strain to challenge virus, vaccine dose ("vaccine quality"), route of administration, management

conditions, and field application method and coverage. Vaccine potency and field application would be ranked first and second in terms of importance (D. Halvorson, personal communication, 31 May 2007).

Vaccine seed strains

Selection criteria

Because the HA is the primary protective protein and induced protection is subtype specific, the vaccine strain must match the HA subtype of the field virus. However, unlike alum-adjuvanted human influenza A viruses, where antigenic drift of field viruses requires changing the vaccine strains every 3–4 years, the inactivated oil-adjuvanted and rFPV-H5 poultry vaccines were far less affected by drift in field viruses until the late 1990s [330]. For example, various H5 LPAI vaccine strains and rFPV-H5 provided broad cross-protection from mortality against diverse H5 field HPAIVs (1959–1997) which differed by as much as 12% in amino acid sequence at the HA1 [312] compared with the challenge HPAIV. However, this subtype-specific broad protection is not absolute within all subtypes and in all field situations, but seems to be most applicable to newly emerged HPAIVs that are antigenically close to predecessor LPAIV in wild birds [323, 325, 327]. Even in the latter scenario, the closer the HA gene sequence similarity between rFPV-H5 vaccine and challenge HPAIV viruses, the greater was the reduction in challenge virus replication and shedding from the respiratory tract [344].

Since the mid-2000s, HPAIVs and LPAIVs that are resistant to AI vaccines have emerged in the field, primarily after long periods of AI vaccine use in the field [325], or potentially in non-vaccinated poultry populations after LPAIVs had circulated, inducing post-infection immunity [71, 132, 190]. The inactivated H5N2 AI vaccine utilized in Mexico from 1995 to 2009 used a 1994 Mexican H5N2 LPAIV strain (A/chicken/Mexico/232/1994 [H5N2]), and provided protection in chickens against the 1995 field HPAIV and the early field H5N2 LPAIV [190]. However, the 1994 vaccine strain was not protective against two later lineages of H5N2 LPAIVs isolated in 1998 from southern Mexico and in 2003 from Guatemala, as evidenced by replication and shedding of the same quantity of either challenge virus from the respiratory tract of vaccinated and non-vaccinated chickens [122, 190]. It is unclear whether the drift in field viruses resulted from immunity following infections in non-vaccinated poultry, or from incorrect vaccination and subsequent infections in vaccinated chickens [103, 190]. The seed strain in Mexican H5N2 vaccines was changed in 2009 to A/chicken/Durango/1558/2006 (H5N2). Additional evidence for the lack of absolute broad protection within a subtype because of antigenic divergence in the HA comes from H7 AIV experimental studies using rFPV-H7 vectored vaccines [190]. Chickens challenged with A/turkey/Italy/4580/1999 (H7N1) HPAIV were only protected if they had received rFPV-H7 vaccines with Eurasian H7 insert, and chickens vaccinated with rFPV containing HA gene inserts from Australian (A/chicken/Victoria/1/1985 [H7N7]) or North American (A/turkey/Virginia/158512/2002 [H7N2]) H7 AIVs were not protected [44]. Because H7 AI vaccines have had no or limited use in Australia and North America, the H7 AIV drift is probably the result of geographic isolation of the H7 viruses in wild birds between different continents and naturally occurring virus drift over long periods of time. Finally, field H5N1 HPAIVs resistant to licensed AI vaccines have been identified in China, Hong Kong SAR, Egypt, Indonesia, and Vietnam, where prolonged AI vaccination has been practiced [334]. Therefore vaccine strains should be reassessed in *in-vivo* and *in-vitro* protection assays on a continual basis, or at the minimum every 2 years, if tested *in vivo* against current circulating field viruses in order to assess protection [2, 86, 344]. If the vaccine strain is no longer protective, a new strain should be selected based on protection studies.

Historically, most inactivated AI vaccine seed strains have been selected from outbreak LPAIVs of the same HA subtype. Many have been used as autogenous vaccines for control of the original LPAI outbreak [332], or used at a later date in other LPAI outbreaks of the same HA subtype [115]. For HPAI, LPAI vaccine strains of the same HA subtype have been protective in poultry, and since 1995 some HPAIVs have been used as vaccine strains in some epidemics – for example, in Pakistan (H7N3 and H5N1), Russia (H5N1), and Indonesia (H5N1, clade 2.1) [314]. Contrary to popular belief, HPAIV strains do replicate to sufficient titer in ECE to

be used in inactivated AI vaccines, but the titer is lower than that of many of the LPAI vaccine strains. However, the safe and biosecure use of HPAIV strains in vaccines requires specialized, high-level biocontainment manufacturing facilities and/or special biosecurity and biosafety personnel procedures. The use of LPAIV strains has fewer biosecurity and biosafety concerns in relation to manufacturing, and is preferable to the use of HPAIVs. When LPAI or HPAI outbreak viruses are used as vaccine strains, they should be a close genetic match to the HA of the current outbreak virus and have high growth characteristics in ECE in order to produce sufficient quantities of antigen to be immunogenic and protective.

During the past two decades, advances in biotechnology have allowed laboratory generation of infectious clone rg AIV seed strains for use in inactivated vaccines or HA gene inserts for use in *in-vitro* and *in-vivo* vectors, or in DNA vaccines. The infectious clone AI viruses are produced by reverse genetics using the six internal genes from an influenza A vaccine strain such as PR8, and the HA and NA genes from the AI outbreak or related viruses [40, 84, 230, 293]. The use of PR8 internal genes imparts the ability to replicate to high virus titers in ECE typically used as the substrate in the manufacturing process. If the donor HA gene is from an HPAIV, the HA proteolytic cleavage site must be changed from a sequence of a HPAIV to LPAIV, thus producing a LPAI vaccine seed strain which can be manufactured at a lower level of biosafety than an HPAIV. The NA gene can be selected from among the existing nine NA subtypes to be different from the outbreak virus, thus creating a vaccine with a heterologous NA subtype which will allow a serological test to identify infected birds within the vaccinated populations (see section on surveillance below). The rg infectious clone vaccines are as efficacious as the existing licensed vaccines based on outbreak LPAIVs and HPAIVs. In the USA, the rg-developed seed strains must be shown to be LPAIV by three tests (i.e. sequencing, failure to plaque in cell culture without exogenous trypsin, and low virulence in chicken IVPI test) before the virus can be excluded from Select Agent regulations as not being a HPAIV [191, 205, 392]. Finally, the advent of biotechnology has allowed rapid selection of the HA gene from outbreak viruses for insertion into vectored vaccines such as adenovirus or rFPV [153], and most recently, with the 2014–2015 outbreak of H5 Gs/GD HPAIV in the USA, using the rdVEE alphavirus, also called the RNA particle system, thus allowing close genetic matching between vaccine and field AIV. In all vectored vaccines, HA should be altered from the HP to the LP cleavage site to maximize replication titers in the *in-vitro* system without being excessively cytolytic on cell cultures or ECE. Recently a simplified single plasmid system for rg generation of AI vaccine seeds was developed [121]. The majority of inactivated H5 AI vaccines manufactured and used across the world have utilized rg LPAIV seed strains [403].

Licensed avian influenza vaccines

A variety of vaccine technologies and virus strains have been licensed and used in the field. In the USA, only four AI vaccine technologies have been licensed: several inactivated whole AI vaccines, an rFPV-H5 vaccine with the H5 gene insert obtained from A/turkey/Ireland/83 (H5N8), an rHVT-H5 vaccine with the H5 gene insert obtained from A/Swan/Hungary/4999/2006 (H5N1) (clade 2.2), and rdVEE with the H5 gene insert obtained from A/Gyrfalcon/Washington/41088/2014 (H5N8) (clade 2.3.4.4) (H5N1) [78, 338]. A variety of HA-subtype vaccines have been licensed by the USDA under autogenous, conditional, and full licensure as inactivated AI vaccines. However, the field application of the licensed H5 and H7 vaccines requires approval of both the state and federal governments, but other subtypes may only require approval of the state government when used in the species listed in the product license restrictions. Historically, vaccines have not been used in the USA in HPAI epidemics, but contingency plans for potential future outbreaks could allow their use. Vaccines against LPAIVs have only been of limited use in the USA. The rFPV-H5 is also licensed and used in Mexico, El Salvador, and Guatemala. In Mexico, an rNDV-H5 vaccine is licensed [164, 332]. An rHVT-H5 is licensed and used in Egypt and Mexico [209], an rNDV-H5 is licensed and used in China [164], and an rDVE-H5 is close to license and use in China.

Globally, the majority of licensed AI vaccines are inactivated whole AI vaccines, principally of the H5, H7, and H9 subtypes. For the current H5 Gs/GD-lineage HPAI panzootic, licensed inactivated

AI vaccines used in Asia, Africa, and Europe have utilized various seed strains for inactivated vaccines and different H5 HA gene inserts for rFPV, rHVT, rDVE, and rNDV (Table 15.6) [78]. Requirements for licensing AI vaccines will be different for each country, depending on the specific requirements of the national veterinary biologics authority in areas of safety, purity, potency, and label approval for species, and age and route of administration.

Field usage of avian influenza vaccine

The quantity of AI vaccine that has been manufactured and used in poultry prior to the past two decades has been poorly documented, but global usage was low until the mid-1990s, and manufacturing expanded geometrically early in the first decade of the twenty-first century. For H5 and H7 vaccines, a survey conducted in early 2002 identified only two manufacturers in OIE member countries that responded to the survey, but by 2006 and 2007 the OIE listed 38 manufacturers of AI vaccines, and the Food and Agriculture Organization (FAO) of the United Nations listed 41 AI vaccines for use against H5 and H7 HPAI available from China, France, Germany, Italy, Mexico, Netherlands, Pakistan, and the USA [125]. By 2012, only 27 manufacturers were recognized as producing H5 and H7 AI vaccines [220, 239, 268]. Since H1–4, H6, H8, and H10–16 LPAI are not reportable diseases to OIE or within most countries, little is known about the availability and use of vaccines for their control. However, vaccines that utilize H9N2 LPAIV seed strains are routinely used in commercial poultry in 10 countries across the Middle East and Asia [73]. Table 15.6 lists the currently available seed strains and those that were historically produced but are no longer available.

Historically, in the USA, AI vaccines have had limited use, most frequently to control and eradicate LPAIVs in small defined geographic areas of high risk, such as the emergency vaccination of Minnesota turkeys to control wild-duck-origin LPAIVs for which, between 1978 and 1996, only 22 million doses of inactivated AI vaccines were used [338]. As a response to the continuing risk, the Minnesota turkey industry eliminated outdoor production in 1997, thereby reducing wild waterfowl–turkey exposure and the risk of infection with wild-duck-origin influenza viruses, and vaccine use against wild-duck-origin LPAIVs has been eliminated [137]. In Utah, during 1995 an outbreak of H7N3 LPAI was eradicated by use of 2.03 million doses of inactivated vaccine over 4 months, along with other components of the control program [137, 140]. In California turkey breeders, 2.3 million doses of inactivated AI vaccine were used between 1979 and 1985 [115].

Another example of emergency and preventative vaccination against LPAI has been in Northern Italy, where 202 140 000 doses of AI vaccine were used between November 2002 and December 2006, mostly in laying hens and meat turkeys, but some in capons, guinea fowls, and cockerels. Most of the AI vaccine was inactivated H7 used in an emergency vaccination campaign against LPAI, but some vaccine was bivalent H5 and H7 used during 2005 and 2006 for a preventative LPAI campaign [223]. As an example of routine influenza vaccination, swine influenza virus (SIV) causes infections and economic losses in turkey breeders located in specific states within the USA where pigs and turkeys are raised within the same geographic areas. For example, 2.6 million and 7.965 million doses of inactivated H1 influenza A vaccine were used in turkey breeders in the USA during 2001 and 2009, respectively, to protect against H1 or H3 SIV [50]. However, the most extensive use of AI vaccine against LPAI has been that of inactivated H9N2 vaccines in the Middle East and Asia, where since the late 1990s billions of doses have been used in layers, broilers, and other poultry.

The use of AI vaccines for control of HPAI was first reported in 1995 in Mexico during the H5N2 HPAI epidemic, and in Pakistan during the H7N3 HPAI epidemic. The H5N2 HPAIV was eradicated from Mexico during 1995, but the precursor H5N2 LPAIV continues to circulate and cause infections in poultry, and has expanded into Guatemala and El Salvador. Between 1995 and 2006, 3.8 billion doses of vaccine were used (1.8 billion doses of H5N2 inactivated vaccine and over 2 billion doses of rFPV-H5 vaccine) [326, 337], and by 2010, 10.1 billion doses had been used (5.8 billion doses of inactivated AI vaccines and 4.3 billion doses of rFPV-H5 vaccine) [44, 334]. Between 1998 and 2014, 7 billion doses of rFPV-H5 were used in Central America in chickens (Michel Bublot, personal communication, 15 May 2014). In Pakistan, use of an inactivated H7N3 vaccine was initiated in 1995, with expanded usage when H7N3 LPAI occurred in

Table 15.6 Current vaccines technologies licensed and used in the field for H5 and H7 avian influenza vaccines (compiled from available information [81, 82, 118–120, 133, 157, 158, 179, 180, 186, 285, 296, 311, 346] (www.nibsc.org/documents/ifu/07-252.pdf, http://onehealth.org.vn/influenza-vaccine-manufacturing-in-viet-namreport-on-the-apaci-satellite-session.new, www.fao.org/3/a-ai326e.pdf, www.navetco.com.vn/vi/sanpham/navet-vifluvac)). Information on seed strains for other subtypes was unavailable.

Technology	Current seed strains or subunit source[a]	Previously available seed strains or subunits	Adjuvants	Countries licensed
Inactivated whole AIV	wtA/duck/Novosibirsk/2/2005 (H5N1, clade 2.2, HPAI) wtA/chicken/WestJava-Nagrak/30/2007 (H5N1, clade 2.1.3 HPAI) wtA/Ck/Mansehra/2006 (H5N1, clade 2.2, HPAI) rgA/duck/Guangdong/S1322/2010 (H5N1: Re-6 H5N1, clade 2.3.2, LPAI) rgA/Vietnam/1194/2004 (H5N1, clade 1, LPAI) rgA/chicken/Guizhou/4/2013 (H5N1: Re-8, clade 2.3.4.4, LPAI) rgA/chicken/Egypt/18-H/2008 (H5N1, clade 2.2.1, LPAI) rgA/chicken/Shanxi/2/2006 (H5N1: Re-4, H5N1, clade 7, LPAI) rgA/chicken/Liaoning/2011 (H5N1: Re-7, clade 7.2, LPAI) wtA/duck/Potsdam/1402/86 (H5N2, LPAI) wtA/chicken/Durango/1558/2006 (H5N2, LPAI) wtA/turkey/Minnesota/3689-1551/81 (H5N2, LPAI) wtA/turkey/California/20902/2002 (H5N2, LPAI) wtA/duck/Potsdam/2243/1984 (H5N6, LPAI) wtA/turkey/Wisconsin/1968 (H5N9, LPAI) wtA/chicken/Italy/22A/1998 (H5N9, LPAI) rg-HA(H5)-A/chicken/Vietnam/C58/2004 plus N3-A/duck/Germany/1215/73 (H5N3, clade 1, LPAI) wtA/chicken/Pakistan/447/4-1995 (also termed A/chicken/Pakistan/34668/1995) (H7N3, HPAI) wtA/chicken/Italy/1067/1999 (H7N1, LPAI) wtA/chicken/Italy/473/1999 (H7N1, LPAI) wtA/chicken/New York/273874/2003 (H7N2, LPAI) wtA/turkey/Oregon/1971 (H7N3, LPAI) wtA/turkey/Utah/24721-10/1995 (H7N3, LPAI) wtA/duck/Potsdam/15/1980 (H7N7, LPAI)	wt and rgA/chicken/Legok/2003 (H5N1, HPAI) wt and rgA/goose/Guangdong/1996 (Re-1)(H5N1, clade 0, HPAI) rgA/duck/Anhui/2/2006 (Re-5) (H5N1, clade 2.3.4, LPAI) wtA/turkey/England/N-28/1973 (H5N2, LPAI) wtA/chicken/Mexico/232/1994/CPA (H5N2, LPAI)	Proprietary mineral oil or alum	Many

(*continued*)

Table 15.6 (*Continued*)

Technology	Current seed strains or subunit source[a]	Previously available seed strains or subunits	Adjuvants	Countries licensed
rFPV	HA-A/turkey/1378/Ireland/1983 (H5N8) HA-A/chicken/Scotland/59 (H5N9)	HA/NA-A/goose/Guangdong/1996 (H5N1, clade 0)	None	China, Mexico, Guatemala, USA
rNDV	HA-A/duck/Anhui/1/2006 (H5N1, rLH5-5, clade 2.3.4) HA-A/chicken/Mexico/435/2005 (H5N2)	HA-A/goose/Guangdong/1996 (H5N1, rLH5-1, clade 0) HA-A/bar-headed goose/Qinghai/3/2005 (H5, rLH5-3, clade 2.2) HA-A/chicken/Shanxi/2/2006 (H5, rLH5-4, clade 7)	None	China, Mexico
rHVT	HA-A/swan/Hungary/4999/2006 (H5N1, clade 2.2)		None	Bangladesh, Egypt, Mexico, Vietnam, USA
rDVE	HA-A/duck/Anhui/1/06 (H5N1, clade 2.3.4)		None	China

AIV, avian influenza virus; rDVE, recombinant duck enteritis virus; rFPV, recombinant fowl poxvirus; rg, reverse genetic (uses PR8 internal gene backbone); rHVT, recombinant herpesvirus turkey; rNDV, recombinant Newcastle disease virus; wt, wild type.

[a]Some seed strains have a veterinary license but are not currently manufactured, while the status of others is unknown.

another region of Pakistan during 2001, and H7N3 HPAI occurred in southern Pakistan during 2004 [338]. Vaccination against H9N2 was implemented in 1998. In 2006, with outbreaks of H5N1 HPAI, Pakistan began using a trivalent inactivated AI vaccine containing H5, H7, and H9 subtypes. The emergence of H5N1 HPAI in Asia with spread to Africa has led to emergency, routine, and preventative AI vaccination, beginning with the use of inactivated H5N2 AI vaccine in Hong Kong poultry in 2002, followed by implementation of an H5 vaccination program in Indonesia in 2003, in China in 2004, in Vietnam in 2005, in Côte d'Ivoire, Egypt, France, Israel, Kazakhstan, Mongolia, the Netherlands, Pakistan, and Russia in 2006, and in Bangladesh in 2012 [230, 231].

Between 2002 and 2010, 113.9 billion doses of H5 or H7 AI vaccine (95.5% as oil-emulsified whole inactivated vaccine and 4.5% as recombinant vaccines [i.e. rFPV-H5, rHVT-H5, and rNDV-H5]) were used to protect poultry, with the majority being used by the world's largest poultry producer and consumer, China, which administered 104 billion doses (91%) in a routine national poultry vaccination program (Figure 15.3) [338]. An additional 8% were used in Egypt (5 billion doses, 4.65%), Indonesia (2.6 billion doses, 2,32%), and Vietnam (1.6 billion doses, 1.43%), with the remaining 1% being used in targeted vaccination programs for high-risk poultry, either as a preventative measure or as a management tool during eradication. The latter countries included Russia (0.37%), Pakistan (0.12%), Hong Kong SAR (0.08%), Kazakhstan (0.03%), Côte d'Ivoire (<0.01%), Democratic People's Republic of Korea (<0.01%; H7N7 HPAI only), France (<0.01%), Israel (<0.01%), Mongolia (<0.01%), the Netherlands (<0.01%), and Sudan (<0.01%) (Figure 15.3). At the time of writing, vaccination is continuing in Hong Kong, China, Egypt, Vietnam, Pakistan, and Bangladesh at levels estimated to be similar to 2009 levels, but most of the other countries have ceased their vaccination programs. By comparison, between 2002 and 2010 less than 300 000 doses (i.e. 0.000003% of the total vaccine used in HPAI programs) of H5 or H7 AI vaccine have been used in zoo, hunting, companion, conservation, or endangered birds, representing HPAI preventative vaccination programs in 20 European and Asian countries on 292 premises [338]. No challenge studies were conducted, but protective HI serological responses of ≥ 32 were reported for 76%, 80.5%, and 81.5% of the birds in the three studies, respectively. The serological response of birds from different orders varied between the individual reports [27, 247, 248, 338].

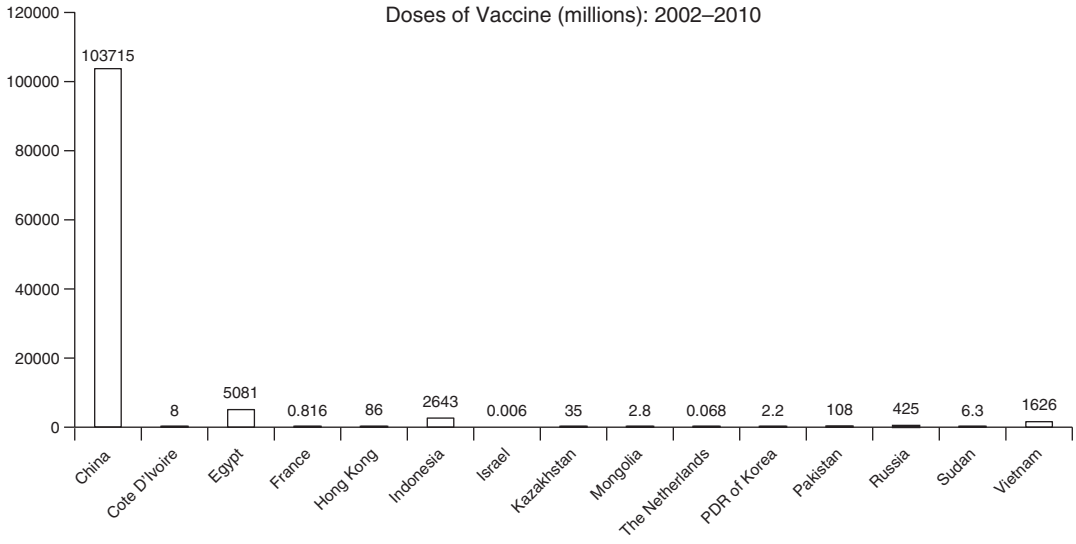

Figure 15.3 Summary of H5 vaccine doses used in poultry by the 15 countries that vaccinated poultry against HPAI from 2002 to 2010. *Avian Diseases* 56(4):818–828, 2012; [341]. Used with permission of the American Association of Avian Pathologists.

The vast majority of AI vaccines have been used in the traditional commercial poultry sector. However, vaccination campaigns have been undertaken in village poultry within countries with endemic H5N1 HPAI. In Egypt, vaccination coverage was low in household poultry, and cumulative annual flock immunity was also low, suggesting that mass vaccination was not an effective long-term strategy for H5N1 HPAI control, but that a targeted approach may be more effective in combination with other control components [27, 90, 247, 248, 279].

Broad homosubtypic protection

Historically, poultry AI vaccines have demonstrated broader and longer-term homosubtypic protection than human seasonal influenza A (H1 and H3) and B vaccine strains. Antigenic mapping has demonstrated that H5 LPAIVs, as they emerge from the wild bird reservoir and infect poultry, have antigenically similar HAs, making them good cross-protective vaccine seed strains against diverse H5 LPAIVs and HPAIVs [104]. For example, studies with the H5N2 Mexican 1994 seed strain have shown broad cross-protection against not only North American but also Eurasian H5 HPAIVs [344]. However, the broad homosubtypic protection is not absolute, and the high prevalence of H9N2 LPAIV and H5N1 HPAIV infections in poultry of some developing countries, and the high usage of and reliance on vaccine in control programs, have been associated with increased immune pressure on field AI viruses and their drift away from some vaccine strains. As a result, field viruses have emerged that are resistant to older, classic LPAI vaccine strains or even the older rg vaccine seed strains [354, 357]. As a result, vaccine strains may need to be changed more frequently and may require increased usage of reverse genetics [357] to generate replacement LPAI vaccine strains, or replacement of AI HA genes in vectored vaccine products such as new rFPV or rNDV vaccines to maintain efficacious vaccines for use in the field. The use of heterologous H7N3 vaccine in Italy during 2002–2004 and H5 vaccines in Indonesia during 2003–2006 was associated with field viruses acquiring specific amino acid changes in antibody-binding sites of the HA [191, 205], highlighting the need to use a homologous seed strain to minimize selection for such antigenic drift variants.

To compensate for minor antigenic differences and optimize homosubtypic protection, increasing the number of vaccinations and/or the quantity of antigen in each dose can broaden the protective immune response. Multiple vaccinations with a killed H5N2 vaccine broadened the antigenic profile in broiler breeders, and counteracted mild antigenic differences between vaccine and field viruses, but such a strategy did not provide protection against challenge when the antigenic differences were broad [23, 344]. In addition, use of high HA antigen content in each H5 vaccine dose has provided some broadening of homosubtypic protection against antigenically divergent H5N1 challenge viruses [2], but this also has limitations.

In addition to antigenic similarity within an HA subtype, the broader and longer-term protection provided by poultry AI vaccines as compared with human seasonal influenza A (H1 and H3) and B vaccine strains may potentially have resulted from the following [365]:

1. Poultry-inactivated AI vaccines use proprietary oil-emulsion adjuvants, which elicit a more intense and longer-lived immune response in poultry than do alum-adjuvant human influenza A and B vaccines.
2. The AI vaccine immune response in poultry appears to be broader than that in humans.
3. Immunity in the domestic poultry population is more uniform because there is greater host genetic homogeneity than exists in the human population.
4. The use of AI vaccine in poultry is targeted at a relatively young, healthy population, whereas AI vaccine for humans is optimized for those groups at highest risk of severe illness and death.
5. Historically, there is less endemic influenza virus infection in poultry than in human populations. For the latter in the developed countries, relatively uncommon AIV exposure along with infrequent use of AI vaccine have exerted less selection pressure on AIVs to drift.

Vaccine quality

Quality control in vaccine manufacturing is critical to ensure the production of a safe and efficacious product [332]. The quality of commercial vaccines

has a major impact on field efficacy in terms of the following:

1. antigenic relatedness of the seed strain to the field virus
2. antigen quantity – sufficient HA antigen must be present (inactivated vaccine) or there must be a high enough titer (live vaccine) to produce a serologically measurable protective immune response, and there should be minimal batch-to-batch variation in antigen content
3. adjuvant – for inactivated vaccine, high-quality adjuvants are needed to enhance the immune response of birds to the HA antigen, and they should be safe to administer
4. sterility – for inactivated vaccines, the vaccine strain must be completely inactivated
5. purity – no fungal, bacterial, or viral contaminants should be present in either inactivated or live vaccines.

Antigenic matching of the seed strain

The seed strain used should antigenically match the field virus to provide maximum protection, and the specific seed strain should be confirmed in manufactured vaccine to match the registration. When seed strains have been used in countries with endemic infection, antigenic drift and reduced protection have been identified, as in Mexico with H5N2 LPAIV [355], and in Egypt [190], Hong Kong [132], Indonesia [86], and Vietnam [344] with H5N1 HPAIV. In some instances, regulatory bodies have been slow to change seed strains. For example, examination of seed strains in H5N2 Mexican inactivated final vaccine products demonstrated that two of 10 seed strains did not match the registered vaccine seed, but these two vaccines were the only ones that provided protection as measured by reduced shedding of challenge virus from oral swabs [74]. Similarly, another study identified three Chinese-origin vaccines which contained seed strains that were not compatible with registered strains for the labelled final product [103].

Antigen quantity

The vaccine should be formulated with sufficient HA antigen to produce a consistent protective response in the target species. In 1998, Garcia and colleagues examined six inactivated commercial H5N2 vaccines from Mexico for protection in chickens against an H5N2 HPAIV challenge, and found that they all provided good protection from mortality, but they showed individual variability in reducing shedding from the oropharynx and cloaca, and in inducing an HI serological response [344]. A single vaccination dose of ≥ 4 μg of HA protein was needed to reduce replication in the respiratory and alimentary tract following IN challenge to an intermediate dose of HPAIV (10^2 mean chicken lethal doses [CLD_{50}]). In another study using experimental vaccines, inactivated AI vaccines containing 3–8 μg of HA protein per dose, or baculovirus-vectored vaccine containing 2 μg of HA per dose, were protective in chickens that had been intranasally (IN) challenged with HPAIVs, compared with doses of 0.1–0.3 μg and 0.2 μg, respectively, which did not provide complete protection [122]. Furthermore, in a contact transmission model, 0.3 μg of HA in inactivated oil-emulsified vaccine, but not 0.1 μg of HA, prevented clinical disease in chickens that had been IN challenged with HPAIV, whereas 0.9 μg of HA in the vaccine provided optimal protection without isolation of challenge virus from respiratory or alimentary swabs [327]. Another study using two vaccinations reported that 0.5 μg of HA was the minimum dose required to produce optimal protection [394]. Adequate HA quantity can be assessed by serological evaluation in SPF chickens immunized with a single dose according to the instructions on the manufacturer's label. Prevention of mortality has been associated with HI antibody geometric mean titers (GMT) of $\geq$ 8–10 [98], and prevention of oropharyngeal shedding of the challenge virus was associated with HI antibody GMT of $\geq$ 128 [182, 323].

A more reproducible and accurate way to establish the required HA antigen content involves the use of a PD_{50} determination. In a chicken experimental study using inactivated experimental AI vaccines containing a standardized generic oil-emulsion adjuvant system and experiencing a high challenge dose of H5N2 HPAIV (10^3 CLD_{50}), the PD_{50} range based on mortality was 0.006–0.156 μg of HA, and the range based on morbidity was 0.008–0.156 μg of HA, which when translated to a standard minimal dose range to achieve 50 PD_{50} would require an HA content per dose of 0.3–7.8 μg [333]. As a summary of multiple experiments, a minimum of 1–5 μg of HA per dose of poultry AI vaccines has been suggested [323]. However, the quantity of antigen needed will vary depending on

vaccine strain, challenge virus strain and challenge dose, and the adjuvant used in the vaccine.

Adjuvant

For inactivated vaccines, the use of oil adjuvants is necessary to produce consistent, broad, and long-lived protective antibody titers. Most veterinary biologics companies use proprietary adjuvant systems for inactivated poultry vaccines. More detailed information can be found in the section on adjuvants earlier in this chapter.

Sterility

During the manufacture of inactivated vaccines, a chemical process is used to inactivate the vaccine strain, and the inactivation should be verified by ECE or tissue culture inoculation. In addition, the antigen should be sterility tested to ensure that no contaminating bacteria or other agents are present.

Purity

For live vaccines, the input products (ECE or tissue culture and seed stock) should be tested to verify that no extraneous fungal, viral, or bacterial agents are present by isolation methods or nucleic acid (e.g. RRT-PCR [332]) detection technologies.

Route of administration

The route of inoculation has a major impact on development of a protective immune response, and is influenced by the type of vaccine, management system, host species, and host age.

Post-hatching parenteral administration

For most AI vaccines that are used in the field, administration is by parenteral injection, and involves the catching, handling, and injecting of individual birds to produce the protective immune response. Parenteral administration is most commonly achieved by subcutaneous (SQ) injection in the nape of the neck, but some vaccines require intramuscular (IM) or wing web (WW) injection. Most licensed inactivated AI vaccines require SQ or IM injection. Although wild-type fowl poxviruses can produce oral, pharyngeal, and tracheal infection and lesions, rFPV-H5 vaccines are more restricted in replication sites with vaccine that is given by the SQ or WW routes, and vaccine given intranasally (IN) or in drinking water will not protect against HPAIV challenge [240]. rHVT is administered via the SQ or *in-ovo* routes, whereas rDVE is given via the IM route.

In the USA, the costs of the vaccine plus parenteral administration are high, with vaccine costs in the range of US$0.05–0.10 per dose and, because of handling of the birds, estimated administration costs of US$0.05–0.07 per bird [19]. However, the actual costs will vary depending on the type and quality of the vaccine, the size and age of the birds handled, and the costs of government control programs, surveillance, and labor for vaccination.

Mass administration

Low-cost methods for mass immunization of poultry against AI would be advantageous, providing a means of reducing the expense of administering AI vaccines, which would translate into an economic incentive to vaccinate, and would thus increase vaccine use in regions or countries where vaccination is needed to control AI. Methods for mass immunization against AI could include *in-ovo* administration, or post-hatching administration to the respiratory tract (via spray) or alimentary tract (via drinking water or feed).

In-ovo administration

In the early 1980s, vaccination by injection into the developing chicken embryo (*in-ovo* administration) was developed to provide early post-hatching protection against Marek's disease herpesvirus (MDV) [332]. The methodology has been automated, allowing mass vaccination of chicken embryos on days 18 to 19 of incubation. Currently, the majority of broiler chickens produced in the USA are vaccinated against MDV by *in-ovo* technology, and this methodology is being widely adopted by the integrated commercial broiler industries around the world. Currently, there are no registered AI vaccines for *in-ovo* administration. However, multiple experimental studies have been conducted and have shown proof-of-concept protection against AI, including an inactivated AI vaccine [286], deficient replicating adenovirus with AIV H5 gene insert [302], rHVT-H5 [226, 359], rdVEE [151], live attenuated AIV with NS gene truncations or other genetic changes [282], and AI-NDV-HN chimera [291, 383]. Another theoretical possibility for *in-ovo* AI vaccine is an AIV-HA-antibody complex vaccine, similar to the infectious bursal disease virus

(IBDV)-antibody vaccine, but this would require use of an inactivated AIV in the antigen–antibody complex [297].

Respiratory administration (via spray and eyedrops)

Infectious bronchitis virus and NDV vaccines are the most common mass-applied live virus vaccines for poultry, with billions of doses used each year around the world in all sectors of poultry production [154]. Most often these vaccines are mass applied in the hatchery by spray cabinet, and in the field by back-pack sprayers in the production house. Such application results in conjunctival and upper respiratory tract exposure, with vaccine virus replication and resulting production of a mucosal and systemic immune response. Because of the widespread use of live NDV vaccines, the concept of using an infectious clone system to produce AIV-vectored vaccines was developed [328]. Such rNDV vectors with H5 or H7 AIV HA gene inserts have been used to immunize chickens, and protection has been demonstrated against challenge for both NDV and HPAIVs [181, 232, 245]. Two rNDV-H5 vaccines have been registered in China and Mexico for field use against NDV and H5 HPAI [125, 209, 244, 329, 374], but maternal immunity in progeny against NDV vector or HA of influenza gene insert has inhibited primary immune response and protection.

In relation to other potential AI vaccines, a spray application of an inactivated H5N2 AI vaccine containing a special adjuvant reduced replication of an H5N2 LPAIV when challenged by eyedrop application in the conjunctival sac [209, 406]. However, a deficient-replication adenovirus-vectored AI-H5 vaccine failed to protect chickens after a single vaccination intranasally (IN), intratracheally (IT), conjunctivally, or *in ovo* from IN challenge by homologous H5N1 HPAIV, indicating that it is unlikely to be used as a topical or spray mass-applied vaccine [211]. By contrast, a prime-and-boost application of deficient-replication adenovirus-vectored AIV-H5 vaccine in the conjunctival sac protected chickens from lethal IN challenge with 1995 Mexican H5N2 HPAIV, while a single vaccination by spray or conjunctival sac only gave partial protection [121, 301]. Temperature-sensitive mutant H5 and H7 rgLPAI vaccines when given topically protected against lethal HPAIV challenge in chickens [360].

Alimentary administration (drinking water and feed)

Currently, no licensed AI vaccines are available for administration in drinking water or feed. Inactivated vaccines have been experimentally administered orally with protection from HPAIV challenge, but the immunization required very high vaccine doses and up to nine immunizations to produce protection in SPF chickens, making alimentary administration unfeasible [291]. Various methods for induction of protective antigens in plants for oral consumption have been proposed, but none have been shown to protect poultry against HPAIV challenge. Such non-live HA AI vaccines may require high doses and special adjuvants to produce a protective immune response.

Recently, an attenuated ΔaroA *Salmonella enteritidis* (SE) vector with AI-M2e gene insert was shown to produce neutralizing antibodies after two alimentary tract vaccinations in chickens, and reduced replication and shedding following H7N2 LPAIV challenge, but did not protect against mortality from H5N1 HPAIV challenge [89]. Similarly, M2e hyperexpression in *E coli* provided only partial protection in chickens against H5N1 HPAIV [85, 185]. By contrast, an attenuated *Salmonella enterica* serovar Typhimurium vaccine strain expressing the H5 HA gene, given orally at 4-weekly intervals, provided partial but not complete protection from mortality from homologous and heterologous H5N1 HPAIV challenge [404].

Management and environmental conditions

Protection induced by a vaccine applied under laboratory conditions in SPF poultry controls all confounding variables and gives the best immune response. However, in the field, vaccine-induced protection will be less than optimal unless all management variables that have a negative impact on host immunity development are controlled. Some experimental AI vaccine studies have reported "sterilizing immunity" at mucosal sites, but, in the field, "sterilizing mucosal immunity" is neither practicable nor achievable. However, vaccines can significantly reduce AIV replication and shedding

from the respiratory and alimentary tracts, which translates into reduced environmental load of virus and reduction or elimination of virus transmission [201]. Management of environmental variables that may inhibit or reduce protection in the field must address the following:

1. failure to vaccinate sufficient numbers of birds in the population to achieve herd immunity
2. administering less than the recommended vaccine dose to each bird
3. use of low-cost, poor-quality vaccines (e.g. with low antigen content)
4. incorrect storage and transport of vaccines
5. incorrect vaccination, with numerous missed injections
6. immunosuppression resulting from specific virus infections (e.g. infectious bursal disease, chicken anemia virus, adenoviruses, and others), exposure to naturally occurring immunosuppressive compounds such as aflatoxins, or exposure to stressful or poor management conditions.

Biosecurity, education, diagnostics and surveillance, and elimination of AIV-infected poultry are essential, because vaccines and their use are not perfect. Vaccination should only be used as one tool in a comprehensive AI control program.

Field application method and coverage

Vaccination programs

The development of a viable vaccination strategy requires examination of multiple facets, including the following:

1. the type of AIV to control – that is, HPAIV, H5/H7 LPAIV, or non-notifiable LPAIV (H1–4, H6, and H8–16)
2. the goal or desired outcome of the control program – that is, to prevent, manage, or eradicate AI
3. the risk level for AIV introduction, and the role that vaccination can play in the control strategy
4. the species of birds to be vaccinated
5. the production sector or system involved
6. the logistic support, including regulatory oversight, availability of trained vaccinators, and surveillance capabilities
7. the types and availability of vaccines.

The use of vaccine is universally accepted for control of economically significant and common endemic poultry diseases, such as infectious bronchitis (IB) and infectious bursal disease (IBD) and, within some countries, zones, and compartments (CZCs), for LPAI control. For example, in much of Asia and the Middle East, H9N2 LPAIVs are endemic in poultry, and vaccines are routinely used to manage the infection and disease in order to make poultry production economically viable, but eradication of H9N2 LPAI has not been the goal (i.e. H9N2 LPAI is endemic in such poultry populations, as are IB and IBD). By contrast, in most developed countries, LPAI of all subtypes is sporadic in poultry and localized in nature, making widespread use of vaccines incompatible with general preventative or eradication strategies [106, 380]. However, with one exception, domestic turkeys are susceptible to H1 and H3 SIV, which is endemic in pigs throughout the world [18]. Therefore, in many developed countries, turkey breeders and occasionally meat turkeys may be strategically vaccinated against H1 and H3 SIV in high-risk geographic areas where both swine and turkeys are produced [101]. Vaccination is less commonly practiced against H5 and H7 LPAI, as stamping-out programs are mainly used for eradication [338].

Vaccination of poultry is very rarely utilized against HPAI in developed countries where early diagnosis and stamping out without vaccination are most commonly practiced for immediate eradication [338]. However, in developing countries, the use of vaccine against H5 and H7 LPAI and HPAI depends on the endemic status and the ability of the stamping-out programs to immediately eradicate the virus. Of 37 HPAI epidemics that occurred between 1959 and 2015, only five epizootics utilized vaccination in combination with stamping out; the other 32 epizootics utilized stamping-out programs alone [338]. AI vaccination was implemented in 1995 for the H5N2 HPAI epidemic in Mexico, in 1995 for the H7N3 HPAI epidemic in Pakistan, in 2002 with for the Gs/GD-lineage H5N1 HPAI epidemic in Asia, in 2005 for the H7N7 HPAI epidemic in North Korea, and in 2012 for the H7N3 HPAI epidemic in Mexico. With H5 and H7 LPAI, the use of vaccines may depend on the local situation, and historically vaccination has been used as an adjunct to controlled marketing.

In situations where vaccine is being considered for use, the risk of AIV infections should be determined, and if the risk is high, correct vaccination

could be used as an effective prevention tool as part of a multi-component AI control strategy. For example, in Hong Kong in 2002, compulsory vaccination was implemented because of the repeated introduction of H5N1 HPAIV in imported poultry from southern China, and because of the presence of endemic infections within the region [344]. This strategy has been successful in preventing further outbreaks in poultry in Hong Kong, and preventing exposure and infections in humans. However, blanket use of vaccination in all situations should not be endorsed, because vaccine use without proper biosecurity and surveillance may make early detection of AIV infection difficult and confound rapid detection and stamping-out programs. Proper vaccine programs should be designed to fit a specific need and the epidemiology of the disease.

Various strategies have been proposed for using vaccines in both HPAI and LPAI control programs [105]. Historically, vaccination against exotic diseases such as foot-and-mouth disease and rinderpest in livestock has utilized a ring vaccination strategy – that is, vaccination of a control or surveillance zone around the outbreak, which is based on the epidemiological concept of short-distance movement of infected large livestock (e.g. cattle and swine) and fomites containing the virus (Figure 15.4). Ring vaccination has been proposed as an emergency vaccination program for HPAI control, but such a strategy may not always be fully effective because of easy and long-distance movement of small poultry to and from live market systems, especially in developing countries where small compact birds are easily and commonly moved in small crates on motor bikes over great distances between farms and markets, and back again. Another strategy would be as a "suppressor" vaccination within the HPAI outbreak zone for non-infected flocks, to increase the resistance of these flocks to AIV infection, and in infected flocks, especially with LPAIV, to induce a consistent high immune level with the goal of stopping AIV shedding and transmission (Figure 15.4). The latter use can have the added benefit of stopping the AIV spread within a premises or barn, even on infected premises, as was demonstrated in field vaccination experiments against H5N2 HPAIV in Pennsylvania during 1983 and against H5N1 HPAIV in Hong Kong during 2002 [58, 61, 64–67, 137, 139, 192, 218, 314, 328, 330]. Alternatively, suppressor

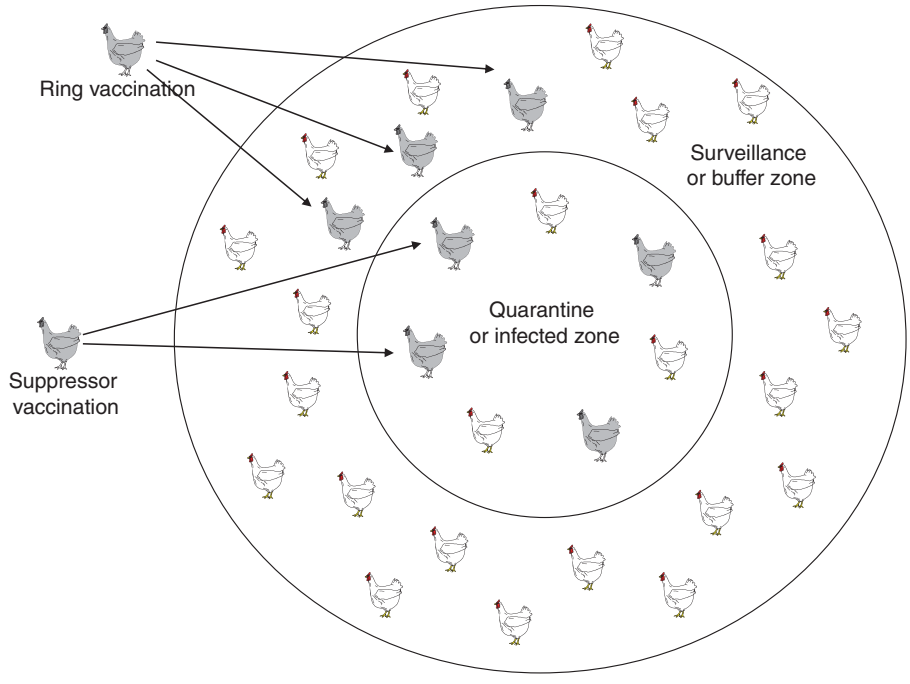

Figure 15.4 Concepts for application of AI vaccine in the field during an emergency vaccination program. Source: David E. Swayne.

vaccination can be used during repopulation by placing vaccinated birds back into the environment when there is still a risk of pockets of residual virus in environments that cannot be properly cleaned or disinfected, or in areas where undetected, low infection rates may still be present in inaccessible village poultry. Repopulation with vaccinated poultry should decrease the potential for AIV resurgence. Similar vaccination strategies can be used with LPAI control. In the case of LPAI, vaccination was practiced on several affected egg-laying chicken farms containing multiple large houses that experienced H7N2 LPAIV infections in Connecticut, USA, during 2003–2005 [105, 106]. In this situation, the LPAIV did not produce consistent infection of all chickens within the large houses, and vaccination was implemented to raise the immunity of all chickens to a consistent and protective level that allowed safe production and marketing of table eggs from the flocks. After 18 months, all vaccinated chickens on the affected farms had been eliminated, and no H7N2 LPAIV was detected during surveillance [314].

Vaccination within a geographic area could be applied as a ring around the outbreak zone, as blanket vaccination in a large endemic infected region, or in the case of a small high-risk area, as targeted vaccination in a defined geographic area, compartment, or poultry species. A detailed strategic plan for AI vaccination includes the following categories: routine vaccination in endemic areas, emergency vaccination during an epidemic, and preventative or prophylactic vaccination when the risk of AIV introduction is high [314]. In an emergency vaccination program, an exit strategy should be developed and implemented as part of the eradication strategy, otherwise the vaccination program will quickly become routine, with acceptance of the AIV infections, and the disease will become endemic.

In the laboratory setting, a single inactivated vaccination at $\geq$ 2 weeks of age typically produces protection from virulent challenge in AIV-antibody-free chickens and turkeys, but in experimental studies of some poultry species, a single vaccination did not produce optimal protection. For example, a single vaccination with Gs/GD-lineage (clade 0) H5N1 vaccine in Pekin-type domestic ducks provided better protection than the same vaccine in Muscovy ducks when challenged with heterologous Gs/GD-lineage (clade 1) H5N1 HPAIV, and similarly a single inactivated vaccination did not provide optimal protection of domestic ducks or domestic geese from H5N1 HPAIV challenge [218]. In addition, the use of of inactivated vaccines at 1 day of age in chickens, with and without maternal antibodies, failed to protect from H5N1 HPAIV challenge [53, 54, 102]. In the field, a single vaccination is rarely sufficient to provide adequate protection for most poultry species, except perhaps for short-lived meat chickens that lack maternal antibodies. At the very minimum, two vaccinations are needed for meat chickens with AIV maternal antibodies, and two or more vaccinations will be required for meat turkeys, and longer-lived breeder and egg production chickens, turkeys, and ducks, to provide immunity throughout their production life. For example, in Italy, clinical infections in vaccinated turkey flocks have been associated with low antibody titers, and multiple vaccinations have been needed to produce a protective immune response that lasts for the entire production cycle (Giovanni Ortali, personal communication, 14 November 2003).

There is less experience of the use of vaccines in chukar partridges, quail, pheasants, guinea fowl, ratites, and zoo birds. In zoo birds, studies in France, Spain, and other European Union (EU) countries identified that 71–81.5% of diverse taxon of birds had protective HI titers (GMT $\geq$ 32) after one immunization, but to improve the number protected and provide long-lasting protection, a minimum of two vaccinations were needed, and a booster at 6 months may be necessary [96]. Vaccination protocols must be developed that blend field experience with laboratory data to produce a viable program that will ensure adequate vaccination of birds, resulting in both individual and population immunity. Such protocols could include inactivated as well as recombinant live vectored AI vaccines.

From a field population basis in a defined geographic area, vaccination can reduce the number of outbreaks and the spread of AIV. For example, use of an AI vaccination program in village poultry, especially in combination with Newcastle disease vaccination, reduced the incidence of H5N1 HPAI and Newcastle disease in Indonesia based on a participatory surveillance program [27, 90, 108, 188, 247, 248, 279, 375]. However, coverage rates

within the population are important for stopping transmission and reducing spread. A minimum of 60% of the birds in the population should be immune in order for a reduction in transmission to be detected, with optimal reduction being seen when an immunization rate of over 80% is achieved [31]. The H5N1 Gs/GD lineage of H5N1 HPAIV is endemic in Egypt despite a national vaccination campaign, but analysis of protective HI titers in the field suggests that effective population immunity was not occurring in poultry, as only 33.2%, 36.4%, and 46% of poultry surveyed had protective titers [33, 369, 370], making effective herd immunity in Egypt elusive.

Priorities for vaccination

When designing a vaccination program with limited resources, the sectors and species for AI vaccine use should be based on economic value and risk analysis. A simple algorithm for AI vaccine use, in decreasing order of application, should be as follows:

1. poultry and other birds in high-risk compartments, sectors, or situations during an outbreak, using a suppressor vaccine when immediate stamping out is not feasible
2. rare captive birds such as in those in zoological collections as a preventative measure when the risk of AI introduction is high
3. valuable genetic poultry stock, such as pure lines, heritage breeds [148], or grandparent stocks whose individual value is high and when there is a high risk of AI introduction
4. long-lived poultry, such as egg layers or parent breeders in endemic areas
5. meat production poultry.

Biosecurity during vaccination

Any movement of people, equipment, or supplies on or off a farm creates a risk of introducing or spreading AIV between premises. During vaccination campaigns, the highest level of biosecurity must be practiced in order to reduce the risk of spreading field virus by vaccination crews as they move from one premises to the next. Such a danger was reported in the eradication efforts of the 1970s during the Newcastle disease epidemic in California. The vaccination crews were epidemiologically linked to prolongation of the outbreak by spreading the field virus from farm to farm [144]. However, any kind of traffic on and off the farm, such as visitors (family, relatives, friends, etc.), utility crews, catching crews, feed trucks, and so on, carries a risk of spreading the AIV [366].

Surveillance

Vaccinated flocks must be monitored to determine whether the vaccination has produced adequate flock immunity, to determine when immunity has declined sufficiently to warrant a booster vaccination, and to determine whether the flock has been infected by field AIV. Such monitoring can be undertaken using virological and/or serological methods, depending on the goal and specific needs of the AI control program.

Assessment of vaccination and immunity

Laboratory assays can be used to assess the effectiveness of vaccination (i.e. the number of birds or percentage of the population that were properly vaccinated with a potent vaccine) and provide an estimate of field protection (i.e. determine the percentage of the population protected against circulating field viruses). First, the use of a homologous antigen to the vaccine in an HI serological test was an effective means of assessing the success of vaccination in chickens by the production of an immune response against the vaccine [139, 344]. Second, humoral immunity produced against the subtype-specific HA protein, as measured in HI or virus neutralization tests using the circulating field virus, provides a positive predictive measure of protection in the individual bird and, when evaluated collectively, the protective immunity within the flock or population [7].

When using a standardized test method, serological results can be compared with *in-vivo* protection studies to establish minimal protective serological titers and determine the point at which booster vaccinations should be administered to achieve an optimal secondary or booster immune response. However, the establishment of a single minimal protective titer for all bird species based on HI results is not possible because HI titers vary depending on HI test procedure, species and breed of bird, the length of time measured post vaccination, and

the type of vaccine and the adjuvants used. In previous experimental studies using inactivated H5 AI vaccines in SPF white leghorn chickens challenged with H5 HPAIV, prevention of mortality was associated with an HI antibody geometric mean titer (GMT) of ≥ 8 (344) or ≥10 [323], while prevention of oropharyngeal replication and shedding of the challenge virus was seen in most vaccinated chickens with an HI antibody GMT of ≥ 40 [182]. Complete prevention of oropharyngeal shedding was only seen in vaccinated chickens with HI antibody GMT ≥ 128 [182]. Developing and implementing minimum serological potency standards would ensure that sufficient antigen is contained in commercial poultry AI vaccines to provide a protective immune response in the field for vaccines with a close antigenic match to circulating field strains, but if the vaccine and field viruses are antigenically divergent, serological protection is less predictable, which may require higher HI antibody titers to predict protection. Furthermore, protective HI titers are usually lower in commercial broilers and breeders, turkeys, ducks, and geese than in SPF laying chickens.

Identification of infected animals within a vaccinated population

Vaccinated flocks should be monitored for AIV infection in order to assess the success of the vaccination program and identify infections in vaccinated flocks which should be eliminated (i.e. DIVA). Conceptually, the DIVA principle of monitoring can be accomplished by a variety of virological and/or serological surveillance methods when using sensitive and appropriate tests based on adequate sample types and numbers of samples (Figure 15.5) [333]. Basic principles for such AI surveillance are available from OIE [314, 315].

Virological surveillance

The most direct and accurate means of identifying AIV infection is through the detection of the virus within the vaccinated flock or population. The simplest and most sensitive surveillance system involves monitoring a highly susceptible subpopulation within the vaccinated population (biosensor concept), because the majority of vaccinated birds will be immune and thus resistant to AIV infection (making most vaccinated and asymptomatic

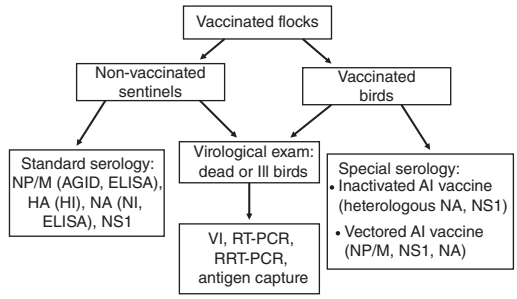

Figure 15.5 Flow diagram for virological and serological surveillance of vaccinated flocks for detection of AIV infection (i.e. DIVA strategies). Source: David E. Swayne.

birds poor subjects for sample collection when attempting virus detection or isolation). Therefore the virological surveillance program should emphasize targeted clinical or syndromic surveillance of highly susceptible individuals within the vaccinated population as follows:

1. permanently marked or identified, non-vaccinated sentinels that originated from an external biosecure source or were obtained from the source flock, but were intentionally left unvaccinated; *or*
2. a subpopulation of unmarked birds that did not develop protective immunity because individuals were missed during the catching and vaccination process, individuals were caught but incorrectly vaccinated (e.g. the vaccine was not deposited in the correct site), or for other reasons individual birds were caught and vaccinated but did not develop immunity.

In HPAI-affected areas, mortality in highly susceptible individual birds such as chickens or other gallinaceous poultry has a syndromic surveillance role as an early warning clinical system or "biosensor" for quick identification of potential cases to be tested for AIV. However, with LPAIV, the infection will not consistently produce mortality, and testing of ill birds along with any dead birds should also be included in syndromic surveillance. The use of mortality as biosensors in vaccinated flocks has been most clearly demonstrated in experimental studies as follows. First, vaccinated birds that did not develop immunity (as demonstrated by lack of HI antibodies) were susceptible to infection and died following exposure to HPAIVs [238], and second, non-vaccinated birds placed in contact with intranasally challenged, vaccinated

birds served as sentinels with detection of HPAIV excretion (Table 15.4) [333]. In the 2003–2005 Connecticut H7N2 LPAI vaccination program, successful surveillance for evidence of infection was conducted through a combination of virological testing of daily mortality in vaccinated birds and serological testing of sentinels [46].

However, virological surveillance should only be undertaken if adequate and appropriate samples are collected from the correct bird subpopulations and tested using the correct laboratory methodology. For example, sampling of ill or dead birds, in which typically there is oropharyngeal and/or cloacal excretion of high levels of AIV, has shown most consistent detection of both HPAIV and LPAIV by using virus isolation, real-time reverse transcriptase–polymerase chain reaction (RRT-PCR), or antigen detection by various antigen-capture ELISA test methods [314]. However, sampling of preclinical infected birds, which shed low levels of virus, will only detect virus if the most sensitive methods are used (i.e. virus isolation and RRT-PCR). The use of antigen capture tests has shown 79–80% sensitivity and 99–100% specificity in detecting virus in samples from ill or dead LPAIV-infected chickens [91, 283]. The RRT-PCR test had 95% sensitivity and 99% specificity for the same data sets. Therefore, to increase the sensitivity of detection of infected birds, daily collection and testing of dead or visibly sick birds should be used for virological monitoring of vaccinated populations for possible AIV infections, and the most sensitive virological tests should be used. As an example of virological surveillance in Egypt during 2007 and 2008, involving commercial and backyard poultry flocks, it identified AIV in 35 out of 3610 (0.97%) and 27 out of 8682 (0.31%) commercial poultry farms, and 246 out of 816 (30%) and 89 out of 1723 (5.2%) backyard flocks, respectively, indicating a low level of virus infection in vaccinated populations [93, 94, 283, 408]. However, H5N1 HPAIV is endemic in Egypt because of low national vaccine coverage [134] and the use of antigenically poorly matched vaccines [338, 339].

Serological surveillance

Serological monitoring provides a historical view of infection within the flock, which can primarily be used to assess the success of the vaccination program, but cannot be used to identify actively infected flocks. Virological surveillance is needed to determine whether the flock is currently infected and what risk, if any, the flock may have as a reservoir, and its transmission risk. A variety of serological surveillance systems have been described, each having specific advantages and disadvantages, but no one system will work for all vaccines and all field situations. All serological surveillance systems are based on differential production of antibodies against various viral proteins following AIV infection as opposed to vaccination (Table 15.7).

The primary challenge with serological surveillance is identification of antibodies in infected birds that differ from those in AIV-vaccinated birds. Antibodies against NP/M proteins, detected by agar gel immunodiffusion (AGID) or ELISA tests, are present in all birds that are infected with any HA

Table 15.7 Positive serological results against specific AI viral proteins following AIV infection or vaccination with different types of AI vaccines.

Group	Serological test results against specific avian influenza viral proteins[a]				
	NP/M	HA	Field virus NA	Vaccine NA	NS1
Avian influenza field virus infection	+	+	+	NA[b]	+
Inactivated avian influenza vaccine: homologous NA	+	+	+	NA	–
Inactivated avian influenza vaccine: heterologous NA	+	+	–	+	–
Vectored or DNA HA vaccine	–	+	–	–	–
Non-vaccinated sentinels	–	–	–	–	–

[a]NP/M, nucleoprotein and matrix protein; HA, hemagglutinin; NA, neuraminidase; NS1, non-structural 1 protein.
[b]NA, not applicable.

subtype of AIV, and cannot be used as a differential test to distinguish infected from inactivated whole AIV vaccinated birds. However, several systems can allow identification of AIV infections within a vaccinated population of birds.

Sentinel birds

Since the late 1970s in Minnesota, sentinel birds have been used successfully for serological detection of LPAIV infections within inactivated AIV-vaccinated turkey flocks [132, 172]. This sentinel system has been expanded for use in other outbreaks of LPAI in turkeys and chickens in the USA and elsewhere in the world [138]. Theoretically, the detection of antibodies against any of the 10 AI viral proteins is indicative of infection, and sentinels can be used as a mitigation step to detect "silent infections" within AI-vaccinated flocks (Table 15.7) [115, 217, 283]. However, the sentinels must be permanently marked or identified so that the surveillance system can be properly managed. First, this allows easy observation of susceptible birds within the flock for syndrome surveillance (i.e. observation of clinical signs or death that would trigger an AI investigation). Second, it allows easy access to birds for swift collection of appropriate samples for serological or virological testing. Finally, it prevents unethical and unlawful tampering with sentinels by unscrupulous individuals who wish to alter the test results by replacing official sentinel birds with other birds. Typically, the state/provincial or federal government should manage the sentinel bird program. During the 2000–2002 H7N1 LPAI outbreaks in Italy, 1% of birds in vaccinated flocks were maintained as non-vaccinated sentinels, and a minimum of 10 birds per barn were bled every 45 days for serological determination of infection [274]. Sentinel birds should be identified by a tamper-proof visual or electronic tag system that uniquely identifies each bird as a sentinel. Management of sentinels is easiest for cage-housed birds such as laying chickens, because the birds can be individually identified and placed within tagged cages that can be physically mapped for quick, repeated observation. Floor-reared birds are more difficult to manage, and may require the development and use of some creative visual identification in addition to the permanent number-tagged system, such as confining the sentinels in several floor cages spread throughout the house or, in the case of free-roaming birds, quick visual identification by using large brightly colored wing tags, painting the feathers of white birds, or using breeds that differ in color from the vaccinated birds as sentinels.

Vaccinated birds

The majority of AI vaccines used in poultry are inactivated AI vaccines. Because protection is HA subtype specific, the inactivated AI vaccine does not require an NA-subtype match between vaccine and field virus in order to be protective [59]. In the mid-1980s and 1990s, serological detection of heterologous NA was proposed as a method to identify infected among vaccinated birds [16]. This concept was used successfully during an outbreak of H7N1 LPAI in Italy during 2000, and in subsequent years against other H5 and H7 LPAIVs [16, 99, 304]. Basically, the AI vaccine has the same HA subtype but a different NA to the field AIV, thus allowing the development of protective HA antibodies, but the vaccinated birds would not have antibodies against the field NA unless they were also infected (Table 15.7). However, to use this strategy as a preventative vaccination program, a pre-existing vaccine bank is needed with a minimum of two different NA subtypes for each HA subtype vaccine, the vaccine should have a rare NA subtype such as N5, or the vaccine could use a synthetic NA from influenza B virus in rg-generated influenza A virus [57–60, 62, 69, 70]. Vaccine seed strains can be LPAIV from an outbreak, can be generated by classic reassortment techniques [20, 113], or can be produced by an rg system [20, 316]. Multiple inactivated AI vaccines for poultry and humans have been developed using the H5 or H7 HA from current field isolates, but engineered to contain various NA (e.g. N1–9) subtypes [191]. In the USA, during the mid-2000s, USDA/APHIS banked poultry AI vaccines against only H5 and H7 subtypes, each with two different NA subtypes, but this bank was discontinued in 2010, citing expiration of the contract and low potential for use. However, after the 2014–2015 H5N8/H5N2 clade 2.3.4.4 Gs/GD-lineage HPAIV outbreak in North America, a new AI vaccine bank was developed. rHVT-H5 containing a clade 2.2 HA gene insert of A/swan/Hungary/4999/2006 (H5N1) and rdVEE-H5 containing a clade 2.3.4.4 HA gene insert of A/gyrfalcon/Washington/41088/2014

(H5N8), with the HA cleavage site altered to low pathogenicity, were first purchased. In addition, an inactivated H5 vaccine seed strain has been developed utilizing the clade 2.3.4.4 HA gene of A/gyrfalcon/Washington/41088/2014 (H5N8) with the HA cleavage site altered to low pathogenicity, and the other seven gene segments from the PR8 vaccine strain. The use of the PR8 N1 gene in the vaccine would allow identification of infection by the H5N8 and H5N2 HPAIVs in vaccinated flocks by the detection of N8 and N2 antibodies, respectively.

Antibodies to the nine specific NA subtypes in a serological DIVA strategy can be detected by various assays, including neuraminidase inhibition [97, 191, 205, 222, 317, 356, 384, 392], indirect immunofluorescence [10, 152], or ELISA assays [60, 70]. However, the heterologous NA system should not be viewed as the only serological DIVA system, as many inactivated AI vaccines use strains that contain an NA subtype homologous to the field virus, so antibodies against the homologous NA cannot be used to identify infection. Experimental studies have been conducted that demonstrated the utility of serological tests to detect antibodies to NS1 and M2e proteins for identifying AIV infections in poultry vaccinated with inactivated AI vaccines [123, 208]. The serological response to the NS1 protein has been proposed as a differentiation test because NS1 protein is only produced in the infected cell, and is not packaged in the AIV [11, 184, 219, 351, 384]. In four separate studies, antibodies against NS1 were not seen in chickens or turkeys that had been immunized with experimental or commercial inactivated AI vaccines unless they had also been infected with an AIV [11]. However, both the NA and NS1 serological tests to detect infection in vaccinated populations only have a moderate sensitivity (63–64%) for detection of such infections [11, 364, 384, 405]. The M2e serological test is expected to have similar sensitivity for detection of infection in vaccinated populations to that of NA and NS1 serological tests. Vaccination with one, two, or three doses of commercial vaccine did not produce antibodies to M2e, but when challenged, such antibodies were seen, especially 6 weeks or later after the last vaccination.

A simpler surveillance strategy for detecting infection in AI-vaccinated birds involves the use of AI vaccines generated by biotechnology which contain only the HA protein, such as *in-vitro* or *in-vivo* expressed HA protein, or an HA DNA vaccine, accompanied by the use of standard serological tests. These AI-vaccinated birds have protective antibodies only against the specific HA protein in the vaccine, but lack antibodies against the other AI viral proteins. Thus the AI-vaccinated birds will be positive for antibodies on the specific HI test, but negative for NP/M antibodies on the standard AGID or commercial ELISA tests used around the world unless the birds have been infected. For example, chickens vaccinated with rFPV-H5 vaccine at 1 day of age had antibodies at 3 weeks of age against the homologous HA, but were negative for antibodies against NP/M as measured by AGID testing [371]. Following AIV infection, some chickens developed antibodies against NP/M as measured by AGID tests. All serological DIVA strategies in vaccinated birds should be interpreted as a flock and not an individual bird test because proper vaccination will increase resistance to infection in the majority of birds [335].

Conclusions

AI vaccines provide protection to birds principally through mucosal and systemic humoral immunity against the HA protein, and such protection is HA subtype specific. However, cell-mediated immunity can play a role in protection, as can antibodies to NA. Protection can be directly assessed by prevention of clinical signs and death, prevention of egg production drops and reduced number of eggs contaminated with HPAIV, a decrease in the number of birds infected, a reduction in the quantity of challenge virus shed from the respiratory and alimentary tracts, and prevention of contact transmission in *in-vivo* experimental studies. Protection can also be assessed indirectly through measurement of protective antibody levels in vaccinated birds, or assaying the quantity of HA protein in the vaccine. AI vaccines should be sufficiently potent to protect birds under a variety of field conditions.

AI vaccines are based on four technology platforms – inactivated whole AI vaccines, *in-vitro* expressed AIV proteins (principally the HA), *in-vivo* expressed AIV proteins in vectored systems (principally the HA), and HA-based DNA vaccines.

However, only inactivated AIV, rFPV-H5, rHVT-H5, rdVEE-H5, and rNDV-H5 vaccines are currently licensed and used in the field in various countries around the world. Historically, inactivated AI vaccines have used seed virus strains predominantly based on outbreak LPAIV, and occasionally on outbreak HPAIV. Such seed strains have been broadly protective within an HA subtype and requiring less frequent change of seed strains than has been necessary for human seasonal influenza A vaccines. In the past 5 years, some seed strains have been produced by reverse genetics utilizing the HA (with low-pathogenicity cleavage site) and NA genes from an outbreak virus and the six internal genes of a high-growth influenza A vaccine strain. AI vaccines have been used in emergency, routine, and preventative vaccination programs.

Several issues are important with regard to field use of AI vaccines:

1. AI vaccine use should be part of a comprehensive AI control program.
2. The vaccine strain must be of the same HA subtype and should be protective in the target species based on *in-vivo* studies against a recent circulating field virus.
3. The vaccine should have sufficient HA content (inactivated or *in-vitro* expressed HA system vaccine with a minimum titer of 1–5 μg HA/dose) or adequate live virus titer (vectored vaccines) sufficient to produce a protective immune response, or sufficient HI serological titers should be demonstrated to indicate protection.
4. Inactivated or *in-vitro* expressed HA system vaccines should be emulsified within a good oil adjuvant system.
5. Manufacturing of AI vaccines must be standardized in order to produce consistent and efficacious vaccine batches.
6. Procedures must be established for proper storage, distribution, and administration of the vaccine.
7. Biosecurity practices must be established to protect and prevent vaccination crews or other service personnel from accidental spreading of field virus.
8. Proper serological or virological surveillance systems must be in place to determine whether vaccination has produced protective immunity, and to monitor vaccinated populations for possible field virus circulation (i.e. DIVA).
9. An exit strategy from emergency vaccine use should be developed to prevent vaccination from becoming a routine program with associated AIV endemicity.

AI vaccines must be periodically re-evaluated in order to determine whether they are still effective against circulating field virus strains, and if they are no longer protective, as has been demonstrated in the field after prolonged circulation of AIV in poultry, vaccine strains should be replaced. Requirements for licensing AI vaccines will vary with each country, depending on the specific requirements of the national veterinary biologics authority in areas of safety, purity, potency, and label approval for species, age, and route of administration.

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SECTION III

Influenza in animals of the class mammalia

SUBSECTION IIIA

Swine influenza

16 The clinical features, pathobiology, and epidemiology of influenza infections in pigs

Susan E. Detmer

Introduction

Influenza A virus (IAV) is a major cause of year-round acute respiratory disease in pigs of any age. In uncomplicated cases, the disease is mild and self-limiting [67]. Similar to the disease in humans, influenza occurs year round with seasonal peaks when the environmental temperatures and humidity are moderate [70].

History

Although clinical signs of influenza were first recognized in pigs during the 1918 Spanish influenza pandemic [36], the first isolation of an IAV from pigs did not occur until 1930 [73]. Influenza A/swine/Iowa/15/1930 H1N1 virus is considered the prototype for classical swine H1N1 (cH1N1) that gradually evolved in North America during the twentieth century, until the introduction of the triple-reassortant internal gene (TRIG) cassette composed of genes from avian, human, and swine IAVs [91]. Since the introduction of the TRIG cassette, there has been an explosion in IAV diversity in pigs worldwide, with the development of several distinct genetic and antigenic clusters (for further information on IAV evolution in swine, see Chapter 18).

Virology

Endemic influenza in pigs is predominantly caused by IAVs of the subtypes H1N1, H1N2, and H3N2. Infections with wholly human and avian-origin IAVs and swine-origin reassortants resulting in unusual subtypes have been demonstrated in pigs [29, 48, 49, 60]. However, these occur sporadically and are unlikely to result in sustained endemic infections. Similarly, antibodies to influenza B viruses have been detected in pigs in the UK and China, but influenza B virus has not been isolated from swine [5, 54]. Although rare, influenza C viruses (ICVs) have been detected in swine [5, 54, 89]. A novel virus that is similar to human ICVs has so far been rare in pigs but may have wider circulation in cattle [24, 71].

On a cellular level, infection with IAVs is initiated by the binding of hemagglutinin (HA) protein on the surface of the virus to sialic acid sugars on the surface of respiratory epithelial cells [26]. Once internalized, the virus replicates and many virus particles are released. The microscopic lesions indicating that this process is occurring can be seen within 24–48 hours of infection. The rate of progression and severity of the respiratory disease vary among IAVs, and can also be modified based on a number of different factors.

The underlying mechanisms of infection remain consistent, and the variability can be seen in the isolation of the viruses from animal samples. Madin–Darby canine kidney (MDCK) cells have the highest sensitivity for growing swine-origin IAVs, and are most commonly used in research and diagnostic applications [55]. Swine testicular cells and chicken embryos are also recommended in addition to MDCK cells. Most IAVs can be propagated in these cell lines under standard culture

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conditions, but there is variability between strains. Some IAVs grow within 48 hours, but others need 5–7 days or a second passage to be isolated. The passage number of the MDCK cells can also play a role. Some IAVs grow to higher titers in MDCK cells of lower passage numbers (less than 100 passages), where other IAVs grow to higher titers in MDCK cells of higher passage numbers (more than 200 passages). These variations that are observed *in vitro* are not observed *in vivo*, and represent one of the incongruences between *in-vitro* and *in-vivo* experimental results.

Pathogenicity

The pathogenicity of IAVs in pigs can be quite variable, and often when an IAV is isolated during an epizootic on a farm, the anecdotal reports from the field have much higher mortality than when the virus is tested in an experimental inoculation, under controlled conditions [50]. The ability of IAVs to cause disease in pigs is determined by a combination of host, virus, and environmental factors. Environmental factors and husbandry issues will be discussed in more detail in the epidemiology section of this chapter. Two of the host factors that need to be considered are the age and immune status of the pig. Immunity and vaccinations will be discussed in more detail in Chapters 17 and 19.

Like most species, neonatal pigs are one of the populations that are most susceptible to IAVs, and they may or may not have maternally derived antibodies (MDAs) provided in the sow's colostrum to protect them. Pigs are usually weaned at 21–28 days of age and moved to a nursery where pigs from multiple litters are mixed together, and moved to the grower-finisher barns at around 10–12 weeks of age. Nursery pigs are highly susceptible to a number of respiratory pathogens, and to endemic infections with a number of pathogens that are associated with porcine respiratory disease complex (PRDC). PRDC is a complex, multifactorial disease that includes IAV. It has both infectious and non-infectious factors contributing to disease, and is seen primarily in grower-finisher pigs between the ages of 3 and 6 months [63]. Like IAV infection alone, PRDC is affected by a combination of host, pathogen, and environmental factors.

One factor specific to IAVs that is dependent on both the host and the virus is the binding of HA to sialic acid sugars. Host specificity for IAVs is determined by the type and distribution of host cell receptors and the protein structure of HA. It is generally accepted that human and swine IAVs bind to NeuAcα2,6-Gal-linked sialic acids (α2,6) and avian IAVs bind to NeuAcα2,3-Gal (α2,3) receptors [26]. A similar distribution of α2,6 and α2,3 receptors has been found in both humans and pigs, with α2,6 being spread throughout the respiratory tract and α2,3 being more specific to the alveoli [57, 77].

The lectin histochemistry (LH) technique, which is used to qualitatively determine the location of these sialic acid receptors in the respiratory tract, has led to discrepancies in the distribution of influenza receptors and specific cell types involved, as well as discrepancies between the cells that were actually infected in culture compared with those predicted by LH in the human airway [53, 61, 62, 72, 87] and in the swine airway [31, 76, 78]. Furthermore, the way in which IAV enters and replicates in cells that have had these sialic acids removed from the surface has not been determined [75].

In addition to host specificity, the characteristics of virulent viruses include an ability to sustain higher and/or prolonged virus replication, replication in the lower respiratory tract, and induction of excessive cytokine expression [32]. The cytokines of interest include interferon (IFN)-α, tumor necrosis factor (TNF)-α, and interleukins (IL) 1, 6, and 8. Induction of excessive expression of IL-1β, IL-8, and TNF-α has been seen with vaccine-associated enhanced respiratory disease (VAERD), characterized by severe respiratory disease with mismatched vaccination and subsequent virus challenge [68].

The inherent capability for virulence of IAVs is related to interactions of viral proteins both with the host and among themselves. The most studied viral protein is the surface protein HA that is responsible for attachment to host cells, internalization, and fusion between the viral capsid and the membranes of endosomes. Human- and swine-origin viruses do not have the multibasic cleavage site that is found on the high-pathogenicity avian influenza viruses (H5 and H7 HA subtypes) and which has been associated with lethal infections in mice and ferrets, but pigs appear to have a lower susceptibility [43]. Two different HA mutations, E119G/V152I/N224K/Q226L and N224K/Q226L, have demonstrated a switch

from α2,3 to α2,6 binding preference in avian viruses [30], but an additional N158D or N158K mutation is needed to improve the virus replication of the mutant, and the T318I appeared to stabilize the HA protein during transmission experiments [28].

The three polymerase proteins, PA, PB1, and PB2, form a protein complex, and their replication of the RNA genome plays a key role in the rate of viral replication and genetic drift. The PB1-F2 protein that results from an alternative open reading frame in PB1 can be identified in some swine-origin IAVs [64]. This protein is most often associated with induction of apoptosis in cells of the innate immune system, but is also associated with suppression of the early interferon response in infected cells, and with increased tissue inflammation [32].

Most of the research examining the functions of viral proteins has been conducted in cell culture and laboratory animal models. Only a limited number of studies have been able to replicate results in the host species of origin, which reflects the relevance of results and other limitations of laboratory animal models for IAV studies.

Laboratory host systems

Ideally, IAVs should be studied within their natural hosts, and the domestic pig is an excellent laboratory model for both swine- and human-origin IAVs [68]. However, the mouse model of influenza is used most often, despite the fact that strains must first be adapted to mice in order to cause disease. Knockout mice can be selected to study specific cellular responses, but caution must be exercised when interpreting these results for other species, as they may not be directly translatable. The guinea pig provides a different rodent model for which adaptation of the viruses is not necessary, but the lung lesions are limited unless the viral dose is high [46]. For swine-origin IAVs in guinea pigs there is restricted contact transmission, which may be a limiting factor for use of this model to study the swine viruses [46].

The ferret model has been used to examine the pathogenicity of IAVs from a number of species, including pigs. It has been used to model direct and indirect transmission, to examine potential virulence factors and immune responses, and to produce standard antibodies for hemagglutination inhibition assays used for human and swine [90]. Compared with humans and pigs, ferrets have a similar distribution of α2,3 to α2,6 receptors, and a similar pattern of lesions and clinical disease [77, 90]. Although the ferret is an appropriate model, it too has limitations with regard to the interpretation of experimental results.

Respiratory tissue explants from pigs can be maintained in incubation chambers for at least 48 hours in order to study infectious agents [17]. Although the full systemic reactions do not take place within the explant, several viruses can be studied on separate sections of explanted tissue from the same animal. Tissue explants offer an excellent intermediate transition from immortal or primary cell lines to animal models of disease. This model also provides the opportunity to decrease the total number of animals required for a study, which is encouraged by research ethics boards.

Clinical disease

Clinical signs of the acute respiratory disease caused by IAV in both naturally infected and experimentally inoculated pigs include fever, anorexia, coughing, labored breathing and "thumping" (a loud noise made by a pig when its whole body shakes due to breathing effort), sneezing, nasal discharge, and poor weight gain. Fever is the most consistent clinical sign, and peaks within 24–48 hours of infection with most IAV strains. Clinical signs start as early as 1 day post infection (DPI) in the majority of experimental infections of swine with both human and swine isolates of IAV [4, 21, 22, 25–27, 34, 40, 41, 51, 66, 74, 79, 85], 2 DPI [3], and 3 DPI [33–35], coinciding with the time of detection of virus in nasal secretions.

The clinical signs cease between 4 and 8 DPI in experimentally infected animals [4, 21, 22, 25–27, 34, 40, 41, 66, 74, 79, 85]. In animals that have robust protection from a vaccine prior to challenge with an antigenically similar isolate, the amount of virus shed is reduced, and shedding ceases by 2–4 DPI [12, 26, 27, 79–81]. Homologous vaccination will ideally result in little to no virus shedding or clinical signs, but this is not guaranteed [1, 69].

Influenza in pigs is a disease with high morbidity and low mortality. Although virus can be circulating on a farm without the presence of

clinical signs [9], the latter can be observed in 80–100% of pigs during an influenza outbreak on a farm, and in 30–50% of pigs on endemically infected farms. Mortality due to uncomplicated influenza infection is rare in pigs, but has been seen in diagnostic cases where coughing led to tracheal hematomas that caused asphyxiation, and where anorexia led to bleeding gastric ulcers and subsequent exsanguination.

More often, mortality from influenza in pigs is due to secondary bacterial infections or co-infections associated with PRDC. Co-infection of influenza virus with *Haemophilus parasuis* or porcine reproductive and respiratory syndrome virus (PRRSV) is associated with more severe clinical disease in swine [82, 83]. Another PRDC pathogen, *Mycoplasma hyopneumoniae*, has been shown to have either a transient effect or no effect on the overall outcome with IAV co-infection [88]. *M. hyopneumoniae* causes ciliary stasis in the trachea and bronchial tree, and thus disrupts the mucociliary clearance defense mechanism. The small particle size (80–120 nm) of IAV means that it evades the mucociliary apparatus, so synergy with this PRDC pathogen is unlikely.

One of the most virulent field isolates had a reported mortality of 10% in finisher pigs, but PRRSV, *Pasteurella multocida*, *Streptococcus suis*, and *Streptococcus* species were also detected in the lung [50]. All of these pathogens are considered part of PRDC [63]. Since IAVs are one of the principal components of PRDC, the clinical signs of PRDC are similar to those described for influenza. The disease complex is characterized by 30–70% morbidity and 4–6% mortality, similar to infection with IAV alone [63].

Pathology of IAV in pigs

Microscopic pathology

The hallmark microscopic lesion of IAV infection is necropurulent bronchitis and bronchiolitis [13, 32]. As shown in Figure 16.1a, a normal bronchiole has a thin layer of epithelial cells that have apical cilia and a small amount of peribronchiolar lymphoid tissue. The initial flu lesions are seen as early as 24 hours post infection (PI), and include vacuolar degeneration and necrosis of the epithelial cells with loss of the apical cilia (Figure 16.1b). This coincides with the ultrastructural findings of abundant virus budding at 24 hours PI [37]. By 48 hours PI, the sloughed necrotic epithelial cells are accumulating within the airway lumen along with neutrophils that migrate across the epithelium, creating the characteristic lesion of necropurulent bronchiolitis. By 72 hours PI (Figure 16.1c), the sloughing of the necrotic epithelial cells is more prominent, along with a small influx of a mixture of inflammatory cells. The remaining epithelial cells spread out to cover the basement membrane (attenuation), and the peribronchiolar lymphoid tissue expands, with increased numbers of lymphocytes admixed with a few macrophages (lymphoid hyperplasia).

Between days 4 and 5 PI, the early signs of recovery include varying degrees of epithelial hyperplasia, mitotic figures within the epithelial cells (Figure 16.1d) and mild inflammation in the bronchi and bronchioles as the inflammation spreads outward, expanding the alveolar septae. By 7–10 days PI there are varying degrees of interstitial pneumonia, perivascular and peribronchiolar lymphoid proliferation, and normal to hyperplastic bronchial epithelia. By 14–21 days PI, the damaged respiratory tissues should be fully recovered at the microscopic level [4, 18, 33, 35, 41, 56, 66, 74]. During the recovery process, bronchiolitis obliterans can result from exposure of the lamina propria and the formation of polyps within the bronchiolar lumen [32].

In some cases, the virus replication and damage is restricted to the bronchial tree, and there are lobules with no lesions, or lesions restricted to the bronchioles (Figures 16.2a and 16.2b). In other cases, the inflammation extends outside the bronchioles into the alveolar walls, causing severe lesions with lobular consolidation (Figures 16.2c and 16.2d). Although the most severe and consistent microscopic lesions are in the primary bronchioles, alveolar lesions are quite variable and can include atelectasis secondary to airway obstruction, consolidation of cells within the alveolar spaces, and interstitial pneumonia, or a combination of these. The pattern of the alveolar lesions tends to be lobular. This is due to a combination of the branching patterns of the bronchial tree that probably result in irregular distribution of virus, and the thick interlobular septae found in pigs and cattle preventing spread to adjacent

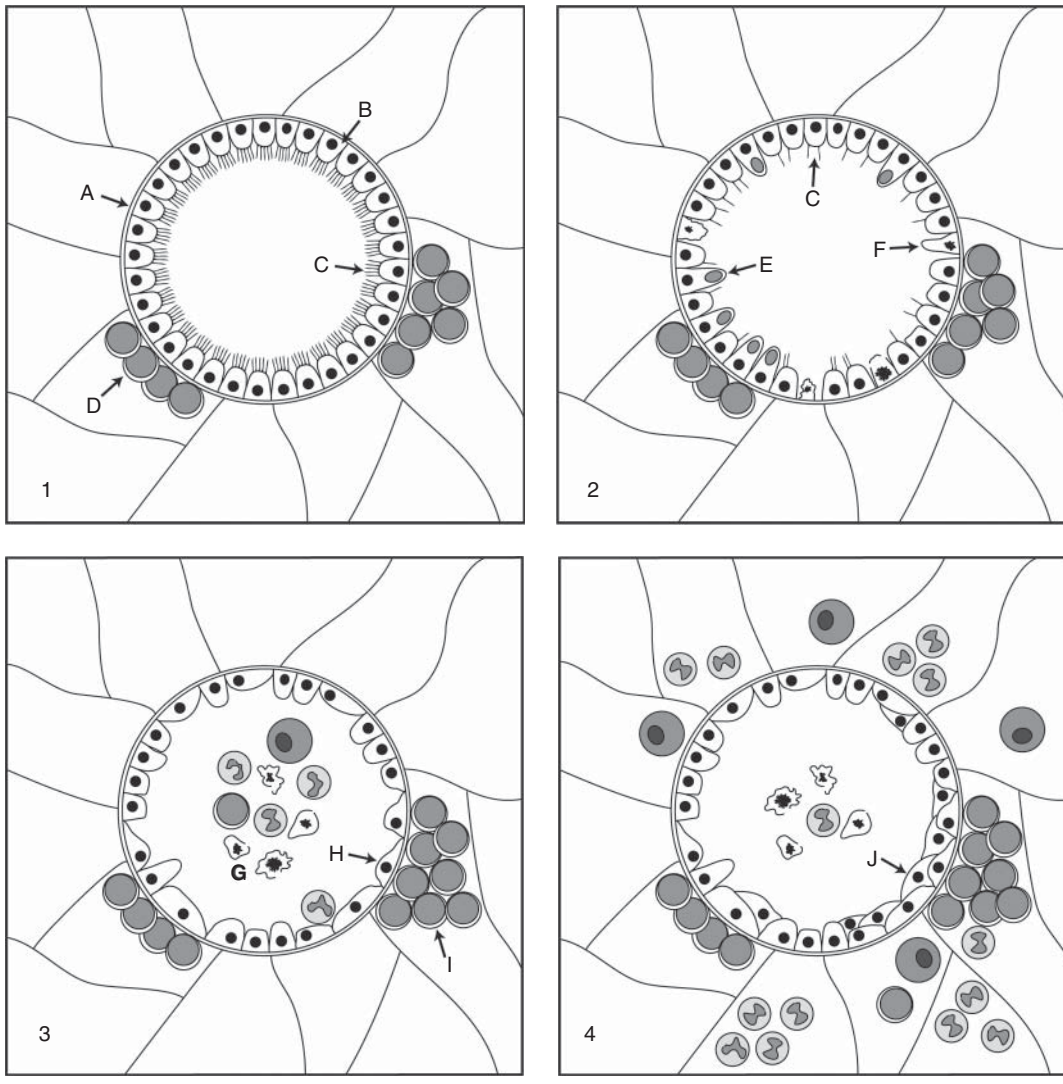

Figure 16.1 Pulmonary bronchioles during different stages of swine AIV infection. Courtesy of Juliane Deubner and University of Saskatchewan. **Figure 16.1a.** Normal bronchiole (A) that has a thin layer of epithelial cells (B) with apical cilia (C) and peribronchiolar lymphoid tissue (D). **Figure 16.1b.** Early influenza lesions of vacuolar degeneration (E) and necrosis (F) of the epithelial cells with loss of the apical cilia are seen as early as 24 hours post infection (PI). **Figure 16.1c.** Influenza lesions at 72 hours PI with necrotic epithelial cells sloughing, a small influx of inflammatory cells (G), attenuation of the remaining epithelial cells (H), and mild lymphoid hyperplasia (I). **Figure 16.1d.** Influenza lesions 5–7 days PI, showing varying degrees of epithelial hyperplasia (J), and mitotic figures in some of the epithelial cells.

lobules. Alveolar collapse or obstructive atelectasis is not always appreciated at either the microscopic or macroscopic level, and occurs when there is significant debris accumulation (or bronchiolitis obliterans) that blocks airflow into the alveolar spaces.

The underlying process for the formation of interstitial pneumonia was identified by ultrastructural examination at 5 hours PI, which demonstrated IAV budding from type II pneumocytes [86]. Alveolar epithelial cell sloughing along with edema and fibrin separating interstitial cells were observed by 24 hours PI [86]. During the first 24–48 hours PI, the destruction of pneumocytes and the migration of neutrophils followed by lymphocytes and macrophages occur, with alveolar consolidation

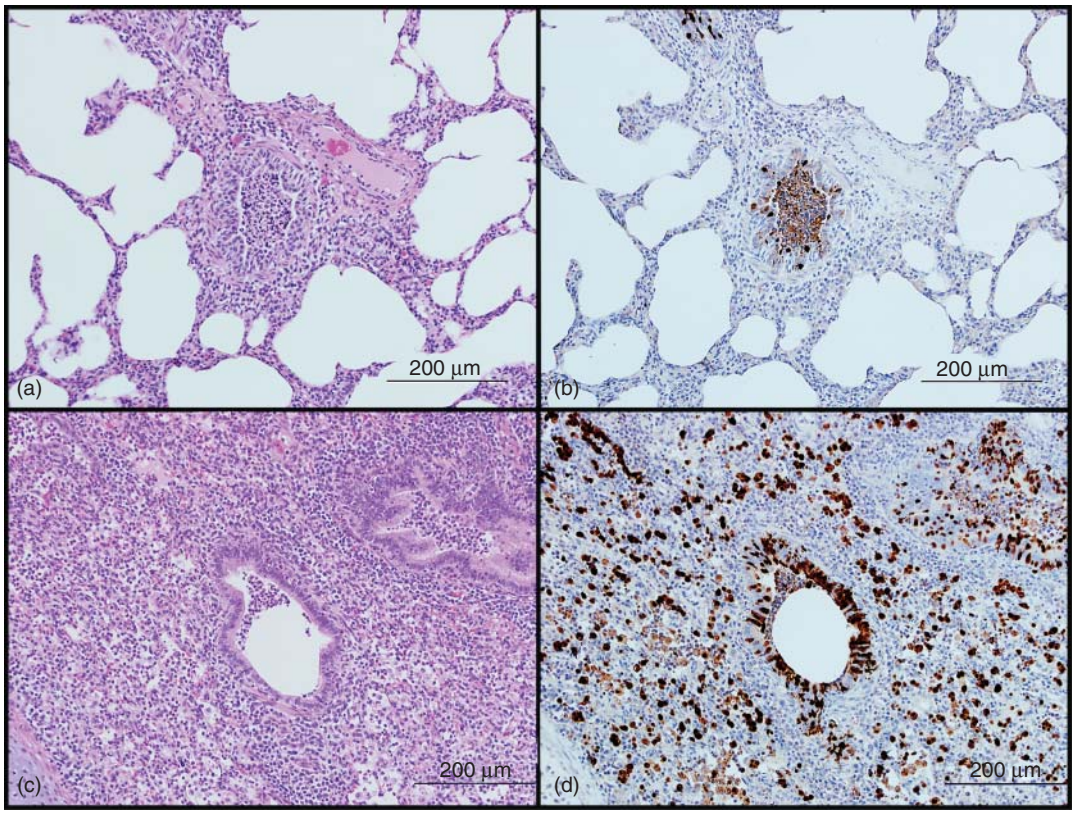

Figure 16.2 Experimental inoculation of a 4-week-old pig with A/swine/Texas/4199-2/1998 H3N2 virus 48 hours PI. Courtesy of Susan Detmer. **Figure 16.2a.** Severe, necropurulent bronchiolitis with mild interstitial pneumonia; hematoxylin and eosin (H&E), 200×. **Figure 16.2b.** Moderate bronchiolar epithelial cell and intraluminal immunoreactivity to anti-Influenza A nucleoprotein; immunohistochemistry (IHC) with diaminobenzidine (DAB), 200×. **Figure 16.2c.** Necropurulent bronchiolitis with severe alveolar pneumonia (lobular consolidation); H&E, 200×. **Figure 16.2d.** Strong bronchiolar and alveolar immunoreactivity to anti-Influenza A nucleoprotein; IHC with DAB, 200×. *See Plate section for color representation of this figure*.

spanning bronchiole to bronchiole within a lobule. Interstitial pneumonia is characterized by alveolar septae thickened by swollen type II pneumocytes, replacement of sloughed type I and II pneumocytes, interstitial edema, and monocyte accumulation.

Gross pathology

The hallmark macroscopic lesion of cranioventral bronchopneumonia reflects the microscopic lesions and the route of infection. The aerosol route of infection results in the movement of the virus through the conducting system (nasal passages and trachea) to the carina, and subsequent spread into the short branches of the bronchial tree within the cranial and accessory lung lobes through gravitational pull. The lesions may also extend to the cranial-most portion of the caudal lung lobes. In addition, the anatomical anomaly of the tracheal bronchus in pigs may result in more lesions within the right cranial lung lobe [13].

Although some lesions can be seen within 48 hours PI, the gross lesions are not fully developed and only partially represent the microscopic lesions [13]. By 5 days PI, the multifocal to coalescing lobular pattern more consistently reflects the microscopic lesions. For instance, the presence of lobular atelectasis is seen grossly as depressed (concave), dark red, polygonal areas that are dense to the touch (Figure 16.3). Lobular and lobar atelectasis is also seen with *M. hyopneumoniae* infections, and can be grossly indistinguishable from IAV infection at the macroscopic level. The raised,

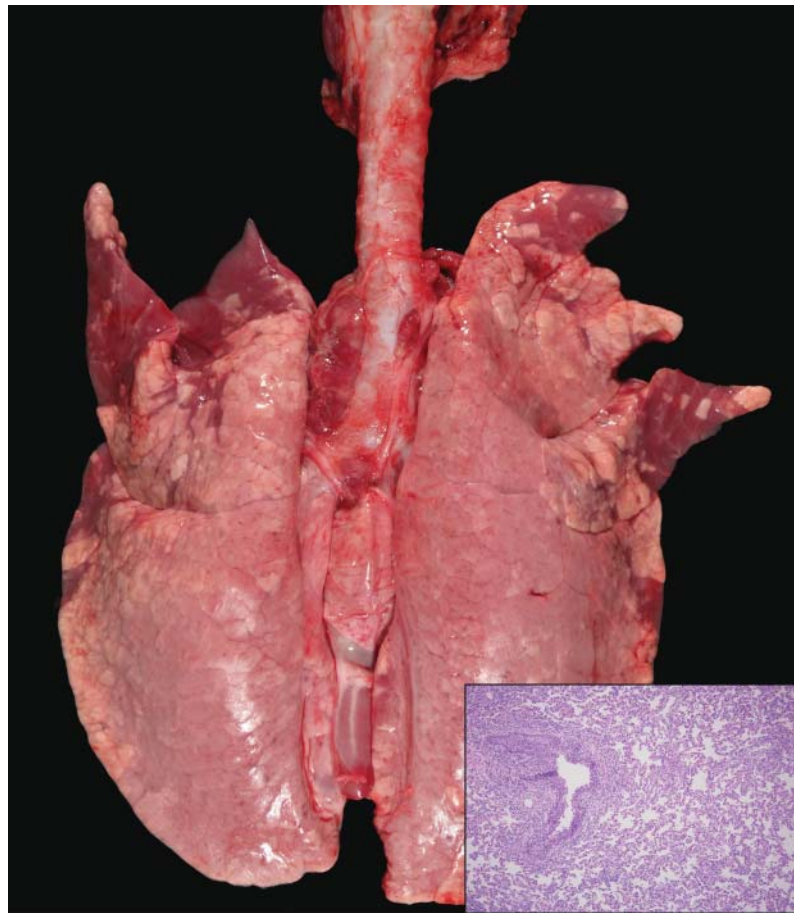

Figure 16.3 Macroscopic lesions in the lung of a 4-week-old pig experimentally inoculated with A/swine/Illinois/02450/2008 H1N1 virus 5 days PI. The depressed, dark red, multifocal to coalescing lobular lesions are in the cranioventral portions of the lungs, and reflect the microscopic lesions (shown in inset) of atelectasis. Courtesy of Susan Detmer. *See Plate section for color representation of this figure*.

dark red to purple, polygonal areas that are often described as feeling "hepatoid" or "meaty" reflect alveolar consolidation (alveolar spaces filled with cellular debris, inflammatory cells, and edema fluid). This gross lesion is more characteristic of aerosol bacterial infections in the lung, and in the case of IAV infections it is often associated with PRDC or secondary bacterial infections.

Immunohistochemistry

Immunohistochemistry (IHC) detects viral antigens within the tissues. The two antigens most commonly detected in either frozen or formalin-fixed tissues are nucleoprotein (NP) and hemagglutinin (HA) protein. Antibodies against NP can be used to detect all subtypes of IAV, but antibodies against HA are subtype specific and can have limited cross-reactivity within the subtype. The NP antigen is located in the nucleus and cytoplasm of infected cells [20, 23, 39, 84], whereas the HA antigen is located in the cytoplasm and along the cell surface [20]. The location of antigens within the tissue will depend on the location of the virus replication. If the infection is primarily confined to the bronchioles, the immunoreactivity will be primarily in bronchiolar epithelial cells and within the neutrophils and necrotic cellular debris in the bronchiolar lumina (Figure 16.2b). If the infection spreads out from the bronchioles into the alveoli, there will also be immunoreactivity, predominantly

in type II pneumocytes that may or may not be sloughed into the alveolar spaces (Figure 16.2d).

Transmission and epidemiology of influenza A virus in pigs

Transmission

In pigs, the incubation period between infection and virus shedding is very short. In experimental infections, the intratracheal inoculation dose of $1 \times 10^{6–8}$ tissue culture infectious dose 50 ($TCID_{50}$)/mL or egg infectious dose 50 (EID_{50})/mL will usually result in nasal shedding with a titer of $10^{2–4}$ $TCID_{50}$/mL within 24–48 hours PI [6, 12, 69]. This varies slightly among different viruses, depending on the ability of the virus to infect the cells, the animal's immune status, and the replication rate of the virus *in vivo*.

Although intratracheal inoculations are more efficient and consistent for experimental models, the actual mode of transmission in natural infection is initially through the nasal passages, down the conduction system, and into the lungs. This is experimentally simulated by both direct contact and shared airspace exposure to infected pigs that are shedding virus [1, 45, 69]. The gross and microscopic lesions produced in transmission models are indistinguishable from those seen in the intratracheally inoculated pigs used for the source infection [1, 69].

Epidemiology

Under field conditions, natural transmission of IAVs can occur year round, and there are a number of risk factors that favor initial virus transmission and continuous circulation of one or more IAVs on a farm or within a farm system. Large herd sizes and large numbers of pigs per pen are well established risk factors [15, 52, 65].

One of the most important risk factors associated with higher rates of detection of IAVs is the farm type, and specifically farrow-to-finish (FTF) farms [10]. In FTF farms, finisher pigs are more likely to have IAV detected in nasal swabs if they are on the same farm as the sow herd. In this situation, all age groups of pigs are on the same farm site, albeit usually separated into several barns, often using continuous-flow management. In a continuous-flow barn, young pigs are introduced to a barn with older pigs, creating a situation where potentially susceptible pigs are continually introduced and maintain an IAV.

For some FTF farms, the pigs are separated on the basis of age in order to create a more pathogen-stable or homogenized group. This homogenization is disrupted when "fall-backs" (pigs that are too small for their group) are moved back a group or two in order to give them more time to grow before they are sent for slaughter. Small room outbreaks of IAV can be seen in these finisher pigs. In another type of farm management, called all-in all-out (AIAO), all of the pigs in a barn are placed at the same time, allowed to grow, and are then removed. In one study, lack of AIAO movements was associated with increased IAV infection risk [15], and another study found that AIAO management was associated with a lower risk than continuous-flow management [10].

The environmental conditions that favor aerosol transmission are low temperature and low humidity [47]. IAV can be detected in the air inside and outside barns both with experimentally infected animals [11] and during outbreaks in the field [8]. The ideal outside temperatures and wind speed for aerosol transmission between barns need to be determined, but virus can be detected up to 2.1 km downwind from infected farms [10]. This finding gives credence to the possibility of aerosol spread between farms. Currently there is only anecdotal evidence in cases where virus strains move between farms and the only epidemiological link is proximity [12].

Other risk factors that need to be investigated more thoroughly include swine vaccine efficacy for the endemic strain, and the vaccination and health status of people working with pigs. Vaccines, both commercial and autogenous (discussed in more detail in Chapter 19), are widely used in pigs. Most often they are used in the sows to produce MDAs to protect the pigs through weaning and early in the nursery. MDAs typically wane between 8 and 12 weeks of age – the age range that is more likely to have virus detected in active surveillance studies [9]. With an exact homologous match, complete protection against IAV infection has been observed [69], but more often the result is partial protection with a heterologous vaccine [1, 12, 69]. It is imperative that we consider whether or not vaccines, and

their correct or incorrect use, play a role in driving evolution, and whether the vaccine protocols that are currently being used have created new risk factors.

In addition to risk factors, other epidemiological concerns are the regional variations that occur in IAVs, and the virus movements that accompany movement of pigs [59]. Regional variations due to antigenic shift and drift are more prominent where pig density is low and the level of movement of pigs into the region is low. One factor that plays a role in determining which strains predominate within a region is pig movement. Within the USA, pig movements play a role in the diversity of IAVs found in the Midwestern ecological sinkhole that is a final marketing destination and the region with the highest hog density [59]. The continual importation of IAVs with pigs from other regions into the Midwest has resulted in multiple genetically distinct variants co-circulating and exchanging segments via reassortment [59]. These include seven genetically and antigenically distinct hemagglutinin lineages – H1α, H1β, H1γ, H1δ1, H1δ2, H1pdm09, and H3 cluster IV [2, 44]. There are several different antigenic groups within the H3 cluster IV that do not follow the phylogenetic subgrouping patterns [42]. Further analysis is needed to determine whether there is a regional prevalence for these new antigenic clusters.

Zoonotic transmission

Zoonosis plays a key role in the epidemiology and evolution of IAVs in pigs. Human-to-pig transmission of seasonal IAVs has been well documented [58, 60]. For this reason, control strategies to mitigate the risks that humans pose to the swine herd should be considered. Although the same issues of partial protection with a heterologous vaccine resulting in reduced clinical signs and virus shedding would still apply, seasonal vaccines should be considered along with N95 respirators to reduce the risk of human-to-pig and pig-to-human zoonoses. Avian-origin viruses are occasionally introduced to confined pigs from poultry and wild birds, but multispecies farms and outdoor pigs are more likely to be at risk [16].

Although most of the documented zoonoses involve human-to-pig transmission of H1N1pdm09, which became established as a human seasonal virus after it first appeared as a pandemic in 2009, other human seasonal IAV strains are sporadically found circulating in pigs [60]. Pig-to-human infections are also sporadically documented. Apart from H1N1pdm09, human infections with swine-origin IAVs usually have limited subsequent human-to-human transmission, and the people infected usually have a recent history of exposure to swine [7, 19, 38]. One exception has been the H3N2 variant virus that was linked to direct or indirect exposure at agricultural fairs in the USA [14].

The greatest risk that these zoonotic infections pose is the opportunity that they provide for reassortment between human and pig strains of IAV. The swine-origin pandemic virus that emerged in 2009 highlighted the importance of understanding the pathogenesis, transmission, and evolution of IAVs in both humans and pigs.

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17 Immunity, diagnosis, and intervention strategies for influenza infections in pigs

Montserrat Torremorell

Immunity

Humoral immunity

Infection with influenza A virus (IAV) elicits an immune response characterized by the production of antibodies and proliferation of immune cells. The humoral or antibody-based response is essential for preventing or reducing infection of the host, while the cellular response is important for viral clearance during the late stages of infection.

Pigs develop a rapid and effective immune response against IAV, as they are able to overcome viral infection within approximately 7 days. They usually remain protected against reinfection with the same or similar strains.

Our knowledge of immunity against swine IAV is limited, and is to a large extent derived from human immunology. Upon infection, antibodies are produced mainly against hemagglutinin (HA), neuraminidase (NA), matrix (M), and nucleoprotein (NP). However, only antibodies against the globular part of the HA protein can block IAV entry to target cells and neutralize virus infectivity. HA protein is responsible for virus attachment to host receptors and subsequent entry into cells. Antibodies against NA act after infection by limiting the release of virions from infected cells. Antibodies to other proteins, mostly NP and M, mediate the destruction of infected cells by antibody-dependent mechanisms.

Most studies have measured the antibody response in serum, but mucosal antibodies in the respiratory tract are most important for protection. In serum, IgM antibodies are produced first, and can be detected by 3–5 days post infection, whereas IgG antibodies can be detected by 7–10 days post infection [19], but usually peak at 15–21 days. IgG antibodies are found predominantly in serum, although they can also be detected in bronchoalveolar lavage fluid (BALF) and nasal secretions, albeit in lower amounts compared with IgA antibodies. IgG antibodies protect the lung parenchyma, and there is some evidence that they can be produced locally. IgA antibodies play a critical role in mucosal immunity, and can be detected by 4–7 days post infection in nasal washes [13, 19]. They reach their maximum titer in serum and mucosal secretions around 15 days post infection, and remain elevated in mucosal secretions for at least 6–8 weeks post infection [13, 18]. Specific IgA activity is higher in BALF and nasal secretions than in serum.

There are differences in the ability of strains to induce cross-reactive antibodies that are able to block virus entry against genetically distinct strains. Cross-reactive antibodies tend to be subtype specific and dependent on antibodies against HA protein. Heterosubtypic protection tends to be independent of cross-reactive HI antibodies in serum [4, 9, 47], and there is weaker cross-protection between H1 IAVs with greater genetic divergence [12, 43]. In addition, T-cell responses are more cross-reactive than antibodies to HA and NA.

Cellular immunity

Cell-mediated immunity (CMI) responses are important both in clearance of the virus and in

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recovery, and are thought to play a role in heterologous immunity and protection against low-dose IAV infection. $CD4^+$ or T-helper cells facilitate antibody responses, and after primary and secondary infection there is an increase in $CD8^+$ cytotoxic T-lymphocytes (CTLs) in the lungs of pigs infected with IAV. A large proportion of these cells recognize conserved epitopes of NP [12]. There is also an increase in natural killer (NK) cells in the lungs. NK cells destroy IAV-infected epithelial cells in the early stage of primary infection in a non-specific manner, but at a later stage of the primary infection, as well as in the early stage of secondary infection, they are possibly targeted to infected host cells by antibodies [12]. Strong T-cell responses, as measured by interferon-γ-producing cells, have been seen in tracheobronchiolar lymph nodes and spleens of infected pigs shortly after infection, and peak at around 21 days post infection [18].

Maternally derived immunity

Transfer of maternally derived immunity (MDI) to piglets occurs through the ingestion of colostrum, which is rich in antibodies, cells, and other factors [36]. Colostrum is of vital importance, as the epitheliochorial placentation of swine prevents the transfer of antibodies and cells *in utero* [16]. MDI is important for protection of pigs clinically, but it can interfere with the development of an effective immune response against IAV infection [16]. MDI is highest after colostrum intake, decays progressively thereafter, and maternal antibodies can survive for around 4–14 weeks [23].

Complete protection has been demonstrated in pigs with MDI following a homologous IAV challenge [3]. However, other studies have only shown partial protection, with a reduction in clinical signs and virus shedding [7, 23]. In fact, pigs with maternally derived antibodies (MDA) shed virus for a longer period after an infection and show reduced growth compared with piglets without MDA [23].

The presence of MDA has been associated with reduced antibody responses and overall weaker immune responses [3, 17, 23, 26, 32]. MDA has been found to affect the proliferative T-cell response after primary infection [23], and to suppress induction of IAV-specific memory T-cells following vaccination [17]. The efficacy of vaccination using an inactivated vaccine or a live attenuated vaccine was reduced in the presence of MDA [48], although the reduction was greater when an inactivated vaccine was used. The presence of MDA at vaccination had a negative impact on the efficacy of the vaccine, as fever and clinical signs were prolonged, and, unexpectedly, pigs with MDA had more severe pneumonia compared with pigs without MDA [17, 48].

The presence of MDA can affect IAV transmission rates. IAV transmission was reduced but not prevented in neonatal pigs with homologous MDI compared with seronegative neonatal pigs and pigs with heterologous maternal immunity [1]. In pigs with MDI, transmission occurred despite the lack of clinical signs. Furthermore, antigenic drift has been documented in pigs with maternal immunity [10].

Diagnosis

Diagnosis of IAV infection in pigs requires laboratory support, as clinical signs are not pathognomonic and must be differentiated from a variety of respiratory diseases. A definitive diagnosis can only be made on the basis of virus isolation, detection of viral nucleic acids or viral proteins, or demonstration of specific antibodies against IAV. Because there are many IAV strains circulating in pigs, it is important to diagnose and characterize the strains, and to understand the limitations of the different diagnostic tests.

Virus isolation provides evidence of viable virus in the sample. IAV can be isolated in embryonating chicken eggs and various cell lines [40], of which the Madin–Darby canine kidney (MDCK) cell line is the most commonly used [27]. Details of the procedures can be found in Chapter 2 of this volume.

Polymerase chain reaction (PCR) can be used to detect genetic material of viruses, but cannot differentiate between viable and non-viable virus. PCR-based diagnostics are widely used in diagnostic laboratories because of their sensitivity, speed, accuracy, and scalability, and have become the method of choice for diagnosing IAV. There are several IAV PCR-based protocols for the detection and quantification of IAV in pigs, including commercial kits [14, 24, 37, 39]. To detect a broad range of IAV subtypes, primers for reverse transcriptase PCR (RT-PCR) are designed to target the conserved matrix (M) or nucleoprotein (NP) genes.

The USDA-validated avian influenza RT-PCR for the M gene [38, 39] has been adapted for routine diagnosis in the USA. RT-PCR based methods can also be used to differentiate between subtypes or strains within a subtype [5, 22, 49], and to characterize strains using sequencing of the genetic material directly from the sample or viral isolate [5, 50].

IAV causes lesions characterized by cranioventral bronchopneumonia with necrotizing bronchitis and bronchiolitis, and variable interstitial pneumonia. IAV can be detected directly in lesions of fresh or frozen tissues by immunofluorescence, or in formalin-fixed tissues by immunohistochemistry.

Antibodies against IAV detect exposure to IAV infection or vaccination, and can also indicate the transfer of maternal antibodies. The most common serological tests for routine diagnosis include the hemagglutination inhibition (HI) test and enzyme-linked immunosorbent assay (ELISA). The HI test is based on the agglutination of red blood cells, detects antibodies against the HA protein of the virus used in the test, and is subtype specific. There may be cross-reactivity within strains of a subtype, in particular among alpha, beta, and gamma clusters of the H1 subtype of swine IAVs, but in general HI is highly specific compared with ELISA methods. There are several commercially available ELISA test kits, including subtype-specific ELISA kits that can detect antibodies against H1N1 and H3N2. These tests often lack sensitivity compared with the HI test [2, 21], and also lack specificity, as they may cross-react with common epitopes found in H3N2 and H1N1 viruses. ELISA tests against the nucleoprotein detect antibodies that are not subtype specific, and can be used as a method of screening for IAV infection. Recently an avian-based kit has been adapted for detecting anti-NP antibodies in pigs [8]. This test is highly sensitive, although antibodies against IAV vaccination may not always be detected. Virus neutralization assays show similar performance characteristics to the HI test, but are more difficult and time consuming to perform, so are more appropriate for use in specialized laboratories.

The most common samples for diagnosing IAV infection in pigs are respiratory specimens, such as lung and tracheal tissues and nasal secretions. Oral fluids are also a suitable sample for diagnosing IAV in groups of pigs [35]. Both PCR-based methods and virus isolation can be performed using oral fluids, but PCR has higher sensitivity. Serum samples are used to detect antibodies but not virus, and more recently ELISA tests using oral fluids have also been validated for antibody detection [30]. The timing of sample collection for antigen detection with regard to infection is important, due to the short duration of virus shedding. Samples taken from febrile animals 2–5 days post infection are most appropriate. However, shedding can be affected by vaccination or the presence of natural immunity, which may in turn have an impact on assay performance [35].

Differential diagnosis should include other common respiratory pathogens of pigs, such as porcine reproductive respiratory syndrome virus (PRRSV), porcine circovirus (PCV2), Aujeszky disease virus, *Mycoplasma hyopneumoniae*, *Erysipelas rhusiopathiae*, and *Haemophilus parasuis*. Co-infections with IAV and *M. hyopneumoniae* have been shown to result in increased severity of clinical signs and macroscopic lesions [41]. Co-infections with PRRSV and PCV2 have been documented, and in some studies their impact has been found to be significant [44].

Intervention strategies

The economic cost of IAV infections in pigs has been difficult to estimate, although it is considered to be significant. Estimates of US$3–10 per animal have been reported for pigs infected with IAV alone or with co-infections. Most of the economic cost is due to decreased growth, increased feed conversion, increased mortality, and higher medication costs [11, 42]. Control of IAV in pigs has become more difficult due to the circulation of multiple genetically distinct strains and the introduction of the 2009 pandemic virus. New reassortant viruses are common, which means that control of IAV with existing vaccines requires constant review.

IAV infections in pigs are also relevant to public health. Although the impact of these infections on humans is difficult to estimate, swine-origin IAV infections have been documented in individuals working with pigs, customers attending live animal markets, and visitors at agricultural fairs [6, 15, 28]. Indeed over 300 human cases of an H3N2 variant virus were reported in 2012 in agricultural fair settings across the Midwestern USA [15]. Although swine IAV is not transmitted through

the consumption of pork [46], reports of cases of pig-to-human transmission of infections, or the emergence of a new strain linked to swine, can have devastating consequences for producers due to the effect on customer confidence, as was the case with the 2009 pandemic virus.

Vaccination is the most commonly used measure for controlling IAV infections in pigs. Vaccines were initially prepared according to conventional methods using the predominant classical H1N1 strain, and were employed primarily to reduce the economic impact of the disease in the breeding herd and neonatal pig populations. However, due to the increasing diversity of influenza subtypes and strains, current vaccines typically include more than one isolate, with the aim of incorporating the most prevalent and cross-reactive strains. Although influenza vaccines are far from an ideal solution, they do offer a valid tool for influenza control. They prevent clinical signs, decrease the number of lesions, lower the economic impact of the disease, and can also reduce shedding [17, 20, 45]. However, the main challenges with regard to use of IAV vaccines are the need to provide cross-protection between strains and to effectively prevent transmission and infection. The first challenge is being addressed by the use of multivalent vaccines and autogenous products prepared from the farm-specific strain or strains. More recently, experimental vaccines using live attenuated influenza strains have been shown to provide greater protection against heterologous strains, with reduced shedding and induction of mucosal immunity [25, 48]. To date there are no commercially available live attenuated vaccines for pigs, although some are currently in development. The effect of vaccination on transmission is still not clearly understood, as it depends on the level of cross-protection between the circulating and vaccine strains. Although some published reports suggest that IAV transmission may be reduced in vaccinated populations [34], others suggest that this may not be the case. Transmission could still occur at lower rates in groups vaccinated with vaccines that were only able to induce partial immunity. This hypothesis is corroborated by a mathematical modeling study which indicated that vaccination alone could not eliminate IAV throughout the breeding herd [33].

Although there is debate about the long-term benefit of IAV vaccination, common protocols for the administration of IAV vaccines in pigs include vaccination of replacement animals, and pre-farrowing or whole herd mass vaccination of breeding animals. The goal of these protocols is to increase the transfer of maternal antibodies to offspring and mitigate IAV infections within breeding herds. Vaccination of weaned pigs is also possible, although the economic benefit of this practice has been difficult to assess, and the timing of vaccination is problematic, due to interference with maternal antibodies [23]. In addition, vaccination against IAV in herds that are experiencing outbreaks of disease such as porcine circovirus-associated disease has been shown to be of questionable value [31]. Finally, vaccines may need to be specific to regions or countries, as the dominant IAV strains differ between countries and continents (e.g. North American vs. European vs. South American strains).

There are no antiviral drugs that are prescribed for food animals to mitigate IAV infections, and there are no treatments specific to IAV. However, viral or bacterial co-infections are frequently diagnosed in animals infected with IAV. Viral co-infections with PRRSV and PCV2 are common, and vaccination/prevention programs against these viruses should be in place. Co-infections with bacterial pneumonia-causing agents such as *H. parasuis, Pasteurella multocida, Streptococcus suis, Actinobacillus suis*, and *Actinobacillus pleuropneumoniae* are also common. In these cases the use of antimicrobial products is recommended. The antimicrobial product of choice may depend on the bacterial species that is to be targeted, but broad-spectrum antimicrobial agents are preferred, and should be administered by injection in the early stages of disease in particular, when only a few animals are affected. Both short- and long-acting antimicrobial products are available. Oral formulations that can be administered in the drinking water are also advisable, particularly when large numbers of pigs are affected and a rapid response is needed. However, one drawback of the use of orally administered antimicrobial products is that the animals may be too sick to access the water or feed. Administration of medicated feed is another option when IAV outbreaks are recurrent and predictable in time and space. However, in the

face of acute disease, when antimicrobial products may be of limited value because the animals are not eating, it is more efficient, although labor intensive, to treat individual pigs that have secondary infections with long-acting injectable antibiotics. Overall, treatment with antimicrobial products should help to reduce IAV-associated mortality and improve clinical signs, especially those associated with secondary respiratory infections.

The general approach to IAV control is similar across different countries and regions, particularly with regard to the use of antimicrobial products. However, there are some differences, depending on whether the cost of IAV is recognized. The approach to vaccination may vary depending on the country or region. For instance, the use of autogenous vaccines (i.e. products prepared from the farm-specific viral strain or strains) is not legal in European countries, whereas in the USA the use of autogenous vaccines is common. In some areas of South America and Asia the use of vaccination may be restricted to commercial products available from other regions, and is dependent on access to diagnostics and general recognition that IAV infections are a problem. General biosecurity practices should also be observed during efforts to prevent IAV infections in pigs. Because IAV can also be transmitted from humans to pigs, and many of the IAV strains that are circulating in pigs are the result of human IAV introductions [29], prevention of IAV transmission from infected farm workers will require the adopting of measures such as vaccination of personnel, use of face masks, and advising personnel to avoid farm work if they are exhibiting influenza-like symptoms.

Although protocols for eliminating IAV from swine herds have been published [42], in view of the self-limiting nature of IAV infections and the fact that herd reinfections are common, discussions about eliminating IAV from swine herds may be premature.

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18 Global evolution of influenza A viruses in swine*

Amy L. Vincent, Nicola Lewis and Richard Webby

Introduction

More pork is eaten worldwide than any other terrestrial source of meat, and the demand for protein by a growing human population means that pork production will inevitably expand [69]. The consumption of meat is anticipated to double in the next 30–50 years, and satisfying this demand will require lean, fast-growing pigs that grow optimally in modern sustainable climate-controlled barns. Not only has expanding pork production and consumption in developing countries been driven by this increased global demand for meat, but also as countries become more affluent the demand for meat increases further. Such a dramatic increase in domestic swine populations will probably lead to changes in influenza virus evolution, with unknown consequences for the overall epidemiology of influenza A viruses (IAVs).

Information on IAV in swine populations is relatively sparse in general. However, most of the public IAV sequences in GenBank [5] from IAVs circulating in this important host species are from developed countries. IAV remains the cause of one of the most important respiratory diseases in animals and humans, but there is a lack of consistency between virological surveillance efforts to keep track of currently circulating strains of IAV in the context of pig population density (Figure 18.1). This impedes our understanding of the global ecology of IAV overall, as well as the relative risk of intercontinental and interspecies spread of IAV from swine. Repeated outbreaks and rapid spread of genetically and antigenically distinct IAVs represent a major challenge for swine production and public health, not only in terms of assessing and controlling currently evolving strains within pigs, but also in characterizing their zoonotic potential [44].

Swine influenza was first recognized as a clinical disease coincident with the human Spanish flu pandemic in 1918, followed by isolation and characterization of swine IAV in 1930 [51, 93]. This classical-swine H1N1 (cH1N1) circulated in many swine populations around the world, in some cases for nearly 70 years with minimal genetic change [25]. Subtypes of H1N1, H1N2, and H3N2 are currently endemic in swine worldwide, exhibiting substantial diversity not only within the hemagglutinin (HA) and neuraminidase (NA) gene segments, but also in the other six gene segments. The global diversity of the HA and NA in swine is summarized in Figure 18.2. These multiple co-circulating subtypes and genetic lineages have different provenances related to their ancestral histories, dating back nearly 100 years.

Much of the genetic diversity of IAV in swine populations is the result of bidirectional transmission between swine and humans [73, 76], followed by periods of onward antigenic and genetic evolution, including antigenic shift mediated by reassortment within pigs. The functional and ecological opportunities for swine to serve as a mixing vessel for the generation of novel IAV has resulted in dynamic evolutionary trajectories of swine-adapted viruses, with periods of rapid

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Animal Influenza, Second Edition. Edited by David E. Swayne.

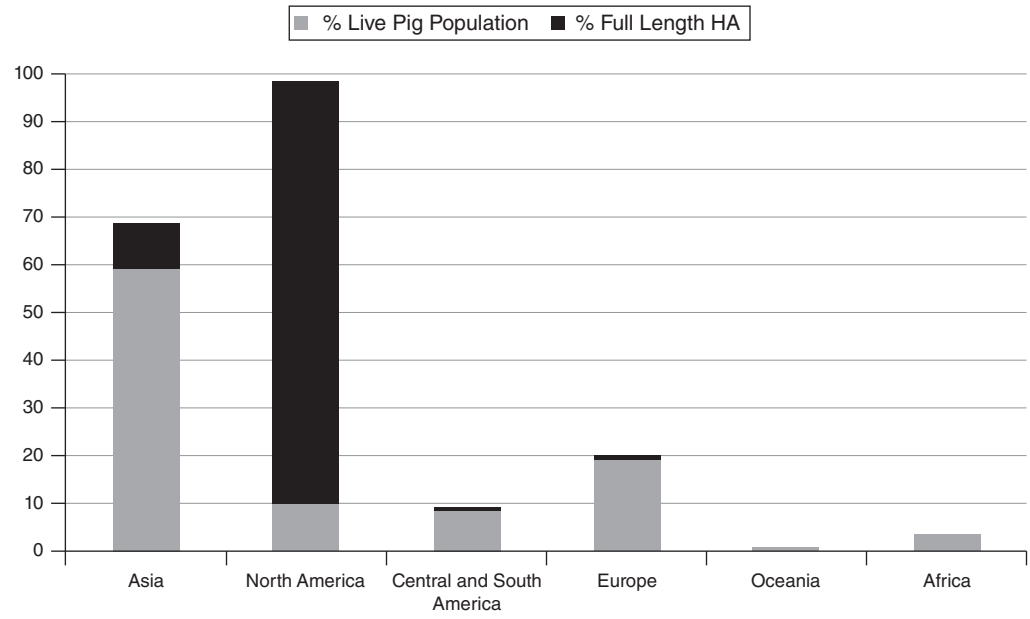

Figure 18.1 The number of hemagglutinin (HA) gene sequences available in the public database GenBank is not consistent with the geographic distribution of live pig populations. Although Asia currently has around 60% of the world's live pig population, only 10% of the HA gene sequences available from IAVs isolated from swine during 2012–2013 were from this region. In contrast, North America has approximately 10% of the world's pig population, but reported around 90% of the global IAV HA sequences from swine. Relatively few sequences were available from pigs in Central and South America, Europe, and Africa. The Oceania region has few pigs and very few sequences from IAVs from pigs.

genetic and antigenic change followed by periods of stability [67]. In humans, swine, and some avian hosts, the expression in the respiratory tract of similar influenza virus-binding sialic acid receptors (α2,6- and α2,3-linked) appears to facilitate this bidirectional transmission [35], and once a variant is introduced into a new host, different population factors in humans and swine drive the virus along non-parallel evolutionary trajectories.

Reintroductions of seasonal H3N2 and H1N1 from humans to pigs at different points in time, in different geographic regions, with reassortment within swine (including the internal genes), have contributed marked heterogeneity to the currently circulating genetic lineages within pigs globally. Although there are often common human seasonal ancestor viruses shared among regionally specific IAVs in pigs, as well as occasional documented transcontinental spread of swine IAV lineages, the complex regional genetic variation allows discrimination of swine IAVs from different regions, as well as discrimination of swine-adapted viruses from contemporary human seasonal influenza. However, the true picture of influenza viruses circulating in the world's pig population is difficult to determine, as many regions lack surveillance at the level that is seen for influenza viruses within the human population. Indeed influenza viruses can remain undetected for decades in under-sampled swine populations, with the potential to emerge and infect other host species.

The most dramatic example of the genetic diversity and often undetermined ancestry of emergent influenza viruses was exemplified by the 2009 H1N1 pandemic virus (H1N1pdm09), a virus with gene segments from two distinct swine IAV lineages – the Eurasian H1N1 and the North American triple reassortant γ-H1 viruses. However, a direct link to an endemic swine IAV was not established, even after more than 5 years of intensified surveillance in swine on a global level. Although the H1N1pdm09 virus arose in people in Mexico and spread in pandemic proportions around the globe, with subsequent human-to-pig transmission in multiple regions [56], the geographic location and host species in which the pre-pandemic ancestor virus evolved remained a mystery for many years, until a recent study identified viruses

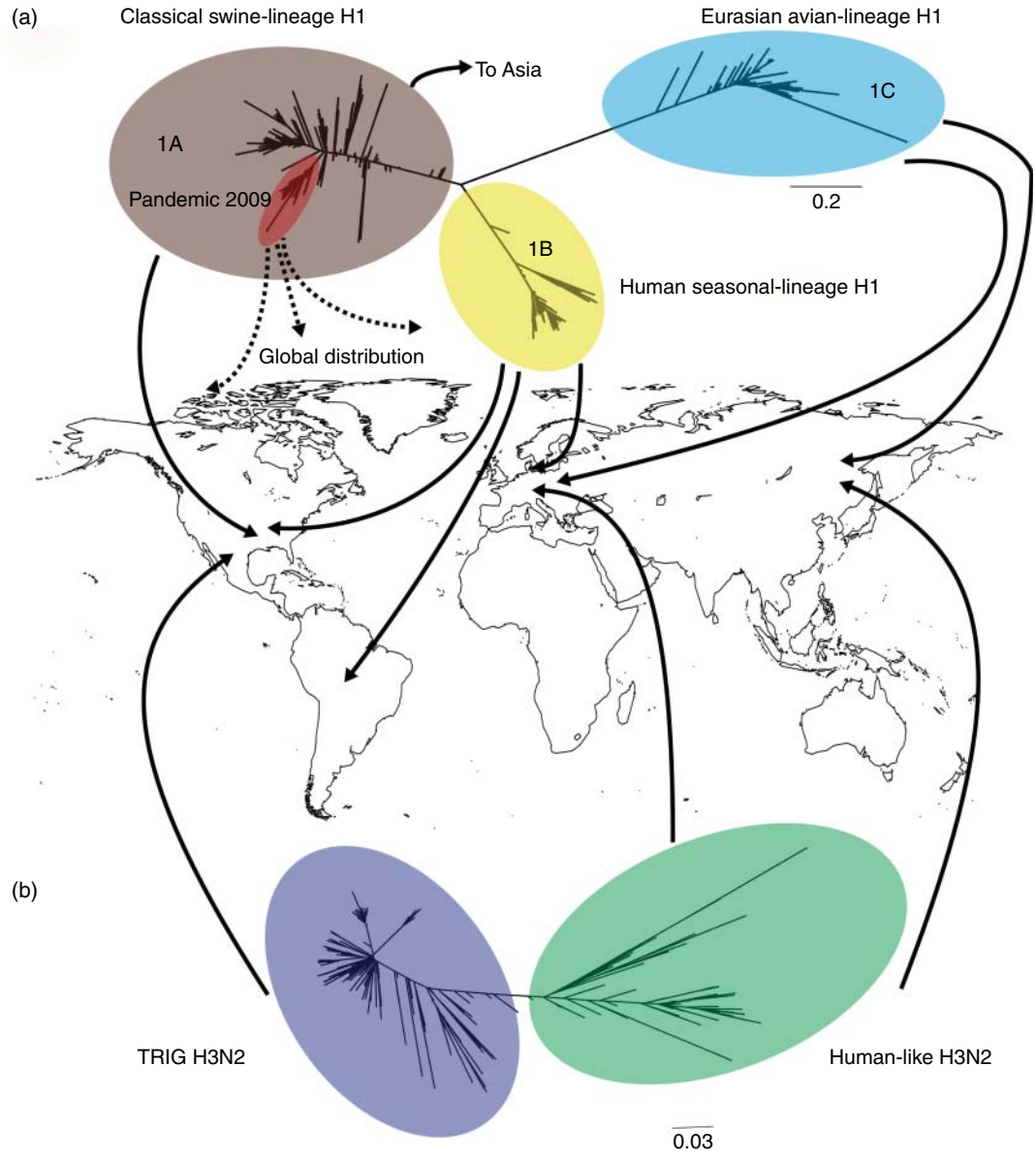

Figure 18.2 The major H1 and H3 genetic lineages and their geographic distribution in swine. (a) The phylogenetic relationships of H1 sequences. (b) H3 sequences are depicted in respective trees and color coded by lineage. In Europe, the HA genes are derived from Eurasian avian-like H1N1 (shown in blue), a human-like H3N2 (shown in green), or a human-like H1N2 (shown in yellow). Classical H1 (shown in brown), human-like H1 (shown in yellow), and human-like H3-TRIG (shown in purple) co-circulate. In Asia, the predominant HA lineages reflect the dynamics observed in North America and Europe, with co-circulating viruses classified as a classical swine lineage, human-like H3, or Eurasian avian-like H1. The H1N1pdm09 arose from the classical swine-lineage H1 (shown in red), and underwent global dissemination through human-to-swine transmission. Used with permission from Vincent, A. L., K. M. Lager, and T. K. Anderson. 2014. A brief introduction to influenza A virus in swine. *Methods in Molecular Biology* 1161:243–258. *See Plate section for color representation of this figure*.

in swine in Mexico that are closely related to a putative pandemic precursor virus [57a].

Geographic distribution of swine IAV genotypes

North America

North America includes three of the world's top ten pork-producing countries. The vast majority of hogs in the USA and Canada are reared in environmentally controlled isolation barns and integrated systems, although outdoor, backyard, and/or small-scale production farms can also be found, and these occur to a greater extent in Mexico [78]. Pork production operations include farrow-to-finish, farrow-to-nursery, farrow-to-wean, wean-to-finish, and finishing (fattening) farms. Although pigs and/or pork production can be found in most regions of the USA, large numbers of weaned pigs are transported to wean-to-finish farms in the Corn Belt in the Midwest, due to the rising cost of feed relative to transportation costs, and limited feed availability in other regions. There has been a significant trend towards consolidation of the industry since the mid-1980s, reflected in a reduction in the total number of operations concurrent with an increase in inventory by a smaller number of operations. Although there were 66 million head reported in 2013, by mid-2014 the swine population was around 62.2 million in the USA, most likely due to an outbreak of porcine epidemic diarrhea virus [107]. The USA imported an average of 5 million hogs per year during 2009–2013, primarily from Canada, and exported an average of approximately 31 000 hogs per year during this same period, with the top three importers over the past 10 years being Mexico, mainland China, and Russia [105]. In Canada, the pig population was 12.7 million in 2013 [101], with around 5 million live pigs exported primarily to the USA, and very few imports [1]. The live pig population in Mexico in 2013 was approximately 16.8 million, with approximately 15 000 pigs per year being imported, and almost no reported live exports [106]. The live swine trade among North American countries and with long-distance trade partners has probably played a dominant role in the migration and evolution of IAV in swine globally [74].

The classical swine H1N1 viruses were genetically stable within the North American pig population until the 1990s [25, 51, 93]. However, in the late 1990s, a novel triple-reassortant H3N2 virus was identified as it swept rapidly through the swine population. It contained HA, NA, and PB1 gene segments derived from seasonal human H3N2, PB2, and PA gene segments from avian IAV, and NP, M, and NS gene segments derived from the classical H1N1 swine IAV [123]. Subsequently, these successful triple-reassortant viruses reassorted with classical H1N1 viruses, resulting in new lineages of H1N1 and H1N2 viruses [41, 42]. The majority of the reassortment events involved only the H1 and/or N1 segments, preserving what has come to be known as the triple reassortant internal gene (TRIG) constellation of swine (M, NP, and NS gene segments), avian (PB2 and PA gene segments), and human (PB1) influenza virus origins [116]. Between these dramatic reassortment events, diversity was further shaped by the accumulation of mutations resulting in changes in viral surface proteins (antigenic drift) [21].

In the early twenty-first century, swine H1N1 and H1N2 viruses containing either the HA gene, NA gene, or both genes derived from human seasonal IAV were detected in the US and Canadian swine populations [115]. The HAs were genetically and antigenically distinct from those of classical swine-lineage H1 viruses as a result of significant subsequent antigenic drift of these H1N1 viruses within humans, an additional H1N1 pandemic in 1977, followed by further antigenic drift within the human host. However, the internal TRIG genes of these human seasonal influenza-derived swine H1N1 and H1N2 viruses were similar to those found in contemporary triple-reassortant viruses. The co-circulation of two lineages of H1 viruses with subsequent genetic variation led to the development of a phylogenetic "cluster" terminology within North America. Viruses with HA genes most similar to those of human seasonal H1 viruses circulating in the early 2000s formed the phylogenetic δ-cluster, and those more similar to the classical H1N1 viruses formed the α-, β-, or γ-clusters [115]. To further complicate the phylogenetic clustering, the HA genes that formed the δ-cluster most probably emerged from at least two separate introductions of human seasonal-lineage viruses, that subsequently diversified within pigs

into two distinct clades termed δ-1 and δ-2 [65], with a predominance of δ-1 detected in recent years [4]. These HA lineages are found with N2 genes derived from human seasonal IAV from either the 1998 or 2002 vintage [75], or the N1 gene as a classical swine lineage or pandemic lineage [4]. It is clear that the USA and Canada share these North American lineages, albeit with regional differences in relative predominance, but reports from Mexico are limited. However, based on the data available it appears that pigs in Mexico have some overlap with at least some of the North American swine lineages [63].

The diversity of IAV in North American swine was further expanded by the bidirectional transmission of the swine origin H1N1pdm09, detected initially in humans in Mexico and then identified around the world [30], and subsequently reintroduced back to swine in North America [34, 119]. Once in pigs, the H1N1pdm09 lineage viruses contributed their internal genes to pre-existing endemic subtypes in multiple combinations via reassortment [24, 48, 60], with the matrix gene found in a majority of endemic swine IAV viruses by 2012 [4]. The resulting reassortant viruses are currently in a period of rapid genetic evolution, most notably in surface glycoprotein HA genes, driving expansion of the formerly stable cluster IV H3N2 viruses into multiple co-circulating sub-clusters, tentatively denoted as A–F [47, 57]. It remains to be seen whether all of the expanded H3 cluster types will continue to undergo sustained transmission and evolution, but the biological consequences of the genetic diversity have already begun to be manifested by antigenic drift [29, 57]. A similar phenomenon appears to be occurring with the HA genes of modern H1δ-1 viruses, but evidence of antigenic drift is pending.

South America

South America reported 62.3 million head of hogs in 2013 [28]. Brazil is the leading pork-producing country in South America, and is ranked third globally, with 38.6 million head. Most of the remaining South American countries have pig populations in the range of 1–5 million. Types of production system vary widely in this region, and more than 50% of the pig population is estimated to be in traditional small-scale outdoor production settings or backyard holdings. As beef consumption is higher than that of pork in many South American countries, intensive pork production tends to be located in regions with less available pasture for cattle [38]. Nonetheless, the pig population has grown by more than 20% in the past 10 years to meet the growing demand for meat in this region and the potential for increased export markets.

Very few publicly available IAV sequences from swine isolates are available from Latin American countries. Argentina reported the presence of distinct human-lineage viruses of H1N1 and H3N2 subtypes [12, 84]. The Argentinian viruses are distinguishable from similar subtypes in North America, and represent independent human-to-swine transmission events. In late 2008, a wholly human H3N2 virus was isolated from pigs with clinical signs of respiratory disease and fever typical of influenza [12]. In 2009 and 2010, Argentina reported the isolation of reassortant viruses with internal genes from H1N1pdm09 and surface genes (HA and NA) from human-like (North American δ2-like) H1 swine IAV [84]. In 2011, another reassortant virus was isolated, with surface genes from the wholly human H3N2 virus first isolated in 2008, and internal genes from the H1N1pdm09 virus [23]. In Brazil, there were few reports of IAV infection in pigs before 2010. Recently, coinciding with the H1N1pdm09 in humans, numerous outbreaks of acute respiratory infection in pigs of different age groups were reported in Brazil, and the H1N1pdm09 virus was identified as being the cause [89, 91]. In addition, an H1N2 IAV identified in a recent study contained H1 and N2 genes of human seasonal origin, and internal genes (M, NP, PB1, PB2, and PA) from H1N1pdm09 [8].

Europe

The European Union (EU) reported 147 million head in 2013, with roughly 500 000 in exports, and with Denmark being the largest exporter. The European countries with the largest pig populations are Germany and Spain, with roughly 27 million and 25 million head, respectively, followed by France, Denmark, and the Netherlands, each with around 12–13.5 million head [28]. Hog production has declined in Western Europe in general, but some EU countries have experienced modest

growth in recent years. The types and size of production systems in Europe vary widely, and the EU has enacted detailed directives on many pig production practices, based on welfare considerations. Pig production ranges from small farms with 10 sows or less to indoor breeding farms with more than 400 sows, but the majority of farms tend to be medium sized, with around 200–400 sows and a farrow-to-finish system [27].

In Europe, there were at least three major contemporary virus lineages distinct from the lineages and evolution of swine IAV in North America (reviewed by Brown [10] and Vincent *et al.* [113]). Although there was extensive circulation of classical H1N1 swine IAV in the mid-twentieth century, these viruses were replaced in the late 1970s by a lineage derived entirely from an avian H1N1. Furthermore, the most recent common ancestor of contemporary H3N2 viruses in European swine populations was the 1968 pandemic human-lineage H3N2 virus (A/Hong Kong/1/68), and thus it is divergent from contemporary H3N2 viruses in North America, as the North American H3N2 viruses were introduced from humans into pigs three decades subsequent to the 1968 pandemic [110]. These H3N2 viruses reassorted with the avian-like H1N1 swine IAV in the mid-1980s, acquiring its internal gene cassette [13]. In the UK, a novel reassortant H1N2 virus was described a decade later that contained the HA gene of a human H1N1 virus similar to A/England-1980, with the remainder of the genes from the predominant circulating H3N2 swine IAV. These three lineages – the Eurasian avian-like H1N1, human-like H3N2, and human-like H1N2 – co-circulated in Europe. Although rare, additional reassortants were detected, such as H1N1 viruses with an avian-like H1 and H1N2 viruses with a human-like H1, with the other genes resembling the prototypical European H1N2 [53].

Following the H1N1pdm09 introduction into pigs, the endemic lineages continued to co-circulate, providing ample opportunity for further antigenic shift and drift. From 2010 to 2013, an extensive virological surveillance program was conducted by the European Surveillance Network for Influenza in Pigs (ESNIP3) partners, a consortium funded by the EU [96]. The intensity of the surveillance programs was highly variable across the countries involved, but most often correlated with the level of pork meat production (see above). Thus areas with intensive production inevitably had a higher total number of investigated herds when specific private or public surveillance programs were in place, as for example in Germany, Italy, Denmark, France, and the UK. Visits to farms where there was acute respiratory syndrome were encouraged by ESNIP3, and visits increased over the duration of the program. Thus an increase of nearly 45% in the number of investigated herds was observed between years 1 and 3 of the ESNIP3 program. These farm visits resulted in the detection of IAV in 31% of cases (2759 positive herds out of a total of 9025 herds). Like the numbers of investigations, the frequency of positive cases was highly variable depending on the country, ranging from 3% to 67%. However, IAV infections were confirmed throughout the year, regardless of the season.

Preliminary subtyping showed that in most countries the European enzootic swine IAV lineages as well as the H1N1pdm09 were the predominant subtypes [96]. Consistent with previous results obtained during ESNIP1 and ESNIP2 projects, conducted between 2000 and 2008 [53, 54, 109], the "avian-like swine H1N1" (H1avN1) lineage that emerged in 1979 was the most frequent lineage in every country, representing 53.6% of the subtypes identified during that period. By contrast, the enzootic "human-like reassortant swine H3N2" lineage that emerged in 1984 accounted for only 9.1% of the identified viruses. These results were consistent with ESNIP2, which reported that this virus was no longer detected in some regions with a high pig population density, while it was still prevalent in other parts of Europe. Thus H3N2 circulated widely in many of the main pig-producing regions, such as Belgium, the Netherlands, Germany, Italy, and Spain, but was almost entirely absent for many years in Denmark, the UK, and France. The enzootic "human-like reassortant swine H1N2" (H1huN2) lineage that emerged in 1994 represented the second most frequent genetic lineage of IAVs in circulation in Europe, and was identified in 13% of the viruses characterized in this study. Furthermore, reassortant viruses between the three enzootic IAV subtypes (i.e. rH1huN1 and rH1avN2) were detected in 7.4% of cases, in several countries. One reassortant ($rH1_{av}N2$)

appears to be established in the swine population in Denmark.

During the period 2010–2013, H1N1pdm09-like viruses were identified in numerous countries, including Germany, Denmark, the UK, Hungary, Poland, Italy, France, and Finland. In contrast, they were not detected in other countries that had significant numbers of detected influenza cases, such as Belgium, the Netherlands, and Spain. In Finland, the H1N1pdm09 virus was detected only sporadically several months after the end of the pandemic in humans, but in most countries this virus has been isolated with increasing frequency over time since 2010. It was also detected in Israel, through an active surveillance program, in a pig herd without any clinical signs. Co-circulation of H1N1pdm09 with European enzootic H1N1, H1N2, and H3N2 IAVs resulted in various reassortment events, leading to the detection of novel reassortant viruses that had mainly exchanged HA and/or NA genes. These viruses accounted for 6.5% of those identified, and therefore were at almost the same levels as other reassortant viruses between old enzootic strains. In total, 16.8% of the viruses were H1N1pdm09-like viruses or reassortant viruses that had acquired one or more genes from the H1N1pdm09 [96].

Asia

Asia contains around 60% of the world's pig population, with 588 million head [28]. In terms of countries, China has the world's largest pig population, with an estimated 475 million head on the mainland and 1.7 million in exports reported in 2013, predominantly to Hong Kong SAR [106]. It is followed by Vietnam and Russia, which reported 26.2 million and 18.8 million head, respectively, in 2013, while other countries such as South Korea, India, Thailand, and Japan each reported approximately 8–10 million. Although production systems vary widely in Asia, more than 50% of the pig population is estimated to be kept in traditional small-scale farrow-to-finish production settings or backyard holdings. In China, the industry has seen dramatic growth in pork production since the 1970s. Government-operated breeding farms may house up to 2000 sows, typically in single-site farrow-to-finish farm systems in hog-dense areas [70]. These farms often supply the smaller commercial or backyard farms. Private or semi-private commercial farms tend to operate in modern two-site systems, and there are estimated to be more than 100 production companies with over 10 000 sows each. Russia has also vastly increased its pork production since 2005, at the same time as the industry has been undergoing consolidation and modernization of its sow farms [52].

Classical swine H1N1 viruses were enzootic in swine populations throughout Asia (e.g. China, Japan, Thailand, Vietnam). IAVs in Chinese swine are the result of intercontinental introductions mediated via pig movement of European and North American lineages along with incursions from human seasonal viruses. After these introductions, reassortment between classical-lineage viruses and the new introductions resulted in similar antigenic subtypes to those in Europe and North America, but combined with unique internal gene constellations and dominant HA and NA genotypes (reviewed by Vincent *et al*. [113]). Prior to 2009 in China, classical swine viruses co-circulated as H1N2 viruses carrying an NA of contemporary human origin, human-origin H3N2 viruses (A/Hong Kong/168-like, A/Port Chalmers/1/73-like, and A/Sydney/05/97-like), and Eurasian avian-like H1N1 viruses [31, 94]. Furthermore, IAV genetic diversity in Chinese swine herds was increased by the intercontinental movement of swine and their viruses [112], resulting in the introduction, circulation, and maintenance of European H3N2 and H1N1 viruses in 1999 and 2001, respectively, along with North American TRIG viruses in approximately 2002. The H1N1pdm09 was also introduced to swine in Asia and reassorted with the endemic subtypes in China [111], Japan [68], and Thailand [49]. The pattern of reassortment with H1N1pdm09 in China mirrors that in North America in that the previously endemic HA and NA lineages have incorporated the internal genes of the H1N1pdm09, while the H1N1pdm09 and its surface genes do not appear to be circulating in the pig population, based on a large long-term surveillance study [58]. The dominant HA lineages in 2012 reported by this study were a human seasonal-derived H3 and the Eurasian avian lineage H1, with sporadic detection of classical swine and H1N1pdm09.

In addition to the North American and European-like viruses circulating in Asia, there are a number of novel genetic lineages, such as the H1N1, H3N2, and H1N2 viruses found in Thailand since the 1980s. Prior to 2009, the Thai H1N1 viruses were clustered as either classical-HA or Eurasian-HA swine lineages with specific genotype reassortment patterns [102]. From 2000 to 2007, H3N2 viruses that contained human-lineage HA and NA genes either of European swine descent or of a more recent human seasonal lineage were detected, with the internal genes derived from Eurasian (PB1, PB2, PA, and M) and classical H1N1 (NP and NS) swine lineages. To add to the novel complexity, the H1N2 viruses contained combinations of genes from the endemic H1N1 viruses and human-like H3N2 [102]. As in other regions, the contemporary Thai H1N1, H1N2, and H3N2 viruses have recently incorporated various internal gene segments from H1N1pdm09 [16, 87]. Likewise, the classical-lineage H1 and human seasonal-lineage H3 viruses in Japan have reassorted with H1N1pdm09 [40], as have the endemic strains that previously circulated in pigs in South Korea [46, 80]. In addition, novel Eurasian avian-lineage H1N2 [79] and H3N1 [80] reassortant viruses were recently identified in South Korea.

Antigenic evolution of IAV in swine

Most swine influenza virus surveillance focuses on the gene segment encoding the hemagglutinin (HA) surface protein because HA is the primary target of the immune response and is the main antigenic component of human and swine influenza A virus vaccines. Assessment of the antigenic relationships between the HAs of multiple subtypes circulating in each host, and a knowledge of the relative HA evolution over time, are key to understanding the global patterns of transmission, assessing the relative risks of new incursions in either direction, identifying future intervention strategies, and ultimately preparing for future epidemics and pandemics. Novel computational techniques have recently been developed to quantify and visualize the antigenic evolution of influenza viruses in a number of hosts [99].

Characterization of the zoonotic potential of swine influenza A viruses, and the development of control methods to minimize risk, both require an understanding of a number of key areas, including up-to-date antigenic characterization of the influenza viruses that are circulating in pigs globally, the effect of swine population immunity and production factors on the evolution of these variants, the antigenic interrelationships between these swine variants and seasonal human influenza strains, and the role that repeated introduction of human seasonal viruses back into pigs might play in modifying the risk of re-emergence of strains with pandemic potential. The close proximity of humans to swine and the propensity of viruses to move between the two species create a complex ecological and evolutionary host–pathogen interface in which viruses introduced into one or the other host might antigenically evolve within this new population on a different trajectory to that within the seeding population. Such evolutionary differences between hosts might also permit antigenic variants to emerge over time to which the other population would be immunologically naive.

Prior to the 2009 pandemic, caused by a virus originating from two swine IAV lineages, the zoonotic risk of H1N1 viruses was considered to be lower than the risk of introduction into the human population of a completely novel subtype such as H5N1. The basis for this assessment was undeniably limited by the relative paucity of swine surveillance data, particularly antigenic data, to assess the relative evolution of H1N1 and other subtypes within pigs and in relation to human population immunity. The swine influenza surveillance that was undertaken predominantly assessed genetic data, and it was well known that the separate introductions of influenza viruses into pigs from multiple hosts since 1918 had resulted in marked genetic heterogeneity in virus diversity among geographic regions. In contrast, within the human population there has for decades been a well-structured near real-time assessment of the antigenic and genetic characteristics of seasonal influenza A viruses, and a rapid and scientifically based vaccine update. The genetic evolution of influenza A viruses in humans is continuous, whereas antigenic evolution is punctuated or clustered, with one cluster circulating at any period in time, and the emergence of a cluster

usually coinciding with the need to update the vaccine strain [99].

Such continuous genetic evolution has also been characterized in influenza viruses circulating in pigs. However, since the 2009 pandemic, research has been conducted within particular geographic areas in order to assess the antigenic characteristics of these currently circulating genetic lineages. For example, human seasonal H3N2 Wuhan95-like viruses were introduced into pigs in the USA and isolated through diagnostic investigations in 1998 and through surveillance efforts to the present day. Within the pig population, these viruses evolved into distinct and co-circulating antigenic clusters, which were not only antigenically different from each other, but also spatially distant from the human evolutionary trajectory and from the likely seeding human strains. The molecular basis for such antigenic drift within pigs bore striking similarities to that observed from 1968 to 2003 in human H3 influenza, but with reduced magnitude of drift over time [50, 57]. Multiple other unclustered antigenic outliers were also identified in swine, which might represent under-sampled antigenic cluster diversity or the emergence of outlier strains. Whatever the host population factors that permit the generation of such antigenic diversity, there are striking differences between the single antigenic subtype variant that tends to circulate at any one time in the human population, and the potential for greater standing antigenic diversity in pigs [57].

Since the 1950s there has been an increasing worldwide demand for meat, which can be partially alleviated by fast-growing species such as pigs, with efficient feed conversion rates. Alongside an increase in pig numbers there has been intensification, with more animals being kept in fewer but larger units. Initially this intensification focused on North America and Europe, where numbers are now increasing more slowly or holding steady. In some parts of the developing world, around 50% of the current pig population is still kept in traditional small-scale subsistence-driven production systems. The effects of disease control interventions (e.g. vaccination), different production systems, previous population immunity, herd age structure and breed on influenza transmission, maintenance, and antigenic evolution within pigs are poorly understood, but it is likely that modern pork production offers multiple key points where interventions occur that may influence the epidemiological dynamics of influenza virus in swine.

Swine in the ecology of IAV and interaction with other host species

Although wild waterfowl are the natural reservoir for IAV, mammalian hosts including swine maintain genetic lineages of IAV that adapt and become distinguishable from other lineages based on genetic and antigenic characterization. The swine lineage cH1N1 was relatively stable at the genetic and antigenic levels in swine in the USA for nearly 80 years, until the establishment of the triple reassortant IAV with human, avian, and swine lineage gene segments in 1998. However, as described earlier in this chapter, alternative lineages of IAV emerged in swine prior to 1998, and persisted as dominant global lineages in other regions of the world.

Multiple independent introductions of avian- or human-lineage viruses were sporadically detected in swine during this time period, but were not always successful at long-term sustained transmission, or remained geographically discrete. However, since the emergence of the triple reassortant H3N2 in 1998 and its subsequent introduction into Asian pigs, the recognized genetic diversity of swine IAV has continued to increase globally, largely as a result of multiple introductions of seasonal human-lineage H1 and H3 viruses followed by reassortment with locally endemic swine IAV [76]. This was followed by the unprecedented human-to-swine transmission events of the H1N1pdm09 virus at least 50 times globally [73]. After the introduction of novel genetic lineages, periods of increased reassortment and diversity ensued [16, 40, 46, 48, 55, 58, 72, 73, 75, 79, 80, 87, 112]. The level of surveillance of swine increased worldwide following the emergence of H1N1pdm09, and continues to reveal the presence of IAV in regions not previously known to have endemic IAV and/or the presence of novel gene lineages not previously recognized in swine. It is critically important to maintain this level of surveillance in addition to expanding it to currently under-represented regions and populations.

Swine play an important role in the ecology of influenza [117], and the interplay between humans and swine [20, 26, 71, 92, 95, 108] as well as between swine and domestic turkeys [7, 77, 85, 86, 121] represents specific niches that exist largely without the interference of IAV from wild waterfowl [76]. It is not only the swine–human interface that potentially poses a risk of cross-species transmission. In Canada, swine triple reassortant H3N2 viruses that possess an internal "triple reassortant" gene cassette are capable of inter-species transmission to turkeys and quail. In these avian hosts they have remained antigenically similar to the reference 2005 virus, unlike H3N2 viruses in Canadian pigs, in which several antigenic H3 variants co-circulate [77]. Investigation of these divergent patterns of evolution of the same subtype within multiple host species provides us with an opportunity to understand the complex ecological drivers that underlie influenza virus dynamics in different hosts, and ultimately provides information that enables us to assess the risk of incursion of these viruses into other host species, such as humans.

Zoonotic transmission of swine-adapted IAV to humans has been documented throughout the years since 1918, and generally results in an influenza-like illness similar to human seasonal IAV, with little evidence of human-to-human onward transmission. One of the fundamental issues with regard to such marked genetic diversity of viruses circulating in the pig population is the increased relative risk of re-incursion of circulating swine IAV into the human population, highlighted by the swine-origin pandemic in 2009 [30], and more recently the emergence of a variant H3N2 virus in the USA in 2012 [26]. Between 2009 and 2013, 348 humans in the USA were infected with an H3N2 variant (H3N2v), and most of these human isolates contained 7 gene segments from the triple reassortant swine H3N2 and 1 gene segment (M gene) from the H1N1pdm09 lineage [15], depicted simplistically in Figure 18.3. Two more cases were identified in 2014, again reassortants between swine H3N2 and H1N1pdm09. The majority of these patients had a history of recent exposure and contact with swine, mostly through agricultural fairs [26, 39]. Although there was an increase in detection of H3N2 in swine with the same genetic signature as the H3N2v in humans during 2011–2012, this cannot be the sole explanation for the increase in human H3N2v, as there were also increases in other genetically dissimilar H3N2 [48, 75] and in subsets of H1 viruses in the USDA surveillance data set [4]. Likewise, there were multiple detections of H3N2 and H1N2 in exhibited pigs at agricultural fairs in Ohio during 2009–2011 [9]. In contrast to H3N2v, there have only been 21 documented cases of H1N1v or H1N2v since 2005.

There may be specific virus properties that conferred a greater capability for H3N2v to infect humans, and the H3N2v was capable of airborne transmission in ferrets [82], the standard laboratory model for human IAV. However, other variant human isolates and swine IAV were also shown to successfully infect and transmit between ferrets in this and other studies [80, 81, 122]. An additional factor is a potential lack of human population immunity against subtypes of IAV that have evolved independently in swine away from their respective human seasonal precursor viruses and the strains used in contemporary human seasonal vaccines [57]. A substantial proportion of adolescents and young adults were shown to have cross-reactive antibodies against H3N2v, but children and older adults lacked such protective antibodies [14, 97]. The current human seasonal vaccines containing H3N2 do not appear to protect against the H3N2v [33, 98]. Since the vast majority of cases of H3N2v have been in children with close contact and long periods of exposure time at agricultural fairs, all of these factors point to a unique set of circumstances that collectively may have increased the odds for H3N2v in these spillover events.

The complex factors that appear to be involved in swine exhibition-associated cases of variant IAV do not diminish the epidemic or pandemic risk of H3N2v or other swine-adapted IAVs if the viruses gained the ability to transmit from human to human, allowing further opportunity to mutate and adapt back to the human host. However, sustained human-to-human transmission has not been observed to date. The H3N2v with segments derived from human seasonal H3N2 nearly two decades ago, and now adapted to pigs, serves as further warning of the importance of pigs and humans in the generation of viruses of concern for both host species. A better understanding of the factors necessary for adaptation of viruses among and between humans and pigs is critical for breaking

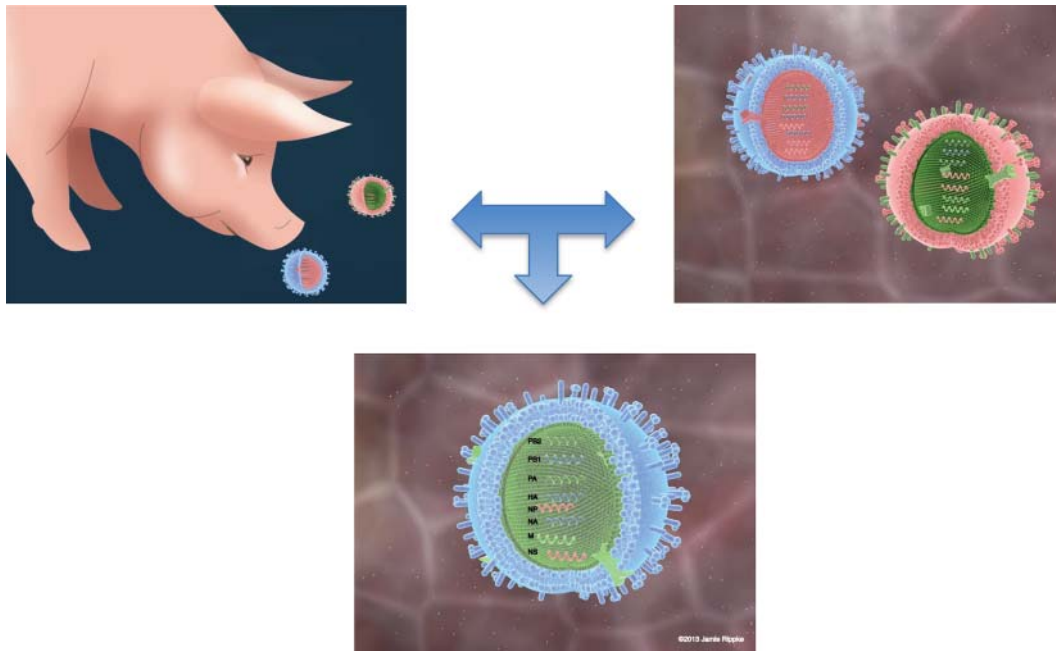

Figure 18.3 Putative generation of H3N2 variant in pigs. Pigs infected with two strains of IAV – H3N2-TRIG (shown in blue) and H1N1pdm09 (shown in pink) – allowed for reassortment of each parent virus's specific whole-genome constellation to generate novel progeny virions. One virus resulting from the potential reassortment patterns contained seven gene segments derived from the H3N2-TRIG and a single gene segment derived from H1N1pdm09 (the M gene shown in light green). An H3N2 with the genome constellation depicted here has been found in almost 350 human cases in the USA since 2010, termed H3N2 variant (H3N2v). Reassortants between endemic swine viruses and the H1N1pdm09 have been detected frequently in pig populations around the world since 2009. Source: Amy Vincent and Jamie Rippke. *See Plate section for color representation of this figure*.

the cycle, and any mitigation measures must be considered in the context of animal production systems, anthropogenic practices, and the ecology of the virus [43].

Determinants of virulence and host range

Pigs infected with IAV show a spectrum of clinical disease, ranging from remaining clinically unaffected to high fevers and severe respiratory signs. The clinical range may be due to prior immunity, properties of the virus, and many other health and environmental factors. Clinical signs of influenza in pigs are similar to those observed in humans, and are manifested as acute respiratory disease characterized by fever, inactivity, decreased food intake, respiratory distress, coughing, sneezing, conjunctivitis, and nasal discharge [37, 90, 110]. The disease incubation period is 1–3 days, with rapid recovery beginning 4–7 days after disease onset. Swine influenza is characterized by high morbidity (approaching 100%) and generally low mortality (less than 1%), and may sweep through a naive herd or spread more slowly through a population with partial or mixed immune status. To date we have only a limited understanding of the kinetics of when and how pigs are infected within a particular livestock production stage. Key to determining the disease dynamics within a pig herd is an understanding of the circulating variants, the age stratification of the herd, the immunological profiles of adult pigs and piglets, the timing of infection within the production cycle, and the role that vaccine-derived or infection-derived immunity might play in driving the burden of influenza within the herd. Influenza viruses are

also commonly found in cases of porcine respiratory disease complex (PRDC), acting in concert with other pathogens [103].

Swine influenza caused by cH1N1 was historically characterized as a seasonal disease, primarily in weaned pigs with waning maternal immunity. Today, clinical disease still peaks at times of the year associated with dramatic fluctuations in temperature and decreased ventilation [37], and was recently shown to have a primary peak in November and December, and a secondary spike in March and April [4]. However, contemporary influenza illness and diagnosis can occur at any time of year, in nearly all age groups of pigs, even suckling pigs from sows with high titers of influenza-specific serum antibodies [3, 19]. This is probably due to the increasing numbers of antigenic cluster types as well as the variability in passive transfer and populations with mixed levels and specificity of immunity. Although passive maternally derived antibody may reduce clinical illness, it is often ineffective in preventing replication and transmission, particularly when there has been antigenic drift of viruses [2, 18, 104], allowing weaned pigs to infect downstream nursery and/or finishing sites [3].

The virological factors that control the infectivity and virulence of swine IAV are poorly defined relative to other IAVs, largely due to the limited capacity for experimental studies in the natural host. Much of the data that has been generated for swine IAV has been obtained from small animal model studies. Rather than aiming to determine the requirements for replication in swine, these studies are typically designed to determine the zoonotic risk of endemic swine IAV. Studies by Ma and colleagues have highlighted the need to be extremely cautious about extrapolating data from small animal models to swine, as they demonstrated that the effects of the well-characterized mouse pathogenicity marker PB2 627 on disease outcomes in mice and swine were not well correlated [66]. However, the accumulated data from such studies have indicated that swine IAVs share many features in common with human IAVs. At the same time, although influenza viruses move between the two host hosts, it is clear that there are subtle differences between them.

By far the most well-characterized regulator of IAV host selectivity is the interaction of HA with the sialic acid receptor on the host cell. It is well documented that swine contain receptors for both avian- and mammalian-adapted IAV, although passage of avian-adapted viruses in swine leads to selection of variants with a greater preference for the typical mammalian virus receptor [36]. While it is clear that, if given in large enough amounts of inoculum, avian IAV can replicate in swine [45], epidemiological evidence clearly suggests that an α2,6-linked sialic acid receptor preference is required for successful establishment, and avian viruses are in general poorly adapted to swine [22, 59]. Although both human- and swine-adapted IAVs recognize the α2,6-linked form of the receptor, it is clear that most zoonotic infections from a swine source are not followed by onward transmission. This suggests that the optimal molecular features required for an influenza virus to infect swine are not necessarily the same as those required to infect humans. In one of the few studies that have addressed the impact of molecular markers on the infectivity of IAVs for swine, Busch and colleagues examined the ability of swine-origin and human-origin H3N2 viruses to infect and replicate in primary swine respiratory epithelial cells [11]. The virus of swine origin was more infectious for swine, and also for the primary swine epithelial cells, compared with the human-origin virus. The genetic differences responsible for this were found to lie within the HA gene. Using a series of mutated viruses this group was able to further assign the differential phenotype to three amino acid residues. Although both viruses bound to the α2,6-linked sialic acid receptor, the HA residues in question appeared to affect more subtle interactions between virus and receptor, including avidity and conformational preference [6]. The authors concluded that it was these differential interactions with sialic acid that were most closely correlated with infectivity of the swine epithelial cells, and that by inference may influence the ability of human IAVs to infect swine, and the ability of swine IAVs to infect humans.

Consistent themes also emerged when H1N1-pdm09 viruses isolated from humans were experimentally inoculated into swine. In one study, differences were noted in the ability of two such viruses to replicate and be transmitted in swine. Using recombinant viruses, an S186P substitution in the HA was found to be responsible for

the increased growth efficiency. The authors concluded that "this efficiency appeared to be more likely through an advantage in cell surface attachment rather than replication efficiency" [64]. The 186P polymorphism was more frequently seen in H1N1pdm09 viruses isolated from the swine, suggesting that it was selected in this host during natural infection and transmission.

Further data suggesting that subtle changes regulate the phenotypes of IAV that are differentially able to infect and be transmitted within swine and humans come from studies examining the balancing activities of HA and NA. Despite considerable variation in the avidity of individual HAs for their cellular receptor within closely related human H1N1 viruses, there was balanced NA activity, such that the more avid HAs were always paired with a more active NA [120]. Similarly, the human viruses with low-avidity HAs were always paired with less active NAs. In contrast to these results for viruses isolated from humans, there was no such balancing of HA and NA activities in the swine H1N1 viruses that were tested. It is not clear why this discrepancy exists, but these data suggest that the factors which regulate virus binding and release are different in humans and swine. Interestingly, it has also been suggested that swine mucins have a reduced ability to interfere with IAV binding to epithelial cells compared with human mucins [17]. As one of the suggested roles for NA is alleviation of mucin-mediated virus inhibition, these data are consistent with a reduced need for optimized HA and NA activity in swine.

Molecular changes in other viral proteins have also been associated with increased infectivity for swine, although the underlying mechanisms are typically poorly defined. For example, Londt and colleagues attempted to adapt an avian H5N1 IAV to swine cells under innate and adaptive immune pressures. Mutations in PB2, PB1, HA, NP, and M were identified after adaptation, with the passaged viruses achieving higher titers in cultured swine cells and explants [62]. Although the important molecular changes were not definitively identified, studies such as this identify the viral proteins that must change in order for an avian virus to more efficiently infect swine. It is noteworthy that in the same study these authors were unable to adapt an H7 virus, which suggests that different IAV subtypes may require different adaptive mutations, and some may be more likely to naturally infect swine. Using a similar strategy, Wei and colleagues passaged a recombinant virus designed to mimic the genotypic constellation of the 2009 pandemic virus multiple times in swine. After passage, substitutions were selected for in the virus population in HA (with different variants appearing in the upper and lower respiratory tracts) and PB1, PA, NA, NS1, and NEP [118]. The functional consequences of these changes were increases in polymerase activity and also enhanced replication and transmission in various animal models, including swine and ferrets. Supporting a role for the importance of non-HA and non-NA genes in IAV adaptation in swine are studies that have created laboratory-derived reassortants and tested their ability to infect, cause disease, and be transmitted in swine. In one such study, reassortants were created between an H9N2 IAV isolated from swine and an H1N1pdm09 virus. In these studies, a reassortant virus in which HA and NA were derived from the H9N2 virus and the remaining gene segments were derived from the H1N1pdm09 virus was able to grow to higher titers *in vitro* and *in vivo* than the parental H9N2 virus itself [32]. These data were consistent with the findings of a similar earlier study [88].

As is typical of IAVs endemic in other host populations, there is considerable variation in the pathogenicity of individual isolates in swine, even within a single monophyletic lineage [e.g. 90, 100]. However, few studies have extended these observations to the level of molecular determinants. A more common approach has been to examine the impact of pathogenicity markers that have been identified in other IAVs, most commonly of avian origin, in swine IAV. Examples of this include studies that have explored the impact of restoring the PB1-F2 open reading frame in the H1N1pdm09 virus (this virus has a naturally occurring truncation) [83]. The PB1-F2 open reading frame is absent in many endemic swine IAVs. In this particular study, restoration of the open reading frame had a minimal but measurable impact on H1N1pdm09 virus replication and pathogenicity in swine, increasing both of these. Lui and colleagues have also examined the effect of polymorphisms at well-characterized positions within the PB2 of endemic swine virus origin [61], and found a measurable impact on viral growth and subsequent disease. Such approaches are important and have

provided insight into swine IAV pathogenicity, but they will not of course reveal swine-specific markers. Although the 2009 pandemic led to increased interest in defining swine IAV infectivity and pathogenicity markers, this field is still in its infancy. Without a basic understanding of specific residues and how they affect viral phenotypes we will be unable even to consider predicting the impact of the enormous IAV genetic diversification that is occurring in global swine populations. This situation must change.

Conclusions

Surveillance, epidemiological investigations, and genetic characterization of IAVs associated with respiratory disease outbreaks in pigs are necessary in order to monitor the evolution of viruses in the pig population. In addition, antigenic characterization is needed to fully understand the relevance of genetic changes for vaccine antigen selection, and vaccine efficacy must be evaluated in the context of serologic cross-reactivity when new variants arise. Increased surveillance for IAVs and new variants of H1N1pdm09 and endemic swine IAVs in the swine and human populations are essential to an understanding of the dynamic ecology of IAVs in susceptible host populations. A cycle of human-to-swine transmission, followed by evolution in swine, and then re-entry into the human population has been established in contemporary human and swine IAVs [72, 75, 76]. The bidirectional interspecies transmission of IAVs and the ongoing evolution of these viruses in swine and humans are unprecedented in the history of IAV.

Although regional surveillance projects are ongoing and are supported by various temporarily funded networks (e.g. the United States DHHS-NIH NIAID Centers for Excellence for Influenza Research and Surveillance (CEIRS), the USDA IAV-Swine Surveillance System, ESNIP3, and the Thailand-Japan Zoonotic Disease Collaboration Center Swine Surveillance Project, among others), most other surveillance in swine has been conducted on a smaller scale by individual investigators. To address the lack of integrated regional and global IAV swine surveillance, the OIE and the FAO formed OFFLU (the OIE–FAO network of expertise on animal influenza) in 2005 to address avian influenza issues, and expanded the network in 2009 to include expertise on IAV circulating in swine, equine and other animal hosts. The mission of OFFLU is accomplished though collaboration and the exchange of information, biological materials, and resources between all influenza sectors. The OFFLU Swine Influenza Virus Group is proactively sharing and analyzing influenza viruses and sequences obtained through surveillance activities from pig populations worldwide [113]. Recent activities include the use of seasonal influenza viruses from humans and circulating endemic swine influenza viruses to assess the antigenic relationships between viruses that are responsible for influenza in swine and humans [70a]. Assessment of the relationships between multiple variants of influenza viruses circulating in pigs and humans, and an understanding of their relative evolution in these hosts over time, are key both to the improvement of vaccines for pigs and humans, and for understanding the relative risk of future interspecies transmission events. The OFFLU Swine Influenza Virus Group is also working to establish a common HA cluster naming system to be used by animal and human health sectors to designate swine influenza viruses on a global scale. This system will enable the evaluation of viruses around the world according to a unified set of criteria, and will allow their genetic relationships to be understood in a common context. Such a system is also important for targeting groups of viruses to study both for their antigenic properties and for developing effective vaccines.

Although IAVs in swine have been documented and studied for nearly a century, many questions still remain unanswered. Similarly, effectively integrated and systematic programs for the monitoring and prevention of IAV infection in swine are lacking at regional, national, and global levels. Recent advances in regional surveillance programs, sequencing methodologies, research, and collaboration through networks have established a framework upon which to continue efforts to control this important virus in swine through production practices and/or improved vaccines. The increase in availability of sequences and use of newer analytic tools have demonstrated the under-appreciated propensity for human seasonal viruses to spill over into swine, as well as the zoonotic potential of swine viruses. The

H1N1pdm09 highlights the importance of monitoring the global evolution of IAVs in swine and assessing the risk of these viruses in human influenza models. Therefore these continued efforts in the swine sector and engagement with the human influenza sector will have the dual benefit of improving animal and human health.

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19 Vaccines and vaccination for swine influenza: differing situations in Europe and the USA

Kristien Van Reeth, Amy L. Vincent and Kelly M. Lager

Introduction

Swine influenza is an acute respiratory disease in pigs caused by influenza A viruses of H1N1, H3N2, and H1N2 subtypes (reviewed by van Reeth *et al*. [82]). These viruses are usually of human or avian origin, and most swine-adapted influenza viruses result from genetic reassortment between already established swine viruses and viruses that were recently introduced from humans or birds. In this chapter we shall use the traditional and well-known designation "swine influenza viruses (SIVs)" for influenza A viruses that have become established in swine populations, although the current recommendation by OFFLU is to use the term "influenza A virus in swine." SIVs replicate in epithelial cells of the upper and lower respiratory tract of pigs, and of the lungs in particular. There is no systemic spread, and virus transmission occurs exclusively via the respiratory route. The infection lasts for only 6–7 days, and respiratory distress, fever, and dullness resolve within a few days. Although infection is rarely fatal, there can be a significant economic impact due to weight loss in growing pigs as well as reproductive failure in sows as a result of high fever. SIV may also contribute to more chronic and multifactorial respiratory disease problems, known as the porcine respiratory disease complex. Many if not most uncomplicated infections are subclinical or very mild. Whether or not the typical signs of "swine flu" develop depends to a large extent on the pig's immune status, the infection pressure, and the resulting viral load in the lungs during the determinative first 24 hours of infection. The primary method of controlling SIV is through vaccination for active or passive antibody immunity, which will either prevent infection or substantially reduce virus titers in the lungs and thereby prevent disease.

The currently available commercial SIV vaccines are mostly traditional inactivated vaccines for intramuscular (IM) injection. The production methods and immunological basis of protection resemble those of inactivated influenza vaccines for humans and horses, but there are also some marked differences. One such difference is the lack of a formal system for recommending SIV vaccine strains. This has become an important issue as several novel SIV subtypes and genotypes have emerged in swine during the last two decades. In addition, antigenic evolution and genetic reassortment between prevailing SIVs has contributed to diverse viruses within the same subtype in many different geographic regions. Several types of new-generation vaccines for SIV have been developed and tested experimentally, but only one of these, an RNA replicon particle vaccine, has been licensed for use in swine in the USA.

Although inactivated SIV vaccines have been used for decades, detailed information about their composition and critical analyses of their efficacy are hard to find. Published vaccination-challenge studies of any type of SIV vaccine should be interpreted with caution, because of multiple differences in their experimental designs and methodology, as well as differences with regard to the field situation. The aim of this chapter is to provide a critical review of the performance of traditional and

Animal Influenza, Second Edition. Edited by David E. Swayne.
 Published 2017 by John Wiley & Sons, Inc.

new-generation vaccines for SIV in experimental studies, and a carefully considered analysis of their strengths and weaknesses. We shall focus on the commercial, inactivated SIV vaccines, and pay special attention to the differences between the situations in Europe and North America, as well as to the issue of vaccine strain selection. The chapter starts with a summary of the pertinent facts related to vaccine use, and will conclude with a discussion of the potential next generation of SIV vaccines.

A primer on SIV evolution and vaccine immunology

An in-depth understanding of the capabilities and limitations of SIV vaccination starts with notions of the antigenic characteristics and evolution of SIVs and of influenza vaccine immunology. We shall therefore recapitulate some basic facts about the current epidemiology of SIVs, as well as the major differences between the immune response following infection with live influenza virus and that following vaccination with killed SIV vaccine. The reader is directed to other chapters of this book for comprehensive overviews of both of these topics.

SIVs in different regions of the world

SIVs of H1N1, H3N2, and H1N2 subtypes are enzootic in pigs all over the world, but their origins as well as their genetic and antigenic characteristics differ between continents and geographic regions [94]. During the last decades, multiple novel H1 and H3 SIV lineages have emerged, most often as a result of virus transmission from humans to swine and further reassortment and genetic evolution of these human viruses in swine populations [61]. Over time, SIVs also undergo antigenic drift in the HA, although this process is generally slower in swine than in humans [16]. One unique feature of the epidemiology of SIVs is that multiple genetically diverse lineages of H1 and H3 viruses may coexist. Table 19.1 presents an overview of the major SIV lineages in Europe and North America [3, 9, 40, 52, 74]. The sequences of the HAs of some H1 SIV lineages differ by as much as 20–25% at the amino acid level. Such large genetic differences exist between the three H1 lineages in Europe – avian-like H1N1 (H1avN1), human-like H1N2 (H1huN2), and 2009 pandemic (pH1N1) – and between North American H1 SIVs of cluster δ versus the other clusters. Smaller genetic differences occur between the α, β, and γ H1 clusters, and between North American H3 clusters, as well as within each of the virus lineages. SIVs in South America and Asia belong to the same HA and NA subtypes as the European and North American viruses, but many of them are different genetic lineages [11, 13, 108]. The 2009 (pH1N1) virus has become established in swine populations worldwide following repeated transmission from humans to swine since 2009. This recent virus has reassorted extensively with almost all of the regionally established SIVs, leading to a further expansion of novel SIV genotypes and an even more complex epidemiological picture. In Europe, regional differences in the prevailing SIVs have become more pronounced since 2009 [9, 74]. In the USA at least 10 H3N2 genotype patterns have emerged, with inconsistent serological cross-reactivity among them [24, 40, 48]. It is clear from this that the epidemiology of influenza is much more complex in swine than in humans, and so is the issue of vaccine strain selection.

The immune response to infection with live, wild-type SIV

The adaptive immune response after infection with live influenza virus includes mucosal and systemic humoral and cell-mediated immunity (CMI). Our general knowledge of the immune response to influenza viruses is discussed in detail in other review articles [15, 20, 79, 106], so it is only briefly recapitulated here.

Antibodies are mainly directed to the hemagglutinin (HA), neuraminidase (NA), matrix (M), and nucleoprotein (NP) proteins of the virus. Antibodies to the most variable viral protein, HA, can block attachment of the virus to host cell receptors and thus prevent the virus from infecting cells. These antibodies are typically measured in hemagglutination inhibition (HI) or virus neutralization (VN) assays. The VN assay is more sensitive than the HI assay, and evaluates antibody that blocks virus entry as well as antibody that may neutralize virus at other stages of the replication cycle. Anti-NA antibodies have not been studied as extensively,

Table 19.1 Major SIV lineages in Europe and North America.

Continent	Year of introduction	Subtype/lineage	Origin of genes	Comments
Europe	1979	H1avN1[a] (avian-like H1N1)	All 8 genes avian-like	Still dominant on European mainland, accounts for over 50% of all SIVs
	1984	H3N2	Human HA and NA (A/Hong Kong/68), internal genes from avian-like H1N1 SIV	Wholly human A/Hong Kong/68-like viruses reported in swine since 1970s, reassortment with avian-like H1N1 around 1984. No longer reported from some European countries since H1huN2 became widespread
	1994	H1huN2[b]	Human HA (A/Chile/1/83), other genes from H3N2 SIV	Second-generation reassortants between three enzootic lineages have also emerged (e.g. rH1avN2 is common in Denmark)
	2009	2009 pandemic H1N1 (pH1N1)	NA and M from European avian-like H1N1 SIV, other genes from γ-cluster H1N2 SIV	Prevalence highly variable in different European countries, but can be regarded as a fourth enzootic lineage
	2010 –	Reassortant viruses with pH1N1 genes	Most viruses have HA and/or NA genes from first three enzootic lineages, and other genes from 2009 pH1N1	
North Amerca	1918	Classical H1N1 (cH1N1, α-cluster)	1918 pandemic H1N1 virus	Virus has probably been circulating in pigs since the human 1918 H1N1 pandemic; first isolated from pigs in 1930 (currently rarely detected in the USA)
	1998	Triple reassortant (tr) H3N2	Human HA, NA, and PB1 (A/Wuhan/359/95 or A/Sydney/97); classical swine virus M, NP, and NS; North American avian PA and PB2	One major phylogenetic cluster (IV) currently circulates with emerging subclades A–F; clade A is dominant
	2000–2002	Reassortant H1N1 and H1N2 (β- and γ-cluster)	HA and NA from cH1N1, triple reassortant internal gene (TRIG) constellation from tr H3N2 SIV (H1N1); or HA from cH1N1 SIV, NA, and other genes from tr H3N2 SIV (H1N2)	β- and γ-clusters are distinct genetic/antigenic clusters of the HA of cH1N1; γ-cluster is currently dominant
	2003–2005	Human-like H1N1 and H1N2 (δ-cluster)	HA and/ or NA from human seasonal H1N1 or H1N2; other genes from trH3N2	Two subclades: δ1 (currently dominant) and δ2
	2009	2009 pandemic H1N1 (pH1N1)	NA and M from European avian-like H1N1 SIV; other genes from γ-cluster H1N2 SIV	Detections are currently sporadic and tend to be new human-to-swine spillover events rather than enzootic circulation
	2010 –	Reassortant viruses with pH1N1 genes	Most viruses have HA and/or NA genes from previous enzootic lineages, and a mixture of other genes from 2009 pH1N1	Nearly all current viruses contain the M gene from 2009 pH1N1

[a] av denotes that H1 gene is of avian origin.
[b] hu denotes that H1 gene is of human origin.

but are also important for protection, particularly when anti-HA antibodies fail to neutralize virus. Although anti-NA antibodies cannot neutralize virus, they reduce the release of newly synthesized virus particles from infected cells by inhibiting the enzymatic activity of the NA, and can be measured in neuraminidase inhibition (NI) assays. Antibodies to the NP and M proteins contribute to the killing of infected cells by antibody-dependent mechanisms, but they too cannot prevent an infection. Continuous antigenic changes in HA and NA epitopes – so-called antigenic drift – allow influenza viruses to escape from the effects of anti-HA and anti-NA antibodies.

CMI is primarily mediated by cytotoxic ($CD8^+$) T cells, which can kill virus-infected cells. Helper ($CD4^+$) T cells are necessary for adequate B-cell activation and subsequent antibody production. Unlike antibodies to the viral surface proteins, T cells are more broadly directed against conserved epitopes in the surface and internal proteins of the virus. The CMI response helps to complete the viral clearance, and may provide broader protection against re-infection with heterologous viruses, but it does not prevent infection. HA-specific, neutralizing antibodies in serum and even more so at the mucosae of the airways are thought to be most important for protection against re-infection with closely related SIVs within subtypes. Due to its polymeric nature, mucosal IgA antibody is believed to be more cross-reactive to drifted influenza viruses than is monomeric IgG. Antibody titers and effector immune cells wane over time, but populations of memory T and B cells are maintained in the airways and lymphoid tissues, and they can quickly be reactivated on re-exposure to influenza virus.

The few studies that have been conducted on the kinetics of the specific immune responses to influenza viruses in swine are reviewed elsewhere [87]. The very rapid and powerful specific immune response can in part be attributed to the excessive production locally in the lungs of a series of pro-inflammatory cytokines, which also have immunostimulatory properties. These cytokines include IFN-α, TNF-α, IL-1β, IL-6, and IL-12 [5, 38, 88, 89]. Consequently, the virus is eliminated within 1 week after the start of infection, usually prior to detection of neutralizing antibodies. Also typical of the immune response to infection with live SIV is that it can often protect against re-infection with distinct influenza variants within the same HA subtype; this is also called heterologous or heterovariant protection. Experimental pig infection studies have provided evidence for cross-protection between the three European H1 SIV lineages listed in Table 19.1, between North American α- and γ-cluster H1 SIVs, and between European and North American H1N1 as well as H3N2 SIV lineages [10, 18, 25, 65, 66, 81, 84, 97]. In these studies, influenza-naive pigs were first inoculated with SIV via the intranasal (IN) and/or intratracheal (IT) route, followed by a second inoculation with the antigenically distinct virus 4–6 weeks later. The second virus used was either undetectable in nasal swabs or lung tissue of these pigs, or virus titers were significantly reduced compared with those in control pigs inoculated with the second virus only. Cross-protection between H1N1 and H3N2 SIVs, so-called heterosubtypic protection, was much weaker [37, 66]. Prior infection with H1N1 or H3N2 could not prevent replication of the other subtype, but nasal shedding was on average 1–2 days shorter than in the control group. In all of these studies, cross-protection occurred in the absence of cross-reactive serum HI antibodies. Cross-reactive immune responses have been detected in serum by VN and/or NI assays, in nasal washes and BAL fluids by ELISA, and in circulating lymphocytes by CMI assays. The role and relative contributions of these immune responses in the cross-protection are still ill defined, as are the target proteins on the viruses.

The immune response to inactivated SIV vaccine

Commercial inactivated SIV vaccines are mostly whole-virus preparations with an oil-based adjuvant that are administered by deep IM injection into the neck. Two doses, 2–4 weeks apart, are necessary to induce robust antibody responses in influenza-naive pigs. For sow herds, the preferred method is to vaccinate gilts twice, pre-breeding, and the sows either quarterly or 3–6 weeks before farrowing, with the primary goal of increasing passive antibody transfer to suckling piglets as well as prevention of clinical disease in the sows themselves as discussed below.

The principle underlying killed influenza virus vaccines in general is the induction of serum antibody to the viral HA (reviewed elsewhere [20]). The antibodies are transferred to the mucosae of the respiratory tract by transudation, where they can contact and neutralize influenza virus. As is the case for other inactivated vaccines, the immune response differs from that induced by infection with wild-type virus in that it fails to strongly induce mucosal or cytotoxic T-lymphocyte responses. Indeed, pigs vaccinated with inactivated SIV vaccines had virus-specific IgG in their serum and bronchoalveolar lavage fluid (BALF), but lacked IgA in their lower and upper respiratory tracts [35, 46, 53, 54, 73, 97, 101]. Lymphocytes in the circulation or spleen of vaccinated pigs may show increased lymphoproliferation and production of IFN-γ [35, 46, 63, 73], but this is likely to reflect activity of NK cells, T-helper cells or the "double positive" $CD4^+CD8^+$ T-cell subset, rather than the traditional $CD8^+$ effector cytotoxic T cells.

Vaccine-induced antibody titers peak at 2 weeks after the second vaccination, and can fall rapidly thereafter [17, 44]. On the other hand, peak antibody titers to the H1 and H3 strains in the vaccine are usually several times higher than those after infection with live virus [35, 97], and HI titers of 160–2560 are no exception [17, 35, 44, 85]. These high homologous antibody titers apparently translate into cross-reactive antibody to heterologous strains, albeit at lower titers. Although there is little information about vaccine-induced antibody responses to NA, NI antibodies have been detected in pigs vaccinated with commercial European SIV vaccines, and they may play a secondary but significant role in protection [21] (Van Reeth *et al.*, unpublished data). Of four vaccines tested, all induced NI antibodies to N2, but two failed to induce antibodies to N1. This may be due to the poor physical stability of NA during the vaccine manufacturing process.

Inactivated SIV vaccines are designed to protect individual pigs against the clinical effects of SIV by reducing lung virus titers. Experimental studies support the hypothesis that a heavy viral load in the lungs is required to induce high levels of several pro-inflammatory cytokines, which in turn induce the typical lung inflammation and disease [5, 38, 88, 89]. Intratracheal challenge studies have shown an inverse correlation between post-vaccination HI or VN antibody titers in the serum of vaccinated pigs on the one hand, and viral and cytokine titers in the lung on the other [89]. In these studies, HI titers of 80–160 or VN titers of 128–192 against the challenge virus could prevent virus replication in the lungs in at least 50% of the pigs. Lower antibody titers may still reduce virus replication sufficiently to give complete protection against disease, and the exact seroprotective titer also depends on the challenge virus dose and route [44, 83, 85]. It should be emphasized that it is not claimed that killed SIV vaccines reduce nasal virus excretion in vaccinated pigs or SIV transmission in the population, although beneficial outcomes with regard to these parameters have been found in experimental studies [41, 43, 46, 47, 55, 70]. Reduced nasal shedding may be due to lower levels of virus in the deeper airways or in the nasal mucosa, or to a combination of both. However, the reduction of virus titers in the lungs of vaccinated pigs is almost invariably greater than that in nasal excretions [17, 21, 39, 51, 53]. This is consistent with the fact that transudation of serum antibodies is less efficient in the nose than in the lungs [31], and that traditional inactivated vaccines cause poor stimulation of mucosal IgA production.

In the field, SIV vaccination of sows is a more common practice than vaccination of feeder pigs. Sow vaccination primarily aims to increase and prolong maternally derived antibody (MDA) levels in young pigs. Piglets from vaccinated sows frequently have significantly higher and relatively uniform HI titers (≥160) compared with those from unvaccinated sows. Consequently, MDAs may be detected for up to 12 weeks in piglets from vaccinated sows, compared with 4–6 weeks in piglets from unvaccinated sows [78]. However, although MDAs will reduce infection and disease with similar strains during a period of high susceptibility to SIV infection [68], they will also interfere with the development of active immunity after infection or vaccination [39, 41, 50]. The conflict between the need to protect suckling piglets by means of MDAs and to be able to effectively immunize growing piglets is a major obstacle to the use of killed vaccines.

Thus, although the absence of detectable serum HI antibodies does not always correlate with lack of protection in immune pigs after infection with live

SIV, HI antibodies are the primary means and correlates of protection with inactivated SIV vaccines. New-generation SIV vaccines, in contrast, may stimulate additional arms of the immune response along with or instead of systemic antibodies. With live attenuated vaccines in particular, the immune response is more similar to that which occurs after natural infection than is the case with inactivated or other non-replicating vaccines. These alternative vaccines are discussed later in this chapter.

Commercial inactivated SIV vaccines

Vaccine characteristics and composition

Commercial inactivated SIV vaccines share similarities with the killed influenza vaccines for humans, but there are also important differences. Whereas the human vaccines generally contain purified viral surface antigens without adjuvant (for a review, see Fiore *et al*. [26]), most SIV vaccines are whole-virus preparations with an oil-based adjuvant. Unlike the human vaccines, SIV vaccines are not standardized for antigenic dose and vaccine strains. Consistent with the antigenic and genetic differences between SIVs in Europe and in North America, the vaccines for each geographic region are produced locally and they contain completely different strains. Within each continent, the vaccine strains may differ between different products, as may the exact adjuvant formulation and antigen dose. This is illustrated in Table 19.2 for European vaccines and in Table 19.3 for North American vaccines.

In Europe, SIV vaccines were initially licensed during the mid-1980s and early 1990s. These vaccines contained the two influenza virus subtypes that were prevalent at that time, namely H1avN1 and H3N2. For most of these vaccines, production was suspended around 2010, but they have been used in many experimental vaccination-challenge studies. A trivalent vaccine including the H1huN2 subtype was licensed in 2010, and is now the main vaccine on the European market. In some countries, monovalent 2009 pH1N1 vaccine is occasionally imported from North America. The products used and vaccination policies vary substantially between countries, but vaccine strains are very rarely updated. As shown in Table 19.2, it is not possible to compare the antigenic mass of various commercial products, because different manufacturers use different methods to measure and express the amount of antigen. In most European countries, only 10–20% of the sow population is vaccinated against SIV, and the vaccine is rarely used in fattening pigs.

The first SIV vaccine in North America was released in 1994. It was a monovalent vaccine developed from a classical H1N1 virus. After the emergence of H3N2 influenza viruses in the US swine population in 1998, monovalent H3N2 and bivalent H1/H3 SIV vaccines were launched. In response to the more recent emergence of antigenically distinct clusters within the H1 and H3 subtypes, vaccine manufacturers have reformulated their vaccines into polyvalent vaccines. Of the four inactivated vaccines that are currently available in the USA, two contain SIVs of multiple H1 and/or H3 clusters (Table 19.3). A monovalent vaccine based on the 2009 pH1N1 virus was licensed in 2009. Vaccine use is higher in the USA than in Europe, with approximately 70% of breeding stock estimated to be vaccinated [99]. The updating of vaccine strains is also considered more important in North America than in Europe. As a result, autogenous vaccines containing herd-specific strains are also very popular in the USA. Excellent information about these vaccines can be found in another review article [72], and they will not be discussed further in this chapter.

It is difficult to obtain a picture of the SIV vaccines used in South America and in Asia. Countries where SIV vaccines are used include Argentina, Brazil, Japan, and South Korea, but these vaccines are unavailable in many other countries, such as Vietnam, Thailand, and the world's largest pork producer, namely China. In general, South American countries mainly use the same commercial products as in North America, whereas locally produced vaccines based on local strains are often used in Asia. In Japan, the local vaccine is based on old H1N1 and H3N2 strains isolated in 1979 and 1969, respectively. In South Korea, two of the three local products are trivalent and all contain SIV strains from 2004–2005. In both countries, SIV vaccines often contain non-oil-based adjuvants, such as aluminum phosphate, Amphigen®, Rehydragel®, and IMS 1313. Vaccine uptake in these countries is

Table 19.2 Major commercially available SIV vaccines in Europe in 2015.

Manufacturer	Product name	Influenza virus strains	Adjuvant	Antigenic content[c] per vaccine dose	Countries where available
Merial	Gripovac	A/New Jersey/8/1976 (cH1N1) A/Port Chalmers/1/1973 (H3N2)	Oil	H1N1: ≥ 1.7 HIU H3N2: ≥ 2.2 HIU	Production stopped
Pfizer Olot	Suvaxyn Flu	A/swine/Netherlands/25/1980 (H1avN1) A/Port Chalmers/1/1973 (H3N2)	Oil	H1N1: 4 μg HA H3N2: 4 μg HA	Production stopped
Hipra	Gripork	A/swine/Olost/1984 (H1avN1) A/Port Chalmers/1/1973 (H3N2)	Oil	H1N1: 3×10^7 EID_{50} H3N2: 2.5×10^7 EID_{50}	Spain, Portugal
Fatro	Fluen-Suivax	H1N1[a] H3N2[a]	Aluminium hydroxide	H1N1: 400 HAU H3N2: 400 HAU	Italy
Izo	Izovac Suiflu	A/swine/OMS/2899/1982 (H1avN1) A/swine/OMS/3633/1984 (H3N2)	Aluminium hydroxide	H1N1: 640 HAU H3N2: 640 HAU	Italy
Impfstoffwerk Dessau-Tornau	Respiporc Flu	A/swine/Belgium/230/1992 (H1avN1) A/swine/Belgium/220/1992 (H3N2)	Aluminium hydroxide–oil	H1N1: ≥ 256 HAU H3N2: ≥ 256 HAU	Production stopped
Impfstoffwerk Dessau-Tornau	Respiporc Flu3 Gripovac 3[b]	A/swine/Haselunne/2617/2003 (H1avN1) A/swine/Bakum/1769/2003 (H3N2) A/swine/Bakum/1832/2000 (H1huN2)	Carbomer	H1N1: ≥ 10^7 $TCID_{50}$ H3N2: ≥ 10^7 $TCID_{50}$ H1N2: ≥ 10^7 $TCID_{50}$	Most European countries

[a]This is an older vaccine; specific strain names are not mentioned.

[b]The vaccine is marketed by Merial under the trademark of Gripovac 3.

[c]HIU = hemagglutination-inhibiting units as determined by measurement of the HI antibody response after administration of the vaccine to pigs; HAU = hemagglutinating units before inactivation as determined in a hemagglutination assay with chicken red blood cells; $TCID_{50}$ = tissue culture infectious dose 50% before inactivation; EID_{50} = egg infectious dose 50% before inactivation.

reportedly rather low, and this chapter will focus on European and North American SIV vaccines.

Regulatory aspects and vaccine strain selection

The authorization process for veterinary vaccines differs between Europe and the USA. In Europe, the evaluation of SIV vaccines relies heavily on their efficacy in experimental vaccination-challenge studies. Challenge studies must be performed with each of the influenza subtypes in the vaccine, according to the requirements of the European Pharmacopoeia. Influenza virus-seronegative pigs must be vaccinated twice according to label directions, challenged with a field isolate of SIV by the IT route, and euthanized at 24 and 72 hours after the challenge. The vaccine complies with the test if the mean virus titers in the lungs of vaccinated pigs are significantly lower than those in unvaccinated controls at both time points. The requirement to

Table 19.3 Major commercially available SIV vaccines in North America in 2011.

Manufacturer	Product name	Influenza virus strains[b]	Adjuvant[c]	Comments
Novartis	PneumoSTAR[a] SIV	α-Cluster H1N1 Cluster I H3N2	Immunstar®	
Intervet/Schering-Plough Animal Health	MaxiVac Excell 3.0	α-Cluster H1N1 β-Cluster rH1N1 Cluster I H3N2	Emunade®	Production stopped
Merck Animal Health	MaxiVac Excell 5.0	β-Cluster H1N1 γ-Cluster H1N1 δ-Cluster H1N1 Cluster I H3N2 Cluster IV H3N2	Emunade®	
Pfizer Animal Health	FluSure Legacy	α-Cluster H1N1 Cluster I H3N2	Amphigen®	Production stopped
Pfizer Animal Health	FluSure XP	A/swine/Iowa/110600/2000 γ-cluster H1N1 A/swine/Oklahoma/0726H/2008 δ1-cluster H1N1 A/swine/Missouri/069/2005 cluster IV H3N2	Amphigen®	Formulation used in Canada
Pfizer Animal Health	FluSure Pandemic	A/California/04/2009 Pandemic 2009 H1N1	Amphigen®	
Pfizer Animal Health	FluSure XP	A/swine/Iowa/110600/2000 γ-cluster H1N1 A/swine/Oklahoma/0726H/2008 δ1-cluster H1N2 A/swine/North Carolina/031/2005 δ2-cluster H1N1 A/swine/Missouri/069/2005 cluster IV H3N2	Amphigen®	This formulation is only available in the USA, not in Canada
Harrisvaccines	Swine Influenza Vaccine	Cluster IV H3N2 HA RNA	None	

[a] PneumoSTAR is the only single-dose SIV vaccine.

[b] Exact strain names and antigen dose are proprietary for most vaccines.

[c] All adjuvants are oil-in-water emulsions, except for Immunstar®, which is water-in-oil-in-water.

demonstrate a beneficial effect of vaccination on fever and weight loss was omitted from the revised European Pharmacopoeia monograph in 2003, because of the difficulty of reproducing the typical flu symptoms by experimental inoculation, and the significant correlation between lung virus titers and disease. Although these changes have occurred, vaccine manufacturers still have to execute the full licensing procedure to simply change vaccine strains, which may partly explain why most European SIV vaccines still have outdated strains. In the USA, the United States Department of Agriculture (USDA) introduced new licensing guidelines for updating strains in current fully licensed vaccines in September 2007. Since then, immunogenicity of a novel strain added to or replacing a strain in an existing license can be demonstrated by serology in pigs rather than challenge. It must also be demonstrated that antigens in a combination product do not interfere with the immune response to each of the vaccine components. Manufacturers in the USA thus have the opportunity to address vaccine updates in a more timely and flexible manner than is possible with the obstacles that are encountered in Europe. Despite this apparent flexibility and the recent observations of expanded genetic [3] and antigenic evolution [48], few updates occurred between 2007 and 2014.

Since inactivated vaccines induce protection by highly specific antibodies against the viral HA, vaccine strains should match with the circulating field strains within each subtype, and the current diversity in these strains complicates control by inactivated vaccines. Several experimental studies in pigs have shown insufficient antibody responses and protection against newly introduced virus lineages with vaccines based on existing strains. In these cases there was usually up to 75% amino acid homology in the HA1 of H1/H3 vaccine and challenge strains. For example, European bivalent H1N1/H3N2 vaccines failed to induce cross-reactive HI antibody responses or protection against the European H1huN2 SIV lineage [22, 90]. Furthermore, the available European vaccines at best induced low or moderate cross-reactive antibody responses to the 2009 pH1N1 virus [22, 45, 51], and protection against challenge was inferior to that obtained with monospecific experimental 2009 pH1N1 vaccine [22, 51]. Because of the importance of genetic and antigenic matching of the vaccine to the challenge viruses, the cross-reactivity of HI antibodies and analysis of HA gene similarity are most often used as predictors of vaccine cross-protection. However, it is not known how much antigenic or genetic distance is required for a change in vaccine strains. Moreover, the location of changes on the HA protein may be more important than the total amount of change. Recent studies of North American H3N2 SIVs identified six amino acid positions near the receptor-binding site of the HA that played a major role in altering antigenic phenotypes [48]. The authors concluded that substitutions in as few as one or two of these amino acids could be sufficient for immune escape and vaccine failure in pigs.

Factors other than the similarity between vaccine and field strains may also strongly affect vaccine efficacy, namely the immunogenicity of the vaccine strains, the quantity of antigen included, and the adjuvant used. This can explain why there may be a poor correlation between vaccine match in laboratory assays and vaccine performance in challenge studies. Commercial European SIV vaccines in particular have frequently shown much broader protection in challenge studies than one would expect based on genetic and antigenic comparisons of vaccine and field strains. The A/NewJersey/8/76 H1N1 strain in the very first European vaccine was a "classical" H1N1 virus that is far more closely related to North American H1 SIVs than to European avian-like H1N1 SIVs. Nevertheless, it offered excellent protection against challenge with H1avN1 viruses from the 1980s and 1990s, which showed only 78–81% amino acid homology to the vaccine strain [33, 85]. Likewise, the A/Port Chalmers/1/73 H3N2 strain in the first European SIV vaccines provided significant protection against more recent European H3N2 SIVs belonging to the same lineage but a different antigenic cluster [17, 35, 83]. These more recent viruses not only showed low HA amino acid homology (84–92%) with the vaccine strain, but also differed from it in three out of the six amino acid positions in the HA that are considered key for antigenic switches of North American swine H3N2 viruses [17]. Several findings provide evidence for a key role of the oil-based adjuvants in the relatively broad serological cross-reactivity and protection observed with these European SIV vaccines, as described in detail in a previous review article [87]. Oil-based emulsions not only increase antibody titers, but also expand the cross-reactivity of the antibody response [20]. Thought-provoking results were obtained in a comparative study involving commercial vaccines containing different H1N1 strains and challenge with a 2007 H1avN1 SIV [44]. Two vaccines containing oil-based adjuvant with H1N1 strains showing 93% and 89% amino acid homology to the challenge virus offered solid protection against challenge. In contrast, the vaccine containing a Carbomer adjuvant failed to offer significant protection, despite having 95% amino acid homology with the challenge H1N1 strain. This is most probably due to the fact that Carbomer, which has the advantage of being better tolerated, is a less potent adjuvant than oil. Similarly, vaccines containing identical strains but different adjuvants and/or amounts of antigen have been shown to differ in their potency and performance in challenge studies [22, 83, 85]. In other words, vaccine efficacy is not always directly correlated with how closely the vaccine strains match the field viruses, and sequence analyses or antigenic data as such are not always reliable predictors of vaccine efficacy. Although cross-protection challenge data remain the ultimate test, the testing of vaccine-induced antisera for reactivity against field isolates in HI or

VN assays is perhaps the most valuable alternative to these costly *in-vivo* studies.

Efficacy of SIV vaccines used in Europe

Most peer-reviewed vaccination-challenge studies have been performed using the first generation of European bivalent H1N1/H3N2 SIV vaccines, which were based on the human A/New Jersey/8/76 and A/Port Chalmers/1/73 strains. In these studies, SIV-seronegative pigs are vaccinated twice with commercial vaccine and challenged with field isolates of SIV 2–7 weeks after the second vaccination. The pigs are usually euthanized during the very acute stage of infection, 1–3 days after challenge. Lung virus titers are the main criterion for evaluating protection. Because of the European Pharmacopoeia requirements, the initial studies used IT challenge with a very high dose (7.5 $\log_{10}$ EID_{50}) of H1avN1 and H3N2 SIVs from the 1980s and 1990s [33, 83, 85, 91]. Under these conditions, unvaccinated challenge control pigs invariably demonstrate high virus titers in the lungs (up to 7.0–8.0 $\log_{10}$ ID_{50}). They also show the severe although transient dyspnea, fever up to 41°C, depression, and anorexia that are so typical of acute outbreaks of SIV. The vaccine offered excellent virological protection against this stringent virus challenge, and the lungs of around 50% of the vaccinated pigs tested negative for the challenge virus. The remaining pigs had reduced lung virus titers, which usually resulted in complete clinical protection. More recent studies have used alternative inoculation routes (aerosol, IN, or IT with a lower dose, namely 5.0 $\log_{10}$ EID_{50}), and they have also evaluated the effect of vaccination on virus secretion [17, 21, 35, 51]. These inoculation methods also result in high virus titers throughout the respiratory tract of unvaccinated pigs, but IN or low-dose IT inoculation largely fails to reproduce disease. Although New Jersey/76- and/or Port Chalmers/73-based vaccines are no longer used in most European countries, the challenge studies using these vaccines provide valuable general lessons about SIV vaccine potency. As previously mentioned, these vaccines have shown the ability to provide protection against H1avN1 and H3N2 SIVs isolated over many years with considerable antigenic and genetic drift compared with the vaccine strains. It is remarkable that a Port Chalmers-based vaccine could still protect against challenge with an H3N2 SIV isolated 35 years later than the vaccine strain, with only 86.9% amino acid homology in the HA1 and differences in as many as 11 amino acids in antigenic sites. This vaccine also induced substantial antibody titers against H3N2 SIVs isolated between 2008 and 2012 [17]. The New Jersey/76-based vaccine was very efficacious against avian-like H1N1 viruses from the 1980s and 1990s, but failed to produce a significant reduction in lung virus titers after challenge with a 2007 H1avN1 isolate [44]. In fact this was also true of the more recent trivalent European H1N1/H1N2/H3N2 vaccine, although it contained a much more closely related H1avN1 virus. However, unlike the bivalent European vaccines, the trivalent vaccine offered 100% protection against challenge with the homologous H1huN2 virus, and it is now the major vaccine on the European market [21, 90]. None of the vaccines mentioned were able to induce optimal protection against the 2009 pH1N1 virus. In some countries, therefore, farms with a diagnosis of 2009 pH1N1 were given special permission to import and use the Pfizer monovalent "FluSure Pandemic" vaccine.

Very few vaccination studies have been performed in pigs with pre-existing immunity to SIVs. However, this scenario is likely to reflect the situation in the field, since most gilts and sows have been previously exposed to one or more SIVs by infection. In one serological study, influenza-naive pigs were first inoculated intranasally with live SIV, or with two or three different subtypes at 3- to 6-week intervals. A few weeks later they were administered a single IM vaccination with a commercial bivalent SIV vaccine [86]. The vaccine H1N1 (A/New Jersey/8/76) and H3N2 (A/Port Chalmers/1/73) strains were only distantly related to the more recent H1avN1, H3N2, and H1huN2 SIVs used for infection and serology. As expected, the single vaccination was insufficient to stimulate robust antibody titers in influenza-naive pigs, and they developed minimal H1avN1 and H3N2 antibody titers and no antibody to H1huN2. In contrast, the primed pigs showed a dramatic boost of HI antibody titers to any of the viruses to which they had previously been exposed, including H1huN2. Two weeks after the vaccination of H1avN1 infection-immune pigs, for example, mean group antibody titers to H1avN1 were as high as

2319 in the HI test and 11 230 in the VN test. This may mean that a close antigenic match between vaccine and field strains is less important in pigs primed by infection with field strains. Furthermore, according to recent intriguing studies in animal models and humans, a broadly cross-reactive anti-HA antibody response is best achieved by consecutive immunizations with strains of the greatest possible diversity within subtypes (reviewed by Chiu *et al*. [12]).

Efficacy of SIV vaccines used in North America

In studies of North American SIV vaccines, pigs are usually challenged 10–21 days after the second vaccination. Challenge is performed via the IN or IT route, or a combination of both, with a moderate virus dose. The pigs are often euthanized toward the end of the course of infection, on day 5 or later, and virus titers in lung tissue are rarely determined. Instead, investigators evaluate nasal virus shedding, virus levels in BALF, lung lesions, and clinical signs. There can be a range of disease severity with the challenge methods used. Some studies have also evaluated the effect of vaccination on virus transmission. There are published efficacy studies of the first, monovalent H1N1 vaccine [46, 55] and of bivalent H1N1/H3N2 vaccines [41, 43]. Challenge was with classical H1N1 SIVs from 1988 or 1992, which were heterologous from the classical H1N1 vaccine strain. The vaccines were shown to reduce clinical scores and macroscopic and microscopic lung lesions, and nasal virus excretion was either reduced [41, 47, 55] or undetectable [43, 46]. From the late 1990s, both H1 and H3 SIVs started to show greater antigenic diversity, and this is seen as a major obstacle to control using inactivated vaccines, even with the newer multivalent formulations. It is often difficult to determine the exact similarity between vaccine and field strains, because specific strain names and genetic sequences are proprietary for most vaccines, and vaccine manufacturers in the USA usually only disclose the phylogenetic cluster of the vaccine strains.

In challenge studies involving more recent H1 SIVs, an experimental vaccine based on the classical α-cluster H1N1 strain A/swine/Iowa/1930 provided incomplete cross-protection against challenge with the recent γ-cluster H1N2 SIV A/swine/Minnesota/2003 [43, 97]. In both studies, the pigs that were vaccinated with whole inactivated A/swine/Iowa/1930 and challenged with the heterologous A/swine/Minnesota/2003 had more severe lung lesions than all the other groups, including the non-vaccinated challenge control group. This phenomenon was subsequently reproduced with 2009 pH1N1 and δ-H1N2, with both of these viruses used as either vaccine or challenge strain [8, 28, 29]. The clinical observation was termed vaccine-associated enhanced respiratory disease (VAERD), and appears to be restricted to whole inactivated vaccines with pairs of viruses that are of the same HA subtype but which have drifted sufficiently far apart to no longer demonstrate cross-reacting HI or VN antibodies. Mismatch of the NA as well as the adjuvant contained in the inactivated vaccine has also been demonstrated to be integral to the VAERD model (Vincent, unpublished data).

Prediction of vaccine protection on the basis of HA sequence similarity or phylogeny is unreliable. Canadian researchers have shown significant cross-cluster protection against the contemporary α-cluster H1N1 SIV A/swine/Illinois/02450/2008 with the trivalent commercial FluSure XP vaccine [19]. The latter vaccine exclusively contains γ- and δ2-cluster H1N1 strains. Between the γ-cluster vaccine strain, which has a common ancestral HA with the α-cluster, and the challenge virus there was only 87.2% nucleic acid sequence homology, and there were as many as 11 amino acid differences in antigenic sites of the HA. Although the vaccine did not have an effect on lung lesions, the amount of virus was significantly reduced in the nasal secretions, lungs, and BALF of the vaccinated pigs compared with the placebo pigs. The authors concluded that a commercial multivalent vaccine helped to protect against challenge with a virus from a swine H1 cluster not represented in the vaccine. The same trivalent vaccine and an experimental monovalent homologous β-cluster vaccine were compared in a transmission study with β-cluster challenge [70]. The challenge virus demonstrated low serological cross-reactivity with the antiserum induced by the heterologous vaccine. Both vaccine regimens significantly reduced transmission from unvaccinated seeder pigs to the vaccinated pigs, but the heterologous vaccinated

group still had transmission to part of the contacts and measurable viral shedding, albeit delayed and reduced compared with naive challenge controls. Soon after the emergence of the 2009 pH1N1 virus in the North American swine population, three licensed vaccines were examined for their ability to induce cross-reactive antibodies and protection. Although the 2009 pH1N1 virus contains an HA of the γ-cluster H1 lineage, limited serological cross-reactivity in the HI test was observed with the two vaccines containing H1 antigen of the same cluster [96]. However, both vaccines demonstrated partial protection against 2009 pH1N1 challenge, but higher antibody titers and complete protection were obtained with an experimental monovalent 2009 pH1N1 vaccine [95].

Challenge studies have also been performed with commercial vaccines that used H3N2 SIVs of heterologous clusters. Three of the initial commercial vaccines that contained only a cluster I H3N2 strain were insufficient to reduce shedding of a cluster III H3N2 virus after challenge infection, although the vaccinated pigs showed relatively mild clinical signs and reduced lung lesions [47]. Since pigs that had been vaccinated with an experimental homologous vaccine had complete sterilizing immunity, the unsatisfactory results were believed to be due to the heterogeneity between the cluster I vaccine strains and the challenge virus, which showed around 93–94% amino acid homology [32]. A more recent experimental challenge study provided similar evidence that polyvalent commercial vaccines containing cluster IV H3N2 can offer better protection against a drifted contemporary cluster IV strain than a commercial vaccine containing only cluster I H3N2 [53]. Two such vaccines containing cluster IV viruses significantly reduced virus titers in BALF, but the quadrivalent FluSure XP vaccine gave the most significant reduction, and only this vaccine reduced the number of pigs with nasal virus excretion. Although the vaccine could not prevent virus transmission to naive in-contact pigs, transmission was more limited, and correlated with the smaller amount of nasal virus shedding by the principal vaccinated pigs. The effects on virus replication and excretion were confirmed in a study with another cluster IV challenge virus with 97.2% amino acid identity with the cluster IV vaccine strain [39].

Since pigs in the USA are often transported to off-site nursery and finisher sites, there is a high likelihood that they will be exposed to viruses that are heterologous to those circulating or used for vaccination on the sow farm. A few studies have attempted to assess protection against heterologous challenge in piglets with passively acquired MDAs through vaccination of their dams. The failure of MDAs to prevent infection and transmission in the face of an experimental heterologous challenge seemed to be even more pronounced [1] than in vaccinated naive pigs with an active immune response in the study mentioned earlier [70]. Corzo and colleagues [14] showed that piglets with MDAs from sows vaccinated with a heterologous vaccine virus were infected by natural exposure and shed virus, and one pig from the homologous vaccinated sows shed virus following homologous virus challenge. Managing SIV in piglets through MDAs seems to be as complex as managing SIV through primary vaccination, if not more so.

Novel SIV vaccines

The ideal SIV vaccine should induce the broadest immune response possible and overcome interference from MDAs, and this has stimulated research into alternative approaches to vaccination. To date, several new-generation vaccines for SIV have been developed and tested in pigs, but only one of these, an RNA replicon particle vaccine, has reached the market. Live attenuated influenza virus (LAIV) vaccines are among the most promising vaccine candidates, and it is likely that they will become commercially available in North America in the near future. We shall therefore focus on LAIV vaccines here, and only briefly review other types of new-generation vaccines.

Live attenuated vaccines

LAIV vaccines administered mucosally (i.e. by the IN or IT route) mimic natural infection and demonstrate the potential for broad cross-protective immunity with heterologous viruses of the same subtype and even heterosubtypic viruses [100]. Researchers in North America have used three distinct strategies to stably attenuate SIVs with reverse genetics technology. These vaccines have

been tested in pigs for immunogenicity and efficacy against challenge infection, which is usually evaluated on the basis of virus titers in nasal swabs, virus titers in BALF on day 5 post challenge, and lung lesions. In addition, the effect of LAIV on virus transmission to contact pigs or vaccine performance in the presence of MDAs has been investigated. Reversion to virulence has not been confirmed in LAIV vaccines for humans, and the temperature-sensitive platform has multiple attenuating mutations, reducing the opportunity for reversion. However, there is concern about the use of LAIV vaccines in swine, and the potential reversion to virulence and reassortment between wild-type and vaccine strains. One factor that may mitigate this risk relates to the fact that the LAIVs used in experimental studies in the USA are from the North American swine lineages, and would thus contribute no extraneous genetic material if reassortment occurred.

Live attenuated vaccine with modified NS1 protein

The non-structural NS1 protein of the influenza A virus is exclusively expressed in virus-infected cells, and is not present in virus particles. One of the major functions of the NS1 protein is the inhibition of the type I interferon-mediated antiviral response. Truncation of NS1 protein in the North American cluster I H3N2 SIV A/swine/Texas/4199-2/1998, from a length of 230 amino acids to 126 amino acids, produced a mutant with restricted replication in the swine respiratory tract, and it had strong immunogenic properties [69, 76]. In addition to modest levels of systemic neutralizing antibodies, this LAIV vaccine elicited mucosal IgA in nasal washes and BAL fluids, and systemic CMI responses [37, 53, 69, 73, 100]. The CMI responses were weaker than those observed after infection with wild-type A/swine/Texas/1998 virus, which can be attributed to the very low level of replication of the LAIV in the respiratory tract [37]. The IN route was more efficient than the IM route in priming the mucosal antibody response, and most studies have been performed using two doses of vaccine administered IN, although a single IN administration was also found to be efficient in a more recent study [100]. The LAIV vaccine provided robust protection against homologous challenge in influenza-naive pigs, and almost complete protection against the antigenically distinct cluster II H3N2 strain A/swine/Colorado/23619/1999 [69, 73, 76, 100, 101]. In contrast, the vaccine was much less efficacious against challenge with a heterosubtypic H1N1 SIV, although there was a slight reduction of virus titers in BALF and nasal swabs at 5 days post challenge, with no effect on lung lesions [37, 69, 100]. The absence of serum HI antibodies to the heterovariant and heterosubtypic viruses in these studies indicates that a complex host response involving both cellular and humoral mechanisms contributes to the broad protection [100], but the protective immune responses require further investigation. According to a more recent study, the NS1 vaccine may also be partially effective in piglets with MDAs [98, 99, 101]. Although it was not fully protective in pigs with matching MDAs, protection against the heterologous Colorado/99 strain was better than after IM administration of an experimental killed vaccine. In control pigs without MDAs, both vaccines provided complete protection against replication of not only the homologous Texas/98 strain but also the heterologous Colorado/99 strain. However, the pigs that were vaccinated with inactivated vaccine and challenged with heterologous virus developed VAERD that was not observed in the LAIV group. Similar findings were reported in a more recent study [73].

Temperature-sensitive live attenuated vaccine

Mutations in the viral polymerase genes were identified in cold-adapted attenuated influenza viruses developed for horses [80] and humans [6]. These mutations were introduced into a well-characterized triple reassortant H3N2 SIV, causing impaired polymerase activity, reduced growth at the temperature of the lower respiratory tract, and an attenuated phenotype in mice and pigs [62]. The temperature-sensitive (ts) LAIV internal gene backbone was paired with several other surface HA and NA via reverse genetic engineering, and such a construct was shown to protect against a homologous 2009 pH1N1 challenge [62]. During 2011–2012, several hundred human infections were detected with an antigenically drifted cluster IV H3N2 from SIV, denoted H3N2v when detected in humans [23, 36]. In the above-mentioned study by Loving and colleagues

[53], a commercial multivalent vaccine with a high degree of serological cross-reactivity to the H3N2v [39] gave significant partial protection as measured by reduced nasal shedding. However, indirect contacts became infected, indicating that the reduction in nasal shedding did not prevent aerosol transmission. By contrast, the tsLAIV tested in the same study provided complete protection, and none of the indirect-contact pigs became infected. Both vaccines provided significantly better protection than two other commercial killed vaccines and the NS1-deleted LAIV vaccine with a more distantly related HA described earlier. This clearly demonstrates that vaccine efficacy depends on multiple factors and may be highly variable for both killed and LAIV vaccines, especially with antigenically drifted HA and NA.

In a series of studies against H1 SIVs, a 2009 pH1N1 tsLAIV was compared with a matched HA protein subunit vaccine when challenged with a heterologous δ1-cluster H1N2 virus [67]. The tsLAIV partially protected pigs, resulting in reduced virus shedding and faster viral clearance, as no virus was detected in the lungs by 5 days post challenge. In marked contrast, the HA-subunit-vaccinated pigs developed more severe lung and tracheal lesions, consistent with VAERD, following challenge. This finding was recently confirmed in another study [27]. The mild post-challenge clinical signs and lung lesions demonstrated by the tsLAIV immune groups in the absence of serum neutralizing antibodies suggest a role for cross-reactive mucosal immunity in LAIV protection from infection and clinical disease.

Elastase-dependent live attenuated vaccine

Other researchers have attenuated influenza viruses by modifying the cleavage site in the viral HA to require elastase enzyme. Although elastase can be added as a supplement during virus cultivation, it is too scarce in the host animal to support significant replication of the virus. Such elastase-dependent mutants have been generated from an H1N1 virus isolated from swine in Canada, A/swine/Saskatchewan/18789/2002 [56]. They were highly attenuated in pigs and still induced systemic and mucosal antibody responses and CMI after two IT or IN doses [57, 58]. In challenge studies, the elastase-dependent LAIV vaccine conferred protection against challenge with the homologous H1N1 virus and antigenically distinct H1N1 SIVs, but only some of the pigs were protected against heterosubtypic H3N2 infection [4, 56, 58]. The same researchers have recently constructed an entirely novel LAIV vaccine candidate [59]. In this genetically engineered virus the N1 protein of the A/swine/Saskatchewan/2002 virus has been exchanged for the H3 of an H3N2 SIV. Theoretically such a virus can serve as a bivalent LAIV and provide complete protection against both H1 and H3 SIVs, unlike all of the other LAIV vaccines examined so far. In a preliminary challenge study, pigs were vaccinated twice by the IT route with this novel vaccine, challenged with H1N1 or H3N2 SIVs 10 days after the booster vaccination, and euthanized for challenge virus titrations on day 5 post challenge. Neither of the subtypes could be isolated from the lungs of vaccinated pigs, but the H3N2 virus was also undetectable in the lungs of 4 out of 5 challenge control pigs. Thus the available challenge data need to be interpreted with caution, and more data are needed. The route of vaccination, vaccine dosage, and regimen also require further investigation, as two IT or IN administrations are not convenient in the field.

Taken together, these studies suggest that IN administration of LAIV vaccines is the preferred method for inducing mucosal secretory antibody responses and for preventing or reducing SIV transmission in the population. Protection may extend to antigenic variants of the vaccine strain, but multivalent formulations or chimeric vaccines would be required to protect against a variety of H1 and H3 SIVs. Furthermore, the presence of matching MDAs reduces the efficacy of LAIV vaccines, and the effects of pre-existing active immunity have not been studied. In humans, LAIV vaccines appear to be more effective in children than in adults, due to interference of pre-existing active immunity with the replication of LAIV vaccines. Live vaccine appears to be more effective than killed vaccine as a priming vaccine, whereas killed vaccine appears to be more effective in boosting pre-existing humoral immunity [2, 20, 26].

RNA replicon particles

Alphavirus-derived replicon particles (RPs) are the first viral vector vaccine technology to have been approved for SIV vaccination in swine in the

USA. The vector is a modified form of an attenuated Venezuelan equine encephalitis virus. It is replication-defective because structural genes have been deleted and replaced by RNA that encodes a gene of interest, such as the HA gene of SIV. The recombinant alphavirus RP can still bind to host cells, deliver genetic material into the cytoplasm, and drive protein expression of the inserted gene(s). However, no infectious progeny virus is produced, and this alleviates concerns about reversion to virulence in vaccinated animals. Alphavirus RPs also have inherent adjuvant properties, which probably contribute to their efficacy. The licensed RP SIV vaccine expresses the HA of a North American cluster IV H3N2 SIV, and the primary vaccination should consist of two intramuscular injections administered 2 or 3 weeks apart. The vaccine has been shown to induce protective immunity against homologous challenge, although it did not provide protection in the presence of MDAs [7]. The same platform that expressed the 2009 pH1N1 HA protein was also protective against homologous infection, and the vaccine that expressed an H3N2-derived NP gene reduced nasal shedding and viral replication following H1N1 challenge in pigs [92, 93]. One advantage of the RP platform is that the HA from any newly emerging strain can be rapidly cloned and inserted into the vector. The technology is therefore frequently used to generate custom-made SIV vaccines. Field data on usage and efficacy of the RP RNA vaccine were unavailable at the time of writing.

Other vaccines

Recombinant protein vaccines

Recombinant protein vaccines based on the conserved M2 minor envelope protein of influenza viruses have been developed and tested in pigs. Such vaccines were very promising in the mouse model of influenza [60, 75], and induced the desired immune response in pigs [34, 42], but there was no significant decrease in virus excretion after challenge. However, anti-M2 antibodies are not neutralizing, which could explain the lack of significant protection in challenge studies in pigs.

More promising results have been obtained with a recombinant, soluble trimer of the HA of 2009 pH1N1 virus [49]. HA trimers are considered better vaccine candidates than HA monomers because they resemble the natural HA more closely and thus induce higher levels of neutralizing antibodies [102]. Pigs vaccinated with this recombinant vaccine developed very high levels of HI and VN antibodies against the homologous virus, which were cross-reactive with a European H1avN1 SIV, but not with H1huN2. They were almost completely protected against homologous challenge. However, this study used a high dose of recombinant HA vaccine and a challenge virus that was very closely related to the vaccine strain. Furthermore, subunit vaccines are unlikely to offer significant advantages over the traditional killed SIV vaccines.

Vector vaccines

A human adenovirus serotype 5 (Ad5) has been made replication defective by the removal of two segments of its genome, and this space in the genome has been used to insert HA or NP antigen sequences of the cluster I H3N2 SIV A/swine/Texas/1998 [77, 105]. Single-dose IM delivery of the Ad5-HA alone or in combination with Ad5-NP elicited high levels of virus-specific HI antibodies. The combination offered better protection against challenge with a closely related H3N2 virus than Ad5-HA alone, and Ad5-NP alone had minimal effects [105]. A prime-boost strategy with the Ad5-HA and Ad5-NP combination followed by a commercial bivalent vaccine 3 weeks later was efficacious against H3N2 SIV challenge in piglets with H3N2-specific MDAs, unlike single administrations of the vector or killed vaccine [104]. The Ad5 antigens could also be delivered with a needle-free device [103]. In a more recent study, pigs were vaccinated once IN with an improved Ad5-HA vector encoding codon-optimized HA of the 2009 pH1N1 virus [8]. This vaccine was able to induce a mucosal antibody response to the homologous virus and complete protection from homologous challenge, but it was much less efficient against challenge with a heterologous δ-cluster H1N2 virus. Recently, recombinant swinepox and equine herpesvirus 1 vectors expressing HA genes of SIV also gave satisfactory results in challenge studies with homologous virus [71, 107]. However, it should be noted that these pig studies used extremely high doses of the vector vaccines (often up to 9.0–10 $\log_{10}$ $TCID_{50}$) and, in most cases, challenge viruses

that were closely related to the vaccine antigens. Furthermore, immunity to the vector virus itself may interfere with booster vaccinations in sows, and with vaccination of their piglets, which are expected to acquire MDAs to the vector.

DNA vaccines

DNA plasmid vaccines that encode protective antigens have the theoretical advantage of expressing antigens in their native form and thus stimulating both humoral and CMI responses, including CTLs. Moreover, several antigens can be combined in a single plasmid, and they are supposed to be able to induce immunity in the presence of MDAs. A number of pig studies with DNA vaccines based on HA genes of SIVs have demonstrated immune responses and moderate protection against homologous challenge infection [30, 46, 55]. Both conventional IM injection and a needle-free vaccine delivery method were successful [30]. A prime-boost regimen consisting of one dose of DNA vaccine followed by conventional inactivated vaccine 4 weeks later gave significantly better results than two doses of DNA vaccine [46]. With DNA vaccines alone, very large doses of DNA were required, often in a series of three or more doses, and protection against homologous challenge was either insignificant or incomplete. It is clear that further efforts will be required to develop DNA vaccines into a viable and practical alternative.

Conclusions and perspectives

Swine influenza has become a very dynamic disease with multiple co-circulating H1 and H3 lineages, which differ between continents and regions. None of the commercial or experimental vaccines discussed in this chapter will be able to provide true universal protection against any of the existing viruses. In fact the immune response after infection with any given wild-type SIV will also fail to offer complete heterovariant and heterosubtypic protection, and cross-protection becomes weaker with increasing antigenic diversity between viruses. This raises questions about the feasibility of "universal" SIV vaccines. Experimental challenge studies with vaccines are usually performed under ideal conditions that differ from those encountered in the field. In most studies, investigators use SIV-naive pigs, short time intervals between vaccination and challenge, and challenge viruses that are relatively closely related to the strains in the vaccine. In the field, swine are vaccinated in the presence of MDAs or, even more frequently, active pre-existing immunity to SIVs that may interfere with vaccine efficacy. Although MDAs have been shown to reduce the efficacy of both inactivated and LAIV vaccines, data on vaccine performance in the presence of active immunity are almost non-existent. Furthermore, there have been few direct comparative studies of the efficacy of commercial and experimental vaccines.

Vaccination policies and attitudes differ greatly between Europe and the United States, as do our views on SIV vaccines of the future. In Europe, vaccine uptake is much lower than in the USA, and researchers still have confidence in traditional inactivated vaccines. This is in part based on the relatively broad protection provided by the first generation of these vaccines, and on the fact that even a moderate reduction of virus titers in the lungs of the vaccinated animal is usually sufficient to alleviate disease. Furthermore, VAERD has never been observed with commercial or experimental killed SIV vaccines in Europe, even if the vaccine strain did not match the challenge strain and there was minimal protection. Some SIV researchers believe that a broad and probably cross-cluster protection can be achieved with inactivated vaccines by optimizing vaccine formulations and regimens. Possible approaches include the use of improved adjuvants, stimulation of anti-NA antibody responses, and the use of antigenically distinct strains for primary and booster vaccinations. For example, consecutive vaccinations with experimental monovalent vaccines based on European and North American H3N2 SIVs, respectively, were shown to induce neutralizing antibody responses and protection against both virus lineages, unlike the traditional primary and booster vaccination with identical viruses [64].

Following the emergence of the H1N1 pandemic virus derived from two lineages of SIV and the outbreaks of H3N2v in humans, there was a dual focus in the USA on reducing disease at the level of the individual pig, and on the prevention of nasal virus shedding and virus transmission in swine populations, as well as between swine and humans. This can in part explain the interest in licensing LAIV

vaccines, which are the only vaccines that have demonstrated mucosal immunity.

SIV researchers worldwide agree on a number of pathways needed to improve SIV vaccines, one of which is increased surveillance for SIVs. Surveillance is essential in order to gain a picture of the dominant virus subtypes and lineages in different geographic regions, to detect changes, and to assist with vaccine strain selection. Unfortunately, surveillance for influenza viruses in swine has been chronically underfunded, and is virtually non-existent in many parts of the world [94]. From a regulatory viewpoint, the rigorous licensing requirements for SIV vaccines in Europe remain an obstacle to vaccine strain updates, should these be needed. Regulators should therefore consider accepting serological data if manufacturers wish to substitute one of the strains in multivalent vaccines. Finally, there are many unanswered questions about influenza vaccine immunology that are hampering progress in the control of swine influenza through vaccination. For example, why do some killed SIV vaccines offer broader protection than others? How much drift is needed before vaccine strains of a given subtype become obsolete? Which amino acid changes are most important? What is the true protective value of immune responses other than neutralizing antibody to the HA? What are the best correlates of protection for LAIV vaccines? To what extent will the combination of multiple strains in one vaccine lead to "antigenic competition" and reduced or biased immune responses? How does pre-existing immunity affect the "take" of different types of influenza vaccine? Many of these questions also apply to human influenza vaccines. Furthermore, studies of the immune response to influenza viruses in the pig may yield insights and information that are important for both veterinary and human medicine.

Acknowledgments

The authors acknowledge grant support from the National Institute of Allergy and Infectious Diseases, National Institute of Health, Department of Health and Human Services (Contract No. HHSN266200700005C), the European Commission (FP7 –GA258084, FLUPIG), and the Belgian Federal Public Service for Health, Food Chain Safety and Environment (RT 09/6227, FLUCROSS). Any mention of trade names or commercial products in this article is solely for the purpose of providing specific information, and does not imply recommendation or endorsement by the United States Department of Agriculture (USDA). USDA is an equal opportunity provider and employer.

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SUBSECTION IIIB

Equine influenza

20 The clinical features, pathobiology, and epidemiology of influenza infections in horses

Gabriele A. Landolt and Thomas M. Chambers

History

Equine influenza (EI) has been observed frequently over the last 1000 years, often in association with human influenza epidemics [91], and the general syndrome of influenza-like equine respiratory disease was described as early as Roman times. All equids (horses, mules, and donkeys) are susceptible. The first virus isolate, an A/H7N7 virus, was obtained during an epidemic of respiratory disease in Eastern Europe in 1956 [139], and viruses of this subtype (equine-1) were subsequently isolated from horses in both eastern and western hemispheres, spreading to the USA and UK in 1963. Coincidentally, in 1963 a second subtype, A/H3N8 (equine-2), was discovered [153], and since then equine influenza virus (EIV) has remained one of the most important respiratory pathogens of equids. From 1963 to 1979, the H7N7 and H3N8 subtypes of EIV co-circulated, and occasionally were both isolated from outbreaks of EI [1, 31, 120]. Since 1979 the equine-1 H7N7 viruses seem to have disappeared from horse populations [57, 150, 155], although limited serological evidence suggests that H7N7 viruses may still be circulating at low levels in the equine population of Central Asia [135], Africa [111], and Eastern Europe [1, 85]. In contrast, the equine-2 H3N8 viruses continue to circulate widely, are considered to be enzootic in Europe and the Americas, and horse populations in much of the world have experienced repeated outbreaks.

It is assumed that both of these virus lineages probably originated from avian influenza A virus ancestors but, evolved to become host-specific and genetically distinguishable from avian influenza viruses of the same subtypes [73, 156]. For the equine-2 lineage, HA and NA as well as genes for some internal proteins, including PA, NP, and NS, probably diverged from avian influenza virus ancestors around 1952–1954 [12, 160]. The "internal" genes of the prototype equine-1 EIV (Prague/56 strain) are remarkably primitive, and are believed to be the closest extant examples of the genes of the common ancestor of type A and type B influenza viruses. The equine-1 H7N7 EIV has triggered considerable interest recently due to a study by Worobey and colleagues [160], who found that by using a host-specific local clock model for phylogenetic analysis, in all 8 gene segments except for the NS "B" lineage, the most recent common ancestor of the equine H7N7 and avian influenza viruses probably dates to between the 1830s and the 1870s. These authors remarked on the coincidence of this dating with the massive EI epizootic of 1872, which affected horses across North America, moving from east to west along the route of the railroad lines [69]. That EI epizootic was temporally and geographically associated with a high-mortality poultry epizootic that is suspected of being high-pathogenicity avian influenza [90]. Supporting this notion is the fact that the HA of equine-1 H7N7 virus is the only known example of a stable lineage of the "high-pathogenicity" genotype of HA in mammals, with a polybasic

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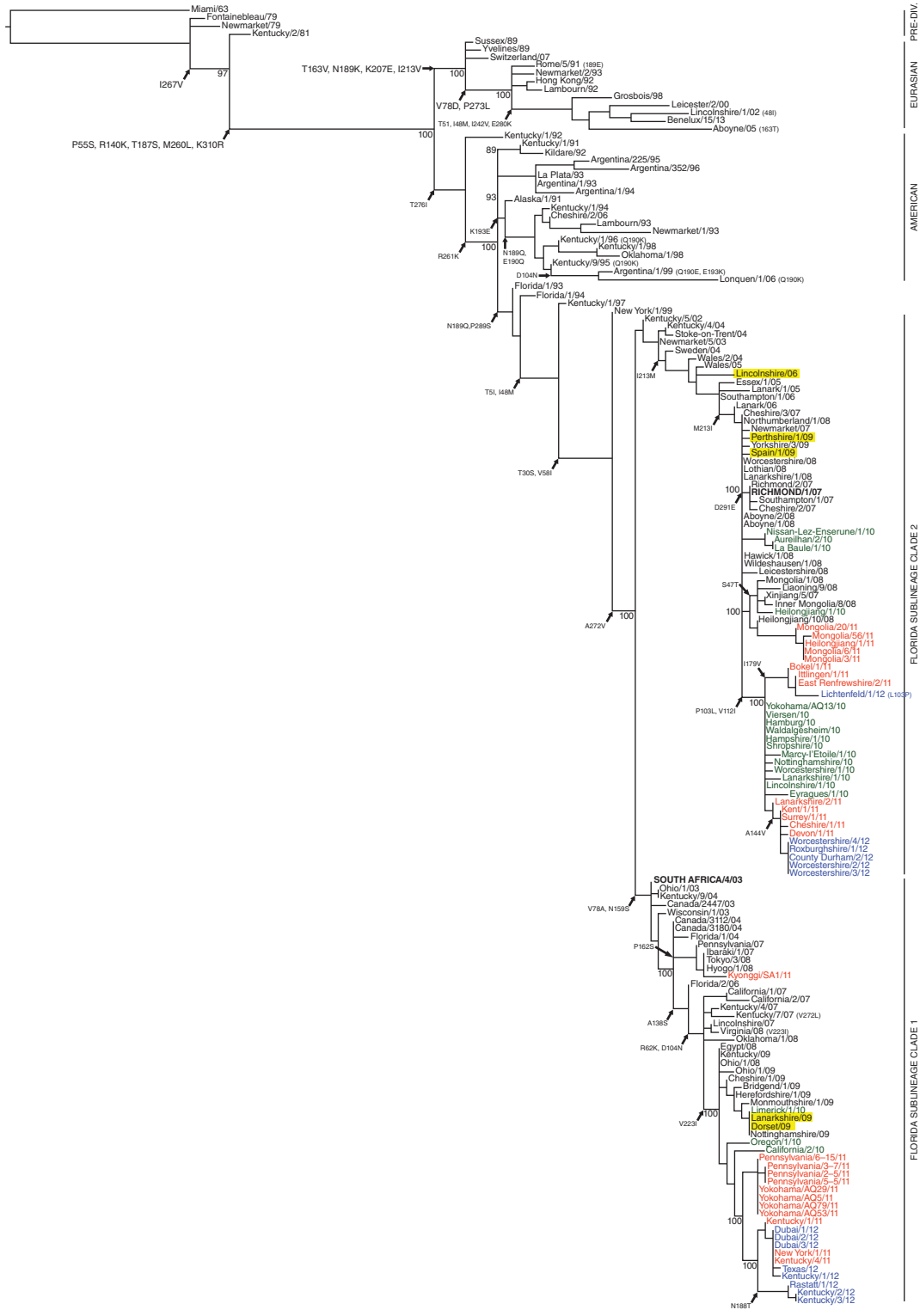
Miami/63
Fontainebleau/79
Newmarket/79
Kentucky/2/81
I267V
97
P55S, R140K, T187S, M260L, K310R
T163V, N189K, K207E, I213V
100
Sussex/89
Yvelines/89
Switzerland/07
Rome/5/91 (189E)
Newmarket/2/93
Hong Kong/92
Lambourn/92
100
V78D, P273L
Grosbois/98
Leicester/2/00
Lincolnshire/1/02 (48I)
Benelux/15/13
Aboyne/05 (163T)
T51, I48M, I242V, E280K
100
Kentucky/1/92
Kentucky/1/91
89
Kildare/92
Argentina/225/95
Argentina/352/96
La Plata/93
Argentina/1/93
Argentina/1/94
T276I
93
Alaska/1/91
Kentucky/1/94
Cheshire/2/06
Lambourn/93
Newmarket/1/93
K193E
100
Kentucky/1/96 (Q190K)
Kentucky/1/98
Oklahoma/1/98
R261K
N189Q, E190Q
Kentucky/9/95 (Q190K)
D104N
Argentina/1/99 (Q190E, E193K)
Lonquen/1/06 (Q190K)
Florida/1/93
Florida/1/94
Kentucky/1/97
New York/1/99
N189Q,P289S
Kentucky/5/02
Kentucky/4/04
Stoke-on-Trent/04
Newmarket/5/03
Sweden/04
Wales/2/04
Wales/05
I213M
Lincolnshire/06
Essex/1/05
Lanark/1/05
Southampton/1/06
Lanark/06
Cheshire/3/07
Northumberland/1/08
Newmarket/07
Perthshire/1/09
Yorkshire/3/09
Spain/1/09
Worcestershire/08
Lothian/08
Lanarkshire/1/08
Richmond/2/07
100
RICHMOND/1/07
Southampton/1/07
Cheshire/2/07
D291E
Aboyne/2/08
Aboyne/1/08
Nissan-Lez-Enserune/1/10
Aureilhan/2/10
La Baule/1/10
Hawick/1/08
Wildeshausen/1/08
Leicestershire/08
Mongolia/1/08
Liaoning/9/08
S47T
Xinjiang/5/07
Inner Mongolia/8/08
100
Heilongjiang/1/10
Heilongjiang/10/08
Mongolia/20/11
Mongolia/56/11
Heilongjiang/1/11
Mongolia/6/11
Mongolia/3/11
I179V
Bokel/1/11
Ittlingen/1/11
East Renfrewshire/2/11
Lichtenfeld/1/12 (L103P)
100
P103L, V112I
Yokohama/AQ13/10
Viersen/10
Hamburg/10
Waldalgesheim/10
Hampshire/1/10
Shropshire/10
Marcy-l'Etoile/1/10
Nottinghamshire/10
Worcestershire/1/10
Lanarkshire/1/10
Lincolnshire/1/10
Eyragues/1/10
Lanarkshire/2/11
Kent/1/11
Surrey/1/11
Cheshire/1/11
A144V
Devon/1/11
Worcestershire/4/12
Roxburghshire/1/12
County Durham/2/12
Worcestershire/2/12
Worcestershire/3/12
T5I, I48M
T30S, V58I
A272V
100
SOUTH AFRICA/4/03
Ohio/1/03
Kentucky/9/04
V78A, N159S
Canada/2447/03
Wisconsin/1/03
Canada/3112/04
Canada/3180/04
Florida/1/04
Pennsylvania/07
P162S
Ibaraki/1/07
Tokyo/3/08
100
Hyogo/1/08
Kyonggi/SA1/11
Florida/2/06
California/1/07
California/2/07
Kentucky/4/07
A138S
Kentucky/7/07 (V272L)
Lincolnshire/07
Virginia/08 (V223I)
R62K, D104N
Oklahoma/1/08
Egypt/08
Kentucky/09
Ohio/1/08
Ohio/1/09
Cheshire/1/09
Bridgend/1/09
Herefordshire/1/09
Monmouthshire/1/09
Limerick/1/10
100
V223I
Lanarkshire/09
Dorset/09
Nottinghamshire/09
Oregon/1/10
California/2/10
Pennsylvania/6–15/11
Pennsylvania/3–7/11
Pennsylvania/2–5/11
Pennsylvania/5–5/11
Yokohama/AQ29/11
Yokohama/AQ5/11
Yokohama/AQ79/11
Yokohama/AQ53/11
100
Kentucky/1/11
Dubai/1/12
Dubai/2/12
Dubai/3/12
New York/1/11
Kentucky/4/11
100
Texas/12
Kentucky/1/12
Rastaff/1/12
Kentucky/2/12
Kentucky/3/12
N188T
PRE-DIV.
EURASIAN
AMERICAN
FLORIDA SUBLINEAGE CLADE 2
FLORIDA SUBLINEAGE CLADE 1

HA1/HA2 cleavage site (R-K-K-R in the prototype Prague/56 strain) and efficient HA cleavage in cell culture in the absence of exogenous trypsin. Equine-1 viruses are highly pathogenic in Balb/c mice without adaptation, and an avian influenza virus reassortant bearing the equine-1 H7 HA is highly pathogenic in chickens [8, 72]. Worobey and colleagues have speculated that the 1872 poultry epizootic is an epidemiological marker for a "global sweep" or replacement of all avian influenza virus internal genes (except for the NS "B" lineage). Furthermore, the unusually high uracil content of equine H7N7 viral genes suggests that the equine H7N7 lineage circulated continuously in horses from 1872 [160].

Between 1964 and 1973, while H7N7 and H3N8 EIV were co-circulating, a one-way reassortment event (or more than one such event) replaced most of these primitive equine-1 internal genes (except for M) with their more modern equine-2-derived counterparts [11, 68, 100]. The avian influenza ancestry of EIV is supported by the emergence in 1989 of a novel influenza H3N8 virus in horses in northern China, a strain (Jilin/89) that was antigenically and genetically distinct from other circulating EIVs. Phylogenetic analysis of this virus showed that it had evolved independently of the existing equine-2 lineage. Its genetic features were entirely of avian lineage, indicating that the virus had spread directly to horses from the avian reservoir without genetic reassortment [59]. Although that virus infected over 20 000 horses and caused around 400 deaths, it did not persist, and was last isolated in 1990. This demonstrated that equids are susceptible to some avian influenza viruses, including those of the H3N8 subtype. Other avian influenza subtypes are replication competent in equine tracheal epithelium, but of the few that have been tested by experimental aerosol infection of ponies (H1N2, H6N2, and H7N2), none produced detectable replication or disease signs [26].

The rate of genetic mutation of EIV is low compared with that of human-lineage influenza A viruses [12, 39, 44, 100], at around 1.8×10^{-3} nucleotide and 1.4×10^{-3} amino acid substitutions per site per year in HA1, compared with rates of 4.6×10^{-3} nucleotide and 6.0×10^{-3} amino acid substitutions per site per year in human H3 HA. Nevertheless, EIVs undergo significant genetic and antigenic evolution. HA has been well studied, due to its importance for effective vaccination (see Chapter 21). Prior to the late 1980s, equine-2 HA apparently evolved in a single lineage [73], but subsequently diverged into two distinct lineages, known as the Eurasian and American lineages [39]. Initially the circulation of both lineages centered largely on their geographic origin [99], but the introduction and maintenance of American-lineage EIV in horses in Europe resulted in the co-circulation of both lineages in Europe, whereas in contrast there has been only a single isolation of European-lineage virus in North American horses [22, 40, 79]. Subsequently, continued genetic divergence of HA of EIV belonging to the American lineage resulted in the formation of three distinct sub-lineages, namely the South American, Kentucky, and Florida sub-lineages [79]. Around 2002, HA of the Florida sub-lineage further evolved into two antigenically distinguishable groups, referred to as Florida sub-lineage clades 1 and 2 (Figure 20.1). Murcia and colleagues [100] grouped the evolution of EIV HA into 12 clades, of which Florida clades 1 and 2 are the most recent. They also found that EIV NA and internal genes have evolved in parallel with HA, with corresponding

Figure 20.1 Phylogenetic tree of EIV H3N8 HA1 nucleotide sequences. This maximum-likelihood tree was generated using PhyML version 3. Bootstrap values obtained after 100 replicates are shown at major nodes. Amino acid substitutions are shown in parentheses or indicated at branch points. Phylogenetic groups (Pre-divergence, American lineage, Eurasian lineage, and Florida sub-lineage clades 1 and 2) are shown on the right. Sequences are color coded by date of isolation for the years 2010 (green), 2011 (red), and 2012 (blue), with the older isolates shown in black. The present OIE-recommended representative vaccine strains A/eq/Richmond/1/07 and A/eq/South Africa/4/03 are shown in bold. Reassortant strains that were identified containing HA from one Florida clade and NA from the other are highlighted in yellow. Reprinted from Woodward A. L. *et al.*, Development of a surveillance scheme for equine influenza in the United Kingdom and characterisation of viruses isolated in Europe, Dubai and the USA from 2010–2012. *Veterinary Microbiology* 169:113–127, 2014, with permission from Elsevier. We thank Dr. Adam Rash for providing the figure. *See Plate section for color representation of this figure*.

lineages and clades, along with evidence of reassortment between clades. While clade 1 viruses predominate in America (and indeed there have been no Florida clade 2 detections in the USA in recent years), clade 1 EIVs have also spread to and caused outbreaks in Europe [22, 23, 53], Australia [154], Africa [75], and Asia [164]. For instance, the viruses isolated from a South African outbreak in 2003, and from a 2007 outbreak in Japan which spread to Australia, were probably of North American origin [22, 154]. In contrast, viruses belonging to the Florida sub-lineage clade 2 have been isolated in Europe [80, 159], parts of Africa [78], and Asia [13, 170, 171]. The impact of this genetic and antigenic evolution on vaccines is discussed in Chapter 21. Another feature of EIV evolution has been "frozen evolution" – that is, the occasional detection of anachronistic viruses, apparently circulating years or decades after their first isolation [18, 19, 44, 86]. These have been unexplained, although laboratory escape is clearly a possibility, and fortunately (as it would confound vaccine updating) these anachronistic viruses do not seem to have persisted for any length of time.

Despite intensive vaccination campaigns in many industrialized countries of the world, severe EI epizootics continue to occur sporadically and highlight the continued threat that EIVs pose to the health of equids worldwide. For example, towards the end of the twentieth century, significant EI outbreaks were reported within a few years of each other in India [149], the People's Republic of China [59, 60, 134], and South Africa [62], where EIVs were not known to be circulating. In India and China, where there is little control, it seems likely that EIVs circulate continuously at low levels, only flaring into large-scale outbreaks when a new antigenic variant appears, whereas the virus that triggered the 1987 South African outbreak was most probably introduced by importation of infected horses from the USA or Europe [74]. In late 2003, a second major outbreak occurred in South Africa, and this outbreak was thought to be due to a combination of an infected, albeit vaccinated, imported horse from the USA and a breakdown in biosecurity measures allowing virus to spread by fomite transmission [61]. In the same year, an outbreak that affected generally well-vaccinated horses occurred in the UK, with infection being confirmed in at least 12 locations and in at least 21 training yards in Newmarket [9, 105]. Since then, substantial EI outbreaks have occurred in South Africa [61], India [152], Japan [164], and, for the first time, in Australia [154]. Like the South African outbreak in 2003, the outbreaks in Japan and Australia are also believed to have been caused by importation of subclinically infected vaccinated horses, from the USA into Japan (the Ibaraki/07 strain) and shortly thereafter from Japan into Australia (the Sydney/07 strain) [25, 154, 164]. In Sydney, upon entering the country the horses were placed in quarantine and remained there, but it was determined that most probably a breakdown in quarantine protocols had allowed fomite transmission of virus outside the quarantine compound, and subsequent spread of virus into the horse population of Australia [25]. Approximately 70 000 horses in New South Wales and in south-eastern Queensland were infected during the course of the outbreak [25, 137]. Promptly initiated control measures, consisting of stringent control of horse movement, quarantine of affected and suspect premises, targeted vaccination, active surveillance, and on-farm biosecurity measures (personal hygiene, equipment hygiene, and access to control measures), were successful in confining the outbreak to these two states [47]. Importantly, these stringent control measures led to the successful eradication of EIV and resulted in Australia regaining its EIV-free status in December 2008 [51, 131].

Economic implications

Even with the availability of efficacious vaccines, EI has remained an economically important disease of equids. In industrialized countries, EI is often managed by vaccination, by implementing movement restrictions on affected animals (quarantine), by resting of affected animals, and, when necessary, by providing supportive medical care. In many other parts of the world, however, horses, donkeys, and mules continue to have a major role as working animals, and influenza virus outbreaks can have severe socio-economic impacts in these countries [134].

Costs associated with EI infections are primarily caused by loss of use of the animal (due to illness and/or quarantine), medical costs (associated with medical treatment, vaccinations, diagnostic testing, and outbreak management), and, rarely, by

the loss of the affected animal due to EI-related complications. Although it is difficult to find reliable data on the financial impact of EI, two recent studies have estimated the costs associated with a small-scale and a large-scale EI outbreak. Von Seeh and colleagues recently published data collected during a small EI outbreak that occurred in 2009 at a boarding facility housing 35 horses [132]. Only five of these horses had recently received an influenza vaccine, and none of the vaccinated horses developed clinical signs of influenza infection despite direct contact with infected stable mates. In contrast, 18 of the non-vaccinated or poorly vaccinated animals developed clinical signs of EI, and ten of these animals required veterinary care. The average cost of veterinary medical care provided was approximately US$450 per horse [132]. This estimate did not include loss of use of the horses housed on the affected premises during the imposed quarantine period. Interestingly, the average cost of vaccination, including the vaccine cost as well as the veterinary visit (call fee, exam fee, procedure fee, and administrative fee) was estimated to be approximately US$60 [132].

An analysis published by Smyth and colleagues described the approximate costs to both the government and the horse industry in Australia incurred by the emergency response aimed at containing and eradicating the virus during the 2007 Australian outbreak [137]. The financial costs of the outbreak included the costs of the emergency response measures and lost income due to interruptions to horse movements and horse events. Assistance packages (e.g. employment assistance, wage supplements) provided by the Australian government to the horse industry amounted to over A$263 million (Australian dollars) in total. In addition, the government provided A$97.7 million to cover costs arising from the emergency response itself. The overall economic impact on the horse industry between August and December 2007 was estimated to be A$381 million (representing a weekly loss of A$21.2 million). This figure was calculated by summing the estimated economic impact of the outbreak on household income, business income, costs to local business, horse association losses, veterinary expenses, and animal losses due to illness and death. Although only 1622 (12.5%) of 13 004 infected horses captured in a survey conducted by the Australian Horse Industry Council required veterinary care, the veterinary costs for these horses totaled A$733 400. Based on data from the same survey, the mortality rate was estimated to be 5% (79 deaths were attributed to EI), and the total value of horses lost due to EI infection was reported to be A$945 000 [137].

Although these figures are staggering, the potential social impact of any infectious disease outbreak should also not be underestimated. In developing countries where working equids are still widely used as beasts of burden, equine disease outbreaks – including EI – can potentially cost human lives. Morens and Taubenberger [91] relate how the massive EI epizootic of 1872 was partly responsible for the severity of the Great Boston Fire that largely destroyed the city. This was one of the most costly fires in history because the Fire Department's horses were incapacitated and fire wagons had to be drawn by hand and were therefore severely handicapped. A localized disease outbreak in Hong Kong in 1992, which affected 352 racehorses (around 25% of the horse population), cost the city of Hong Kong 10% of its tax revenue for the duration of the outbreak, and this had to be made up later [121]. The 2007 Australian EI outbreak disrupted numerous horse races, performance and pleasure events, and also qualification and selection events for the 2008 Beijing Olympic Games [66]. In addition, Taylor and colleagues documented the substantial psychological distress experienced by horse owners during the 2007 Australian EI outbreak [143], particularly younger horse owners, whose principal source of income was from horse-related industries, as well as those with a lower level of formal education. Interestingly, despite the fact that the prevalence of severe psychological distress was higher in areas where infection had been reported (i.e. New South Wales and Queensland), horse owners nationally experienced increased levels of psychological distress [143].

Transmission to other mammalian hosts

Prior to 2004, the horse was viewed as an isolated or "dead-end" host for influenza A viruses, as cross-species transmission of EIV had been documented in only a handful of reports. For example, experimental infection of human volunteers with

equine H3N8 virus resulted in influenza-like illnesses associated with virus shedding and subsequent seroconversion [34, 70], although the virus was only moderately infectious and quite weakly pathogenic. Conversely, occasional human-to-equine transmission of H1N1, H2N2, and H3N2 viruses has been reported [64, 148], and experimental infection of horses with human H3N2 viruses demonstrated their susceptibility to infection with human viruses [70]. Despite these reports, as well as reports from the twelfth to the nineteenth centuries of repeated association between equine and human outbreaks of "influenza" [91], since the start of the modern virological era there has been no evidence that either horse-to-human or human-to-horse transmission routinely occurs under natural conditions. Serosurveys of individuals who are exposed to horses have found only infrequent and low-titer positive results [24] (K. R. Leedom Larson, personal communication, 2009), and the only published report of a naturally occurring human infection with EIV was not supported by a typed virus isolate [14].

However, the notion of the horse as an isolated, "dead-end" ecological niche for influenza A viruses has been undermined by the transmission of H3N8 EIV to dogs in the USA [36, 116], the UK [38, 104], and Australia [76]. In contrast to previously reported sporadic infections of dogs with human influenza viruses that resulted in neither clinical disease in dogs [15, 16, 30, 67, 144] nor virus spread among dogs [109], infection of dogs with equine H3N8 virus has been associated with clinical signs of respiratory illness, including fatal pneumonias [36, 38, 76, 104]. In addition, recovery of virus from dogs from across the USA indicates spread of virus, and supports the apparent maintenance of the equine-lineage H3N8 virus within the canine population of this country [36]. In addition to these naturally occurring cross-species transmission events, EIV was also reported to have spread from an experimentally infected horse to a dog that was housed in the same stall [163], and experimental inoculation of dogs with H3N8 EIV resulted in nasal virus shedding and subsequent seroconversion [117].

In the USA, the emergence of the existing canine influenza virus lineage is thought to have occurred following a single whole-virus H3N8 equine-to-canine transmission event in around 2004 [36]. However, serological evidence [2] suggests that a canine influenza-like virus was circulating in racing greyhounds in the USA before 2004, and possibly from 1999. As several additional equine H3N8 transmission events have been documented since the early 2000s, it has been postulated that genetic evolution of viruses of the H3N8 equine lineage resulted in mutations that facilitated infection of canine respiratory cells by these "mutant" equine viruses. This hypothesis appears to be supported by the recent finding [56] that infection of canine tracheas with a 2003 H3N8 EIV isolate (A/equine/South Africa/2003) mimicked the infection and replication characteristics of a canine-lineage H3N8 virus. In contrast, infection of canine tracheal explant cultures with two equine H3N8 viruses isolated in 1963 (A/equine/Miami/1963 and A/equine/Uruguay/1963) showed reduced replication efficiency, and the viruses were less pathogenic [56]. In evolutionary terms, canine H3N8 influenza viruses are most closely related to the equine Florida clade 1 sub-lineage, and their HAs are distinguishable from the HA of the equine H3N8 viruses by five amino acid mutations (N54K, N83S, W222L, I328T, and N483T) [36, 127]. The tryptophan (W) to leucine (L) substitution at residue 222 located near the receptor-binding pocket is of particular interest, as it influences the binding specificity of the HA protein for receptor analogs. Interestingly, in this regard, several studies show that equine and canine isolates show preferential binding to sialic acid (SA)α2,3-gal [32, 38, 117, 142], which is mirrored by a predominance of SAα2,3-linked residues throughout the canine and equine respiratory tract [38, 117, 142]. However, in the tracheal epithelium of horses the predominant moiety is N-glycolyl SAα2,3-gal [142], instead of the N-acetyl SAα2,3-gal found in avian species. Interestingly, too, Yamanaka and colleagues [168] reported that whereas EIVs display a clear binding preference for the N-glycolyl SAα2,3-gal receptor moiety, canine H3N8 isolates did not appear to show a preference for N-glycolyl SAα2,3-gal, and one canine strain tested showed reduced binding to the N-glycolyl form.

In view of these similarities in receptor-binding preference, one might expect that H3N8 viruses would also spread in the opposite direction – that

is, from dog to horse. Intriguingly, independent studies found that two distinct isolates of H3N8 canine influenza viruses were unable to infect, replicate, or spread among influenza-naive equids [124, 168]. Moreover, inoculation of horses with these canine isolates did not result in clinical disease in either of these studies. These findings suggest that factors other than receptor-binding preference are likely to contribute to the species specificity of canine and equine H3N8 influenza viruses. This notion is supported by a recent study of the structural consequences of amino acid substitutions between canine and equine H3N8 viruses, which indicated that a single amino acid substitution (T30S) in the fusion subdomain of recent H3N8 canine and equine influenza virus HAs may modulate HA stability or membrane fusion activity [32]. This substitution apparently arose in EIV in 1999, coincidentally the same year to which serological studies have traced the possible emergence of canine influenza in the USA [2]. Another possibility is that the canine-specific I328T substitution at the HA1/HA2 cleavage site may be involved in species-related protease recognition of that site [32]. It is therefore possible that a combination of mutations, such as an altered receptor-binding specificity and membrane fusion, may be necessary for successful interspecies transmission between horses and dogs.

EIVs have also been isolated from pigs during swine influenza surveillance in China [147]. The affected pigs showed signs of respiratory disease, including coughing and depression. Sequencing of the virus isolates revealed that these were closely related to true EIV (98.6–99.5% identity), but with amino acid changes in all gene segments, including two changes in HA (W222L and I328T) that are characteristic of the canine influenza viruses. Whether canines or equids (or another species) were the source of virus introduction into these swine is not known. Once such viruses enter swine, could further adaptation allow an EIV-derived virus to be transmitted to humans? It is clear that the potential role of equids in the global ecology of influenza A viruses is not yet fully understood.

Clinical features of infection and disease

The clinical presentation of EI infection has been extensively described elsewhere [52], and has remained unchanged since its first description more than 40 years ago. In a group of susceptible horses, EI is characterized by the rapid spread of an acute, febrile respiratory illness that is accompanied by a dry hacking cough and nasal discharge (Figure 20.2). The incubation period is short, and

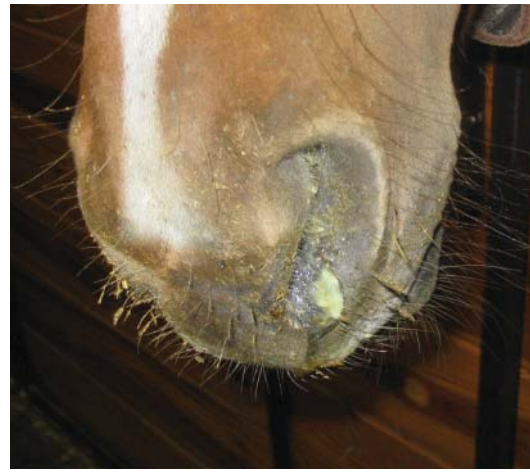

Figure 20.2 Equine influenza virus-infected horse exhibiting typical mucopurulent nasal discharge. Experimental infection with A/equine/Ohio/2003 (H3N8) virus, 5 days post infection. The horse was not febrile at this point, but had a cough and was still shedding detectable virus. Some horses in this study developed secondary spikes of pyrexia, and mucopurulent nasal discharge persisted as late as 9 days post infection. Photo courtesy of Thomas Chambers. *See Plate section for color representation of this figure*.

clinical signs as well as nasal virus shedding can be detected as early as 24–48 hours after exposure to an infected horse or following experimental infection [37, 65, 138, 145]. The disease is rarely fatal, but deaths have been reported during some epidemics, particularly in donkeys and, rarely, in neonatal foals [1, 54, 118]. Although influenza morbidity rates may be as high as 60–90% in susceptible populations, mortality rates are usually less than 1% [60]. An exception was the 1989 influenza outbreak in the People's Republic of China that was caused by an avian-lineage H3N8 virus, and was associated with a mortality rate of 20% in some herds [59]. Morbidity rates within large groups of horses with varying degrees of previous exposure to influenza antigen may range from 20% to 37% [93, 121].

The typical clinical presentation of EI consists of pyrexia, anorexia, lethargy, nasal discharge, and cough. Pyrexia is often the first symptom to be manifested, with body temperatures that can occasionally exceed 41°C. Following experimental inoculation, a first peak of fever is often observed at 48–96 hours after infection, with a possible second peak occurring between days 4 and 7 after infection. Nasal discharge is usually serous during the first few days of illness, but may become mucopurulent by 72–96 hours after infection (Figure 20.2). Concurrently, a dry cough develops. In a similar manner to that in other species, coughing tends to persist long after pyrexia and nasal discharge have resolved, and horses may still be coughing 3 weeks post infection. Submandibular and retropharyngeal lymphadenopathy is a variable but common finding.

Most affected horses become anorexic at the time of the initial pyrexia (donkeys may be an exception), although this typically resolves within 1–2 days. As a consequence, weight loss is well documented in horses with EI infection. These negative effects of EIV on the well-being of the horse can be significantly exacerbated by even moderate exercise [106]. Hemogram abnormalities include a normocytic, normochromic anemia and leukopenia. Leukopenia may be due to both a neutropenia and lymphopenia. Monocytosis during early convalescence is a variable finding. Uncomplicated cases of equine influenza resolve within 7–14 days post infection, although coughing may persist for 21 days.

In severe infections, tachypnea with concurrent detection of abnormal thoracic auscultation findings and ultrasonographic evidence of pulmonary consolidation may be detected. Pneumonia is a common consequence of severe infection, especially when complicated by bacterial co-infection, and typically occurs 7–14 days after infection (Figure 20.3) [58]. In adult horses, most fatalities are probably due to secondary bacterial pneumonia. As virus replication leads to cell death [81, 82, 130] and loss of the the ciliated respiratory epithelium in the trachea and bronchial tree [158], reduced mucociliary clearance [31, 52, 157], as well as the disruption of the superficial layers of

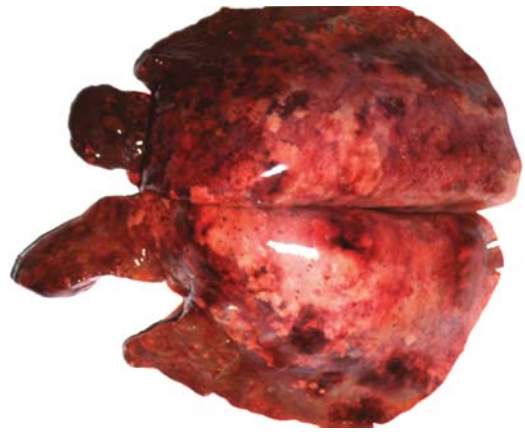

Figure 20.3 Acute severe bronchointerstitial pneumonia with edema and hemorrhage in a horse with equine influenza A virus infection and secondary bacterial infection. Photo from Noah's Arkive, University of Georgia. *See Plate section for color representation of this figure*.

the respiratory epithelium predisposes the affected horse to the development of secondary bacterial infections [158, 161]. Secondary bacterial infections of the airways are largely due to proliferation of β-hemolytic streptococci [71, 83, 92, 108, 128], and are considered important in the pathogenesis of bacterial pneumonia of horses [3, 4, 10, 98]. Myositis, myocarditis, and limb edema have also been described as potential sequelae of EI infection [52, 120, 157]. Furthermore, it has been speculated that EI infection may predispose horses to the development of inflammatory airway disease, recurrent airway obstruction, or exercise-induced pulmonary hemorrhage [52, 120, 158]. Finally, neurological deficits were noted in two horses during the 2003 outbreak in the UK [42]. Necropsy results for one of these horses revealed the presence of a non-suppurative encephalitis. Although influenza-associated encephalopathy is an uncommon complication, it has been reported to occur in humans, particularly in children [103, 141]. The pathogenesis of influenza-associated encephalopathy is unclear. As the virus is rarely detected in the central nervous system, direct neuroinvasion by influenza is not thought to be the cause of the encephalopathy [141].

Epidemiology

EI is the most frequently diagnosed and economically important cause of viral respiratory disease of the horse throughout most parts of the world [93, 96, 133]. With the exception of New Zealand, Iceland, and Australia, EIVs are thought to be enzootic in horse populations throughout large parts of the world. In contrast to the seasonality of human influenza, outbreaks of EI can occur at any time of year, although seasonal outbreaks have been reported [93, 107]. All ages and breeds are susceptible to infection [63, 110, 152]. However, although infections of foals with EI have been described [118], large outbreaks are an uncommon occurrence in this age group [136]. The highest incidence of disease is found in young horses [92, 157]. This is probably due to commingling of young animals that lacked previous exposure (e.g. at racetracks and sales barns) [92]. Outbreaks of EI occur most often when susceptible animals are congregated and kept in close contact with each other, and the disease is of greatest economic importance in large populations in enzootic areas where horse movements and commingling are common practice.

Spread of virus among susceptible animals is thought to occur through three modes, namely direct physical contact with infected animals or virus-contaminated fomites, droplet transmission (contagious droplets more than 10 μm in diameter and capable of being projected over moderate distances by coughing and sneezing), and aerosol transmission (droplet nuclei less than 5 μm in diameter, and capable of being widely disseminated and reaching the lower respiratory tract) [20]. However, although aerosol transmission unquestionably has the greatest impact with regard to infection control, the importance of bio-aerosol transmission in the spread of influenza A virus has not been well characterized. Human studies show that influenza virus can remain infectious for days on contaminated surfaces, with the exact duration being dependent on the prevailing environmental conditions (e.g. humidity, temperature, exposure to sunlight) [17]. Meteorological factors that have been associated with an increased risk of spread of equine influenza virus between premises during the 2007 Australian equine influenza outbreak were relative humidity of less than 60% and wind speeds of greater than 30 km/hour from the direction of a barn containing infected horses [46]. If susceptible animals are in close contact with each other, disease may spread rapidly (anecdotal evidence suggests that spread may occur within as short a period as 2–3 days). In larger populations with varying levels of immunity, disease spread can be considerably slower, and disease outbreaks may last for several weeks [93].

EI outbreaks in naive populations present a different picture from those that occur in horse populations in enzootic areas. Investigations of the 2007 outbreak in Australia have provided new information on the epidemiology of the disease in naive populations [35, 45, 48, 89]. Overall, the outbreak followed a classic epidemic curve, peaking at 6 weeks and lasting for 18 weeks. Early in the outbreak, spread across long distances was associated with transport of infected horses [35, 45, 48]. Local spread was due to direct contact, to spread via respiratory secretion droplets generated by coughing or indirect contact (fomites and human assisted)

[35, 45, 63, 77, 89], and to an unknown contribution of aerosol spread [45, 88]. The most important risk factor for virus introduction was proximity to the nearest infected premises [47]. After virus introduction, clinical signs of disease were observed in 75–100% of horses on infected properties within 5–9 days [63, 95]. Mares on thoroughbred studs were most severely affected [63], whereas foals and yearlings generally showed only mild disease signs. Despite one cluster of fatalities in foals, deaths due to EI were extremely uncommon [54]. Disease spread across the country was prevented by enforced movement restrictions, cancellation of equestrian events, and biosecurity measures [35, 77, 89]. The impact on disease occurrence of vaccination, which was implemented 6 weeks after the start of the control program and after the epizootic had reached its peak, has not been precisely determined [35]. Modeling indicated that starting vaccination earlier (1 week into the control program) would have reduced new infections by 60% [50]. However, it has been established that strict biosecurity measures were associated with a significant decrease in the likelihood of a premises becoming infected [47]. For example, the use of footbaths prior to introduction of infection onto the premises was associated with a fourfold reduction in the risk of infection [47].

The modeling of EIV outbreaks has been recently reviewed [41]. Early models using SEIR (susceptible–exposed–infectious–recovered) methodology produced estimates of the basic reproduction number R_0 of 10.89 in an unvaccinated population, with a latent period of 1.25 days and an infectious period of 5.5 days [55]. Vaccination reduced both R_0 and the infectious period considerably, as expected. Other studies [e.g. 113] have found more conservative values for R_0 of around 2–5. These models have shown that factors including viral antigenic drift and vaccine mismatch, heterogeneity in antibody titers and presence in a herd of low responders, the timing of vaccination in relation to exposure, and vaccination in the face of an outbreak all affect the outcome [5, 113–115].

Diagnosis, treatment, and control

Diagnosis of EIV has two main stages, namely presumptive diagnosis on the basis of typical clinical signs, and laboratory confirmation by testing of diagnostic specimens. A third stage, prospective diagnosis as part of active surveillance, features mainly in the horse importation programs of the influenza-free countries. The value of prospective diagnosis is that it may detect horses that are subclinically infected. A horse that had been vaccinated several months previously may have partial immunity that is sufficient to prevent or minimize disease signs, but insufficient to prevent virus shedding and contagion. Presumptive diagnosis is of value to the horse owner or manager because it enables control measures to be implemented, such as isolation and supportive care, that are generally appropriate regardless of the correctness of the diagnosis. However, for epidemiological purposes its value is limited because other equine infectious agents, notably equine herpesviruses, can produce disease signs that are similar enough to those of EI to sometimes mislead experts. Laboratory confirmation of a diagnosis of EI is therefore essential for accurate epidemiology, but not so necessary for the horse owner or manager. Difficulties associated with laboratory confirmation can result in many EI cases never receiving laboratory confimation. Such difficulties include the following:

1. There is no existing treatment option specific for EI, so owners and practitioners may adopt the attitude that "there is no need to know."
2. In many countries, including the USA, EI is not a notifiable disease.
3. The preferred diagnostic specimen is the nasopharyngeal swab, and many horses strongly object to this procedure. As a result there is often little incentive for practitioners to collect these specimens.

Serological methods are briefly discussed in Chapter 21. Serology-based diagnosis is retrospective and of little use at the time of an outbreak. Nasopharyngeal swabbing of horses has been recently described in detail [28]. The quality and timeliness of the swab specimen are of critical importance for the accuracy of diagnosis, but these factors are also the most difficult to ensure or assess. The swab specimen can be tested for EIV by a variety of methods, including most of those used for human, swine, or avian influenza. The classic method consists of inoculation of embryonating chicken eggs or Madin–Darby canine kidney (MDCK) cell culture to test for virus growth

[27], which is too time-consuming to be useful for disease control. A variety of influenza rapid detection kits developed to detect, for example, NP antigen of human or avian influenza, are satisfactory for detecting EIV [29, 87, 166]. Thus, in principle, stall-side diagnostic testing is possible, although this has not yet been widely adopted. An NP-ELISA is also in use [33], which is more sensitive than the rapid detection kits. The reverse transcriptase–polymerase chain reaction (RT-PCR) has become the method of choice in many diagnostic laboratories, and has been demonstrated to be the most sensitive technique [122]. A number of PCR-based methods for EIV detection have been reported [e.g. 6, 43, 84, 112, 123]. Moreover, PCR methods for other influenza A viruses have been successfully applied to EIV detection [e.g. 49, 151]. In the USA, the Spackman protocol [140] is widely used by veterinary diagnostic laboratories. Recent technological advances, such as isothermal amplification, are also now being applied to EIV detection [7, 101, 102].

Treatment of EIV infections of horses consists of stall rest and supportive care, including the use of anti-inflammatory, anti-pyrexic, analgesic agents such as phenylbutazone or flunixin meglumine [119]. Antibiotics may be used to combat secondary bacterial infections and inhibit the development of pleuropneumonia. Antiviral agents have been studied for use against EIV in horses. Amantadine and rimantadine both inhibit equine H3N8 virus replication and significantly reduce virus shedding in experimentally infected horses [21, 125, 126], but massive doses are required due to the low bioavailability and rapid excretion of these agents. Oseltamivir is also effective for reducing pyrexia and virus shedding [165, 167], although the required dosage (2 mg/kg at least twice a day, but three times a day is better) [169] may still be prohibitively expensive. Peramivir, another neuraminidase inhibitor, has also been studied. When administered intravenously (7.8–9.3 mg/kg body weight) to horses that had been experimentally infected with EIV on the first day of pyrexia, peramivir significantly reduced the severity and duration of pyrexia and other clinical signs compared with those observed in saline-treated controls. Furthermore, a single dose of peramivir was sufficient, as plasma concentrations at 36 hours post administration were still more than 30 times higher than the measured *in-vitro* virus-inhibitory concentration [162]. However, so long as these agents remain first-line antiviral choices for use in humans, they are unlikely to be approved for veterinary use.

Control of EIV infection often relies heavily on the use of vaccines. In addition to immunoprophylaxis, the risk of introducing virus onto a premises can be substantially addressed by husbandry procedures. If premises were to use the same procedures as described in the OIE *Terrestrial Animal Health Code* (www.oie.int) for qualifying as influenza-free countries, it is likely that the introduction of EIV onto premises could be avoided. In other words, new or returning horses should be isolated for 4 weeks before entering the resident population. During their stay in quarantine, no clinical signs of EI should be detected and no additional horses should be introduced into the quarantine facility. Finally, the new or returning horse should be fully vaccinated prior to entering the quarantine facility. Virological testing at the beginning of the quarantine period may also be undertaken in order to increase the level of confidence in the animal's influenza virus-free status.

Unfortunately, these measures can be challenging to implement for many barn owners. Therefore the control of EI often remains heavily dependent on vaccination. Other husbandry procedures, including segregation of horses into smaller groups, can be another valuable tool for control of the spread of virus through a population. Segregation may allow for potential containment of disease, as infection moves more slowly through facilities where horse populations are separated [93]. In contrast, shared equipment, such as grooming tools and tack, increases the risk of infection [94]. Monitoring of the immune response to vaccination by serology has been reported to be useful for prevention and control of EI outbreaks [107, 146]. In large equine populations, routine surveillance for EIV can provide the opportunity for early detection of outbreaks and also for detection of new virus strains that may not be controlled by existing vaccines [97].

Additional measures that can be useful in the control of an EI oubreak are highlighted by data collected during the Australian outbreak. For example, the use of footbaths reduces the level of risk, as discussed earlier [47]. Studies have shown

that farm owners who believed that these measures were effective were more likely to demonstrate a high level of compliance with the recommended biosecurity measures [129], and horse owners who received infection control information from a veterinarian (as opposed to receiving this information from the media, government sources, other horse owners, etc.) were more likely to perceive such biosecurity measures as effective [129].

Vaccination for EIV is discussed in Chapter 21.

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21 Vaccines and vaccination to control equine influenza

Thomas M. Chambers

Goals of vaccination

The essential goal of vaccination is protection from disease. For owners of equids and for those who depend upon equids for their day-to-day livelihood, only protection from disease is important. There are, however, a variety of factors that affect the use of vaccines and some strategic goals.

Horses travel internationally, as do their diseases. There are several examples of the introduction of equine influenza viruses (EIVs) into naive equine populations which resulted in high morbidity and mortality and severe disruption of equine-related commerce, even though the viruses themselves were not unusual in their pathogenicity under experimental conditions. Countries differ in their regulations with regard to the vaccination status of imported horses. Those countries and regions which are believed to be free of equine influenza (e.g. New Zealand, Australia, South Africa, Hong Kong) have stringent requirements for recent booster vaccination of imported horses, whereas countries such as the USA where equine influenza is considered enzootic often have no such requirements. Furthermore, it has been confirmed that some past outbreaks resulted from introductions of live virus in imported horses that had been vaccinated. Horses with partial immunity can become subclinically infected although still shedding virus, and may be unrecognized sources of contagion. Thus a theoretical goal of vaccination is the achievement of "sterile" immunity (i.e. prevention of infection and virus shedding as well as prevention of clinical signs).

Young foals, especially newborns, lacking antibody protection are at high risk of morbidity and mortality from equine influenza infection. Therefore another goal of vaccination is to boost antibody titers in pregnant mares, so that maternally acquired antibodies will protect their foals. The American Association of Equine Practitioners (AAEP) recommends vaccination or boostering of pregnant mares to be completed by 4–6 weeks before foaling (www.aaep.org/-i-166.html) [132]. Maternal antibodies are effective, as has been demonstrated in countries where equine influenza is enzootic and mares are well vaccinated, and foals rarely contract influenza. The horse has a six-layered placenta, so maternal antibodies are transferred exclusively via colostrum, and foals that fail to ingest sufficient colostrum (due to failure of passive transfer) are at risk.

When equine influenza is diagnosed at a farm or track with many horses, practitioners sometimes elect to vaccinate non-affected horses in order to boost their immunity in the face of the outbreak. There is evidence that this can be effective if the horse has already had a primary course of vaccination (i.e. is not naive), and if the interval between booster vaccination and exposure to infecting virus is sufficient to permit new antibody production (about 7 days) [7]. An extension of this concept is "ring vaccination" to prevent an outbreak from spreading. This was part of the containment strategy adopted by the government of Australia to limit the 2007 equine influenza outbreak and ultimately eradicate the virus [106].

Herd immunity refers to the concept that if a sufficient proportion of a population is immune to an infectious disease, the disease agent is less likely to find a susceptible host and so less likely to penetrate into the herd at all. In the case of equine influenza,

Animal Influenza, Second Edition. Edited by David E. Swayne.

evidence suggests that if 70% of an equine herd are fully immune, this may be sufficient to confer herd immunity [6].

Criteria and models for measuring efficacy and protection

Licensure of EIV vaccines requires demonstration that they are "fit for purpose" – that is, safe for use in horses and efficacious against disease and infection caused by EIV. In the first decades of the history of EIV vaccines, demonstration of efficacy could be based on immunogenicity (i.e. horses that received the vaccine would seroconvert). Currently, in both the USA and Europe, the regulatory authorities – the US Department of Agriculture/Veterinary Services (USDA) and the European Medicines Agency (EMA) – have accepted the validity of experimental-challenge studies comparing vaccinated and unvaccinated horses. The standard, as described in the European Pharmacopoeia [3], is that there are statistically significant differences in clinical signs and virus excretion between vaccinates and controls. There is no specific requirement for an allowable threshold in either category [135]. Thus vaccines are labeled as reducing clinical signs of disease, not preventing them, and/or reducing EIV replication and shedding. For a variety of reasons that will be discussed later in this chapter, EIV vaccines that are highly efficacious under experimental conditions are likely to have more limited effectiveness in the field.

Experimental challenge of vaccinated horses with wild-type (*wt*) virus is required for new products, or for changes in the vaccine technology or manufacturing process. Under the relevant USDA guideline (Veterinary Services Memorandum No. 800.111, 2007), introduction of a new virus subtype into a vaccine also requires experimental challenge as proof of efficacy. Experimental challenge has become widely accepted as an indicator of the clinical and virological protective efficacy of EIV vaccines, and is now used as a model system to address a variety of questions not required for licensure. Early challenge efforts seem to have produced infections but not clinical disease (J. T. Bryans, personal communication), but with current experimental requirements, influenza-naive horses can reliably be made sick. This author's view is that a low-passage virus is essential, as is the need for a high dose of challenge virus (approximately 10^6 or more egg infectious doses per horse). This dose is probably much higher than natural exposure would supply, but it ensures the consistent appearance of clinical signs in susceptible horses. The model was described by Mumford and colleagues [83], and is fundamentally similar to that of Holmes and colleagues [56]. Vaccinated and control horses, or more typically ponies, are exposed to aerosolized virus by inhalation, either through a face mask or within a tented stall. EIV is a Biosafety Level 2 agent, although it is not generally hazardous to human health, and therefore this procedure is performed in dedicated animal facilities with equipment and procedures in place to prevent spread of the aerosolized virus outside the facility. Influenza-naive control animals exhibit the usual influenza disease signs of pyrexia up to 41°C, cough, and nasal discharge (initially serous and later mucopurulent), usually starting on the second day post challenge, and these signs can persist for 1–7 days. Lung congestion, detected by auscultation, is also indicative of disease severity, although quantification is problematic. The author's laboratory uses a 0–5 scale that progresses from normal to minor inspiratory wheeze, pronounced inspiratory wheeze, inspiratory plus expiratory wheeze, wheeze plus coarse crackles, and muffling by pleuropneumonic fluid fill. However, the scoring is subjective, and the two lungs or different regions within each lung may have different scores, so this system is not widely used. Leukopenia of 5 days' duration following challenge of susceptible ponies has been reported [72]. Plasma levels of cardiac troponin I have been examined as a potential biochemical marker of disease severity, but only transient increases were observed, and no overall correlation was found [34]. Virus shedding is measured by collection of nasal or nasopharyngeal swabs and testing for live virus in embryonating eggs or Madin–Darby canine kidney (MDCK) cells, virus antigen by ELISA for viral nucleoprotein [22], or viral RNA using quantitative or non-quantitative RT-PCR methods [e.g. 71]. For this purpose, nasopharyngeal swabs have been shown to be more sensitive than nasal swabs, yielding higher virus titers for longer periods [102]. Live virus shedding begins at or even before the

onset of pyrexia, reaching a peak on day 2 or 3 post challenge, and gradually becoming undetectable between days 6 and 10 [e.g. 102]. Viral RNA may remain detectable by PCR for longer periods (up to 35 days in one report) [112]. In comparison with naive control horses, horses that have been effectively vaccinated show reduction or elimination of pyrexia and other clinical signs, and reduction or elimination of detectable virus shedding.

This model is effective and versatile. The challenge virus strain can be varied to demonstrate vaccine protection against heterologous as well as homologous virus, the test animals can be challenged at various times following vaccination to test the duration of immunity, and the model can differentiate between clinical protection and virological protection. One drawback is that because horses are large and costly to maintain, the number of animals per experiment is generally low, which reduces the statistical power of the experiment. Indeed there are few sources of influenza-naive horses and ponies available to researchers, and the animals have little or no genetic uniformity, most clearly demonstrated by the fact that horses and ponies are sometimes used interchangeably.

To overcome this problem, small animal models have been developed using mice or hamsters to evaluate the immunogenicity of EIV vaccines [31, 85, 131]. Hamsters are a more sensitive model than ponies for detection of antigenic differences based on both serum single radial hemolysis (SRH) antibody titers and virus excretion. However, the small animal models are not useful for evaluation of clinical protection, because *wt* H3N8 EIVs without adaptation generally produce minimal clinical signs in mice and hamsters. The equine H7N7 viruses are an exception, causing lethal disease in mice without adaptation [63], but it is believed that this subtype no longer circulates in horses, so it is not now recommended for vaccines.

In one other respect the regulatory standard for EIV vaccine licensure has improved since the twentieth century. The national regulatory authorities now accept that it is necessary to periodically update the virus strains in EIV vaccines without considering the updated vaccine to be a brand new product (i.e. without a requirement for a full licensing dossier). The aim is that the updating process can be speedily accomplished so that in the event of emergence of an antigenically new EIV strain, appropriate vaccines would become available within 1 year instead of the 5 or more years that are now typically required for strain updating. According to the USDA guideline (No. 800.111), if the update does not change the virus subtypes in the vaccine or the manufacturing process, USDA will not require full-scale efficacy and field safety studies. Manufacturers must demonstrate that the updated vaccine produces a similar immune response to the original formulation in the target host animal or in a suitable laboratory animal model (i.e. serological studies are presumptively adequate for the most usual kind of updating), although national regulatory differences of opinion sometimes arise as to the requirements for updating vaccine seed strains. Similarity of immune responses is generally shown by comparison of mean serum antibody titers. When comparing the results of different trials performed at different times and possibly under different experimental conditions, a simple comparison of maximum mean titers can be potentially misleading. Heldens and colleagues [54] have recommended an area-under-the-curve calculation to improve such analyses, but the effect of this is unknown. The specific serological tests used are described in the following section.

Tests and strategies for detecting EIV-specific immune responses to vaccines and infections

Immunity-measuring techniques are seldom applied to privately owned horse populations, as these populations are generally decentralized in terms of both geography and management. Where EIV vaccination is required, as for example by the UK Jockey Club since 1981, documentation of vaccination is usually accepted in lieu of actual testing of serum samples.

Where routine disease surveillance of an established resident population is needed (e.g. to ensure freedom from disease), periodic serum collection and testing of influenza-naive sentinel horses may be used. However, in a changing, mobile, largely seropositive population, serosurveillance is of little value, and virological surveillance by testing of nasopharyngeal swabs must be employed to detect infections. This surveillance can be either

targeted or randomized, but ideally will incorporate elements of both. In addition, horses that are observed to be sick with respiratory signs should be tested in every case, but only in exceptional situations will this latter kind of surveillance be undertaken. Following the 2007 Australian outbreak, to achieve a status of provisional freedom from infection, random surveillance by collecting nasal swabs was designed to detect EIV infection on at least 1% of the high-risk premises of the control (Amber) zone and 1% of horses on an infected property. In the low-risk premises where horses had been vaccinated but there was no evidence of disease, serological surveillance could be undertaken using a DIVA (differentiating infected from vaccinated individuals) test, namely nucleoprotein-ELISA (see below), because the only vaccine permitted – recombinant canarypox hemagglutinin – did not express nucleoprotein (NP), and the surveillance was designed to give 95% confidence that infection of 5% of premises would be detectable. Eventually, for formal recognition of freedom from equine influenza by the World Organisation for Animal Health (*Office International des Epizooties*, OIE), active surveillance by nasal swabbing was designed to detect EIV if present at 0.01–0.5% prevalence. A total of around 44 000 horses on more than 9700 properties in New South Wales and Queensland were tested (i.e. about 5% of the horse population of these states) [77].

The serological tests used to detect equine EIV-specific antibodies are typically the hemagglutination inhibition (HI) test and the SRH test. These tests are essentially the same as those used for other species. Cross-laboratory studies have demonstrated that for EIV serology, the SRH test has greater reproducibility than the HI test [28], and primarily for this reason it is preferred by many laboratories. The HI test is technically simpler and remains widely used, but neither test is useful for differentiating between related virus strains, or for distinguishing between naturally infected and vaccinated animals (DIVA). Virus neutralization assays are occasionally used, with results that correlate well with SRH and HI data for the same cohorts [79].

Two modifications are often used in conjunction with the HI test, namely pretreatment of sera and pretreatment of antigen. Pretreatment of equine sera is essential for removal of non-specific inhibitors of hemagglutination, which if present will produce false-positive results. Equine sera contain high levels of α2-macroglobulin [109, 114], a heat-stable, virus-neutralizing 720-kDa glycoprotein that binds hemagglutinin (HA) through its sialic acid residues and blocks HA binding to erythrocytes. The most commonly used serum pretreatments are receptor-destroying enzyme, periodate or trypsin periodate, and kaolin/erythrocyte adsorption. Heat treatment (56°C for 30 minutes) alone is insufficient. The author's laboratory has shown that kaolin treatment, which is widely used in diagnostic laboratories, is effective when testing for equine antibodies against H3 EIV, but fails to eliminate false-positive results when testing for antibodies against H7 EIV [12].

Pretreatment of antigen with detergent (Tween-80) and ether [62] was introduced to solve the problem of equine post-vaccination titers against H3 EIV appearing much lower than post-vaccination titers against H7 EIV. This was attributed to more extensive carbohydrate side chains on the equine H3 HA making it a poor antigen, whereas the equine H7 HA is an effective antigen. Modifying the H3 HA by Tween-80 and ether treatment, creating subviral HA-coated particles, raises the observed titers to levels similar to those from unmodified H7 HA (i.e. it increases the sensitivity of the HI test). However, the amount of increase is inconsistent because of batch-to-batch variation in the treated antigen. Therefore the practice of such treatment probably contributes to the poor reproducibility of the HI test for equine serology studies.

Two serological tests have been developed specifically to distinguish between infected and vaccinated animals (DIVA), namely an immunoblot, or alternatively ELISA, which detects antibodies specific to the viral NS1 protein [11, 95] with an experimental sensitivity of more than 70%. NS1 is present in infected cells but absent from virus particles, so anti-NS1 antibodies are produced to detectable levels after infection but not after vaccination using conventional vaccines. Modified-live virus (MLV) EIV vaccines probably do induce an anti-NS1 response, but this has not been confirmed. Anti-NS1 antibodies in the horse are relatively short-lived, but for samples collected within 1–2 months after an outbreak this method provides a DIVA capability.

In the 2007 Australia outbreak of H3N8 EIV the only vaccine approved for use, namely recombinant canarypox expressing only the EIV HA, was in part chosen deliberately for DIVA purposes because it would not stimulate responses to other EIV proteins, such as neuraminidase (NA) [106]. In the event, instead of NA, ELISA for detection of antibodies to EIV NP protein was used [39, 64, 115]. This ELISA was the same as or a slightly modified form of assays designed to detect NP-reactive antibodies in avian or swine sera. The assay was less sensitive than HI or SRH at detecting seroconversions, and also usually lacks DIVA capability with regard to conventional or MLV EIV vaccines. However, in the special situation that utilizes recombinant canarypox with HA but lacks NP, as was presented by the 2007 Australia outbreak, it was useful as a DIVA test.

Cell-mediated immunity

For many years, measurement of equine cell-mediated immune (CMI) responses was rarely undertaken, because of lack of equine-specific reagents and the laborious nature of the ^{51}Cr-release assays. Hannant and colleagues showed that whereas EIV infection of horses induces MHC-restricted antigen-specific cytotoxic T-lymphocyte (CTL) responses, vaccination using conventional adjuvanted inactivated virus vaccines does not induce detectable CTL activity [45, 47]. Vaccinated ponies did show lymphocyte proliferation as detected following *in-vitro* stimulation of peripheral blood mononuclear cells (PBMCs) with inactivated EIV. This CTL assay has since been refined by using live EIV for *in-vitro* stimulation of CTL precursors, and equine dermal cells as target cells, but remains laborious [98]. An alternative approach has been the analysis of cell-associated or serum cytokines, in particular interferon-gamma (IFN-γ), which is a pro-inflammatory cytokine associated with Th-1 responses. Cell-associated IFN-γ is measured by flow cytometry of antibody-stained PBMCs following *in-vitro* stimulation with EIV and treatment with brefeldin A to block IFN secretion [96]. Significant increases in the percentage of IFN-γ-expressing cells following *in-vitro* re-stimulation with EIV have been observed following either experimental challenge or vaccination of naive animals [1, 97–101], but not when horses had pre-existing immunity to EIV [1]. Bioassays for equine cytokines, including IFN (primarily IFN-α), IL-6, and TNF-α, have been described using serum or nasal secretions as samples for systemic or local responses, respectively [129]. As measured by bioassay, EIV infection of naive ponies induces local (nasal) IFN and IL-6, and low levels of systemic (serum) IL-6, but not IFN. Levels as well as duration of secretion appeared to be positively correlated with the severity of disease, making them markers of pathogenicity. TNF-α was not detected [129].

The advent of quantitative real-time RT-PCR and the availability of the equine genome [127] have provided a convenient new tool for analysis of many equine cytokine responses by measurement of cytokine transcripts in immune cells. Generally the cell sources are peripheral blood leukocytes, which are lysed immediately upon collection in tubes containing an RNA-stabilization buffer. These samples are conveniently obtained as often as needed and can be processed at leisure. However, in view of the bioassay results [129] it can be questioned whether peripheral blood leukocytes are an adequately revealing sample for EIV-associated cytokine activity. Transtracheal wash or bronchoalveolar lavage techniques can produce respiratory tract cell populations that are suitable for analyses of cytokine transcripts or *in-vitro* stimulation of CTL precursors [e.g. 15], but these sampling methods are much more laborious than peripheral blood collection and, as the methods themselves induce local inflammation, should not be used on consecutive days on the same animal. Primer/probe sets have been developed for mRNAs of over 50 equine cytokines and immune cell markers, including those specifically associated with Th-1 (IL-2, IFN-γ, and Tbet), Th-2 (IL-4, IL-13, and GATA-3), Treg (IL-10, TGF-β, and FoxP3), and surrogates for CTL (perforin and granzyme B) (a partial list is available at www2.ca.uky.edu/gluck/HorohovDW_EIRClonedCytokines.asp). Quinlivan and colleagues [110] compared plasmacyte cytokine mRNA levels post challenge in vaccinated and unvaccinated horses, and detected significantly elevated expression of IFN-α and IL-6, but not TNF-α, and only slightly elevated IL-1β on day 2 post challenge in unvaccinated horses, whereas recently vaccinated horses showed no changes. Another study reproduced the day

2 post-challenge elevation of IL-6 mRNA and also showed significantly elevated IL-1β mRNA [21]. These expression patterns are consistent with those found in other species after influenza challenge [e.g. 49]. IFN-γ has also been assessed. Post-challenge elevation of IFN-γ mRNA has not been consistently observed, but when it has been reported it was associated with protective immunity [5]. Following primary vaccination, Gildea and colleagues [41] observed significantly increased IFN-γ mRNA, peaking on day 7 post challenge, which corresponds to the above-mentioned findings from *in-vitro* CMI assays. Those researchers also observed increases in IL-1β and IL-4 mRNA which peaked on day 14 post challenge. As detected by real-time RT-PCR of plasmacyte samples, the magnitude of these increases is typically small (often less than twofold).

Correlates of protection and evaluation of vaccination success

Correlates of protection

Inactivated and subunit vaccines are primarily intended to stimulate serum antibodies. Stimulation of other arms of the immune system (e.g. CMI, mucosal immunity) is a bonus, but is also more difficult to measure. Serum antibody titers have been correlated with clinical or virological protection using the SRH test, providing a useful gauge of the effectiveness of vaccination. When protection against a virus that is antigenically similar to the vaccine strain is assessed, a serum SRH titer of 85 mm^2, or an HI titer of 64, is correlated with clinical protection (i.e. absence of disease signs). In order to achieve virological protection (i.e. complete resistance to infection based on the absence of detectable virus shedding), SRH titers higher than 120 mm^2 are needed, and a titer of 154 mm^2 is recommended [84, 86, 87, 91]. However, in the field, vaccinated animals may be exposed to *wt* viruses that are more or less dissimilar to the vaccine strains, particularly as the strain-updating process for EIV manufacturers can take more than 5 years from the time a strain update is recommended. Evidence from the 2003 EIV outbreak in the UK, the first introduction of the Florida clade 2 sub-lineage, points to the need for higher SRH titers for protection against heterologous viruses. In two yards with ongoing surveillance, horses with mean titers of 156 mm^2 still seroconverted upon exposure, indicating that they were susceptible to infection, whereas the mean titer of horses that did not seroconvert and were thus resistant to infection was 203 mm^2 [90].

Host factors

In addition to management factors, host factors play a role in vaccine effectiveness. A well-known problem is that some horses, particularly young animals, are poor responders to at least the primary course of vaccine, although they may respond to subsequent doses [40, 91]. Low responders in a population can elevate the risk of disease for the whole population, by providing a portal of entry for virus, and susceptible hosts for its amplification and dissemination, and managers may have unjustified confidence in the safety of their herd. Horses aged 3 years or older have been reported to be more susceptible to infection than 2-year-olds, even though serum antibody titers would have predicted that there would be no difference [90], and this has been associated with the length of time that has elapsed since the most recent vaccination [7]. "Original antigenic sin" may also play a role [90]. In that study, too [7], male horses were found to be at significantly increased risk of infection compared with females, even though serum antibody titers were comparable, and this was attributed to the immunosuppressive effects of testosterone.

Maternal antibody interference

The issue of maternal antibody interference with EIV vaccination of foals was raised in 1991 by Van Oirschot and colleagues [126]. EIV vaccination of pregnant mares is recommended in order to raise EIV-specific antibody titers in colostrum, but there is considerable evidence that maternal antibodies interfere with the foal's active immune responses to conventional EIV vaccines [26, 55, 125, 133], preventing the development of adequate serum antibody titers until multiple doses of vaccine have been administered. Holland and colleagues showed that maternal antibody interference in foals was specific for the EIV subtype to which their dams had immunity [55]. Wilson and colleagues showed that when vaccination was started at 3 months of age,

foals were unresponsive to two doses of vaccine and required one to three additional boosters to achieve the same titers as yearlings after two doses [133]. Analysis of the 2003 EIV outbreak in the UK showed a significantly increased risk of infection associated with first vaccine administration at less than 6 months of age, compared with administration at 6–18 months of age [7]. EIV vaccination of foals from vaccinated dams, using conventional vaccines, is not recommended before 6 months of age. EIV is rarely a problem in foals with maternal antibodies, so some delay in foal vaccination is biologically feasible, but once weaned these animals may require EIV vaccination in order to be sold or transported, and therefore management needs may prompt earlier vaccination. The foal age at which maternal antibodies have waned sufficiently to permit effective vaccination is unclear. On the basis of the HI test these antibodies frequently become undetectable in serum by around 4 months of age [125], but interference may continue well after this age [55]. At one time it was thought that maternal antibody interference might signify the induction of a state of immune tolerance (i.e. vaccination in the presence of maternal antibodies inhibits responses to future doses as well as to the immediate dose) [26], but apart from serum antibody responses there is no other evidence for immune tolerance, and the poor responses might be due to use of vaccines with weak potency. In foals that have not acquired maternal antibodies (e.g. due to failure of passive transfer, or as in the 2007 Australia outbreak in which many dams were influenza-naive), foals may be vaccinated much earlier – for example, at 1 month of age [132].

Vaccination schedule

A typical recommended schedule for primary vaccination using conventional inactivated virus vaccine (e.g. the American Association of Equine Practitioners [AAEP] *Risk-Based Vaccination Guidelines*, www.aaep.org/-i-166.html) suggests starting a three-dose series at 6 months of age, with the second dose 4–6 weeks after the first (the primary course), and the third dose (first booster) 5–7 months later. In young horses the antibody titers raised by the primary course of conventional vaccines are short-lived and fall below the 85 mm^2 threshold for clinical protection well before the administration of the first booster [e.g. 40]. This period of clinical susceptibility has come to be known as the "immunity gap" [53]. In older horses that are regularly vaccinated, the serum antibody titers are relatively stable, and modeling has shown that revaccination at 6-month intervals is expected to significantly lower the risk of EIV outbreaks compared with revaccination at 12-month intervals [104], although the longer period may still be appropriate for horses at low risk (i.e. those that do not travel or that are not exposed to newly introduced horses). In young horses, however, it is difficult to close the immunity gap using conventional vaccines. Heldens and colleagues [53] explored a strategy using an accelerated booster at 8 weeks after the primary course, and found no benefit. Vaccination in the presence of vaccine-induced antibodies seemed to have much the same result as vaccination in the presence of maternal antibodies, with only modest and short-lived spikes in serum titers. An immune stimulating complex (ISCOM)-Matrix vaccine was more successful in inducing titers that remained adequate for clinical protection for 5 months after the primary course [51]. Reducing the interval between the first and second doses to 3 weeks has no detrimental effect, whereas increasing the interval to 13 weeks did not change the boosted titer level but merely increased the period before the second dose during which titers were inadequate for protection [25].

Mucosal immunity

Although serum antibody levels are strongly correlated with protection, mucosal immunity probably plays an important role, as shown in other species, including humans [e.g. 122]. Key evidence for the importance of mucosal immunity or cell-mediated responses is the finding that in young horses following experimental infection, the duration of clinical immunity against re-infection is far longer than the duration of detectable serum antibodies [48]. Hannant and colleagues tested a prime-boost strategy using a conventional alum-adjuvanted vaccine for priming, followed by two intranasal (IN) vaccinations using inactivated EIV adjuvanted with cholera toxin B subunit [44]. They found that this efficiently primed antibody responses, primarily IgA, in the nasal mucosa as detectable in nasal

washes, and was both clinically and virologically protective. Anamnestic responses of nasopharyngeal mucosal antibodies in horses are faster than serum anamnestic responses [46], so stimulation of mucosal immunity is a promising vaccination strategy.

One such strategy using a commercial ISCOM vaccine, featuring a priming vaccine dose by intramuscular (IM) injection followed by a boosting dose by IN administration, was explored by Crouch and colleagues [24]. In their study, the IM–IN regimen was not as protective as a standard IM–IM regimen, but their results indicated that the IM–IN regimen is more effective than IM vaccination alone in priming the mucosal immune system for a secretory IgA response. This may have the benefit of heightened cross-protection against heterologous challenge strains, and such a prime-boost strategy is of continuing interest, but requires further examination.

Surveillance and vaccine updating

Antigenic drift has been slower in H3 EIVs than in human influenza A viruses (only one-third to one-fourth of the speed) [9]. Consequently, the vaccines for EIV have not required annual reassessment and 2- to 4-year seed strain replacement as has occurred with human influenza vaccines. Yet the occurrence of major outbreaks among vaccinated herds (for example, in 1989 in Europe [69], in 1992 in Hong Kong [108], and in 2003 in Europe again [90]) demonstrates the necessity for tracking EIV antigenic drift and when necessary instituting the updating of EIV vaccines. International surveillance for EIV outbreaks has been much improved as a result of more effective diagnostic procedures (PCR, rapid detection kits) replacing the use of virus isolation in embryonating eggs. Dedicated surveillance systems have been established in countries such as the UK, Ireland, and France, where funding is available to enlist practitioners and sentinel practices into the systems [38, 67]. However, surveillance still lags behind in many countries, including the USA, where equine influenza is not a notifiable disease and equine practitioners are not incentivized to collect nasopharyngeal swabs from suspect cases.

An important step forward was the formalization of an international surveillance review process by the establishment of the OIE/World Health Organization (WHO) Equine Influenza Expert Surveillance Panel (ESP) in 1995 [82]. This group consists of the designated experts from the OIE reference laboratories for equine influenza in England, Ireland, Germany, and the USA, and equine influenza experts from several other laboratories representing all continents. At an annual meeting the pooled data on disease surveillance and virus characterization are reviewed with special regard to instances of vaccine breakdown, or emergence of novel antigenic variants. On these bases an annual recommendation on vaccine strain selection is agreed and published in the *OIE Bulletin* [e.g. 4]. In many years there is insufficient evidence to warrant any change in the panel's recommendation. Until around 2000, most EIV vaccines contained both an H7N7 strain and an H3N8 strain. The H7N7 subtype was considered epidemiologically irrelevant and ceased to be recommended after 2000, although at the time of writing the equine/Prague/56 H7N7 virus is still found in vaccines in some parts of the world. Starting in 1995 [92], the ESP has recommended two H3N8 strains. From 1995 until 2010 these were representatives of the American and Eurasian lineages [30, 138], and since 2010 they have been representatives of the two clades descended from the American lineage (Florida clades 1 and 2) [4]. From 2010 the Eurasian lineage was no longer recommended, as it had become epidemiologically unimportant, although it may possibly still circulate at low levels. When a new strain is recommended, the OIE reference laboratories are able to supply that strain or antigenically similar strains to vaccine manufacturers. Regulatory authorities (USDA, EMA) have accepted the role of the ESP as the preferred advisory body to justify the strains selected for updating by vaccine manufacturers.

Molecular characterization of virus isolates focuses upon the amino acid sequence of HA1. Although whole-genome sequencing is a welcome addition, it has not yet had an impact on vaccine strain recommendations, as there has been no appearance of a new EIV subtype since 1963 (or arguably 1989, the avian-like H3N8 equine/Jilin/89 virus [43]), no confirmed reappearance of the EIV H7N7 subtype since 1979 [130], and no evidence for inter-subtype reassortment since the 1970s [8], and although there have been intra-subtypic

reassortment events [88, 136], these have not necessitated changes in strain recommendations. Several phylogenetic trees of EIV H3 HA evolution have been published, most recently by Woodward and colleagues [136]. A method for quantitatively relating HA amino acid substitutions to vaccine strain effectiveness, the $P_{epitope}$ value, has been proposed [29], although it needs stronger corroboration, and there is evidence that critical single amino acid substitutions can be more relevant to vaccine cross-reaction than accumulations of non-critical changes [68, 137]. Alongside the nucleotide sequence evolution, antigenic relatedness of virus isolates has been analyzed by comparative HI testing using an established panel of ferret reference sera. These analyses consistently show 2- to 16-fold differences in reactivity between Florida clade 1 and clade 2 strains [18, 19, 136]. Antigenic cartography [68, 136] has greatly facilitated these comparative analyses, and demonstrated that while genetic evolution continues in circulating strains, the most recent strains continue to map to the same clusters that were identified by the Florida clade 1 and clade 2 lineages. However, as Woodward and colleagues [136] have pointed out, cross-reaction does not necessarily correlate with cross-protection.

Types of vaccines and methods of administration

The available commercial EIV vaccines that have been licensed are listed in Table 21.1, and are based on various vaccine platforms and technologies.

Conventional vaccines

Adjuvanted, inactivated whole-virus EIV vaccines were first developed in the 1960s by Bryans and co-workers [17]. Their initial studies used viruses grown in embryonating chicken eggs and inactivated by formalin. An adjuvant was soon incorporated, as without it the inactivated H3N8 EIV, administered by IM injection, induced no detectable serum HI antibody response after two doses, whereas the unadjuvanted H7N7 EIV did induce HI antibodies. Adjuvanted, inactivated whole-virus vaccines remain the most common type of EIV vaccine. Of the eight product lines of commercial EIV vaccines currently marketed in the USA, five are of this type and use carbomer or lipid-based adjuvants. Most are multivalent vaccines containing two EIV strains, such as A/equine/Ohio/03 (Florida clade 1 lineage) and A/equine/Richmond/07 (Florida clade 2 lineage), and in addition these may be formulated as combination vaccines for other equine diseases, including tetanus, equine herpesvirus-1/4, eastern, western, and Venezuelan equine encephalomyelitis viruses, and West Nile virus. These vaccines rely upon stimulation of serum antibodies for immunity. The adjuvant is essential for successful vaccination, and the nature of the adjuvant strongly influences the resulting immune response. Vaccination with killed antigen typically leads to the antigen processing through the exogenous pathway and preferential presentation to $CD4^+$ T cells in association with MHC class II molecules. The first generation of EIV vaccines often used alum (aluminum hydroxide or aluminum phosphate) as adjuvant, which is known to drive Th2 responses. Although these could stimulate lymphocyte proliferation responses in vaccinated ponies, they were poor inducers of CTL responses [45]. By contrast, the use of lipid-based or polymer-based vaccine adjuvants such as Carbopol (polyacrylic acid) can increase antigen processing through the endogenous pathway and presentation in the context of MHC class I [121]. Evidence that this heightens CMI as well as humoral responses is based on observed elevation of IFN-γ production by peripheral blood lymphocytes [41, 101].

Adjuvants also influence the specific antibody isotypes produced by vaccination [89]. Whereas antibody responses to experimental infection of naive ponies are primarily mucosal IgA and serum IgGa and IgGb isotypes (also known as equine IgG1 and IgG4/7 [128]), the isotype that predominated following vaccination with an alum-adjuvanted commercial vaccine (two doses, 3 weeks apart) was IgG(T) (IgG3/5), best known for neutralizing tetanus toxin and responses to equine intestinal parasites [105]. These vaccinated ponies also failed to generate serum antibody responses detectable by HI, so the absence of protection when vaccinates were subsequently challenged with *wt* EIV could be explained by either a qualitatively inappropriate antibody isotype or a quantitatively insufficient response [89].

Table 21.1 Licensed commercial EIV vaccines available throughout the world.

Vaccine type	Manufacturer	Trade name	Virus strains	Region	Adjuvant	Number of combinations
Inactivated, whole virus	MSD Animal Health	Equilis Resequin	Prague/56, Newmarket 1/93, and Newmarket 2/93	Europe, South Africa	Aluminum hydroxide and Immunostim®	1
	Merck	Prestige line and Encevac line	KY/93, KY/02, and Newmarket/2/93	North and South America	Havlogen	6
	Elanco Animal Health	Duvaxyn line	Prague/56, Suffolk/89, and Newmarket/1/93	Europe, South Africa	Carbopol 934P and aluminum hydroxide	2
	Zoetis	Fluvac Innovator line	KY/97	North and South America	Metastim	6
	Nisseiken Co., Inc.	Equine Influenza Trivalent	Avesta/93, Ibaraki/1/07, and La Plata/93 *or* Yokohama/aq13/2010	Japan	?	1
		JIT 08	H3N8	Japan	Aluminum hydroxide	1
	Bioveta, Inc.	Fluequin line	Prague/56, Morava/95, and Brno/97 *or* Brno/08	Czech Republic	Aluminum hydroxide *or* Algeldrat *or* Montanide ISA 35 VG	3
	Boehringer Ingelheim	Vetera line	KY/95, OH/03, and Richmond/07	North and South America	Carbimmune	8
	Boehringer Ingelheim	Calvenza-03 line	OH/03, KY/2/95, and Newmarket/2/93	India, North and South America	Carbimmune	2
	National Research Center on Equines	Indigenous Equine Influenza Vaccine (egg and cell culture based)	Ludhiana/87 and Katra-Jammu/06/08	India	?	2
	Vencofarma	Vacina Influenza Horse Vencofarma *or* Vacina Lexington 8 Vencofarma	Prague/56, KY/94, and South Africa/4/03	Brazil and most of South America	Aluminum hydroxide gel	1
	Hertape Calier	Tri-Equi	A1 and A2 (including KY/92)	Brazil and most of South America	? likely aluminum phosphate gel	1
ISCOM, subunit	MSD Animal Health	Equilis Prequenza line	Prague/56, Newmarket/1/93, and Newmarket/2/93 *or* South Africa/4/03 and Newmarket/2/93	Europe, South Africa, Central America, and Colombia	ISCOM Matrix	3
	Zoetis	Equip line	Newmarket/77, Borlange/91, and KY/98	Europe	ISCOM Quil A	2
Modified live virus	Merck	Flu Avert	Cold-adapted KY/91	North America	None/not needed	1
Recombinant canarypox vector	Merial	Recombitek rFLU	OH/03 and Richmond/1/07	North and South America, China	None/not needed	1
	Merial	Proteqflu line	OH/03 and Richmond/1/07	Europe, South Africa, Morocco, Russia	Carbomer	2

Although the possibility of an insufficient response cannot be excluded, evidence for an inappropriate antibody isotype includes the finding that, in the horse, IgGa and IgGb are superior to IgG(T) in terms of complement fixation and antibody-dependent cellular cytotoxicity [74], and mouse models have also shown evidence of superior lower respiratory tract protection against challenge when the vaccine-induced isotypes (IgA and IgG2a) match the infection-induced isotypes [e.g. 10].

Conventional EIV vaccines are administered by IM injection, typically in the brachiocephalicus/serratus cervicis muscles of the neck. Well-managed horses receive a variety of injections at this site and can become accustomed to the procedure with minimal protest. This neck location has the additional benefit that adverse reactions to vaccines – typically local swelling or edema, with local heat and pain – are visible to inspection. When they occur, such reactions may be visible within 48 hours and persist for several days. Adverse reactions to EIV vaccines are associated with the nature and quantity of adjuvant, and avoidance of adverse reactions is a critical factor governing the selection of adjuvants for commercial equine vaccines, more important even than the quality of the immune response. In general, to avoid adverse reactions, a maximum IM-injectable volume for a single injection is 2 mL, which puts a physical limitation on the antigenic potency of combination vaccines containing multiple antigens. There is conflicting evidence on the relative performance of monovalent versus combination vaccines for EIV [26, 52, 58], and it is possible that the particular antigens in the combination are critical, although this requires further investigation.

The performance record of conventional vaccines has been patchy. A double-blinded, randomized, controlled field trial conducted in the 1990s [80] found that vaccination did not decrease the risk of disease, although the duration of disease was reduced. A more recent trial [58] comparing several conventional vaccines in adult non-naive horses concluded that none of them delivered antibody responses at the levels associated with protection, but another study conducted in weanlings [40] found that a different conventional vaccine showed performance superior even to that of vaccines based on more modern technologies (including ISCOM and canarypox-HA, described below). It must be emphasized that even when vaccine efficacy is supported by experimental-challenge data, those challenges are administered under optimum conditions which cannot necessarily be extrapolated to field protection, where horses that differ in health status or have different histories of management, vaccination, prior exposure, underlying chronic conditions, or stress (e.g. due to transport) may be co-mingled.

ISCOM-based vaccines

Subunit vaccines for EIV are ISCOMs containing purified HA and NA antigens. The antigens may be either mixed directly with the matrix components (saponin Quil-A®, phospholipid, cholesterol) (ISCOM vaccine) or integrated into pre-formed ISCOM micelles (ISCOM-Matrix vaccine). Both types have been developed into commercial EIV vaccines. Quil-A® saponin has adjuvant properties, and ISCOM vaccines are believed to process antigens through both the exogenous and endogenous pathways, and present them in association with both MHC class I and class II molecules [78], potentiating innate immune responses and also Th1 immune responses [119]. Vaccination of naive ponies with an ISCOM EIV vaccine induced primarily IgGa and IgGb antibodies, especially following the first dose [23]. An ISCOM-Matrix EIV vaccine has been shown to also induce CMI responses based on IFN-γ production [100]. An ISCOM/tetanus combination vaccine was reported to cause significant short-term elevation of serum amyloid A, plasma fibrinogen, and white blood cell levels, and significantly decreased serum iron concentrations [2]. Several experiments have demonstrated protective responses of long duration against experimental challenges with *wt* EIV [23, 50, 51, 97].

DNA vaccines

DNA vaccines have the potential advantages of safety and ease of updating. For EIV, an experimental vaccine consisting of DNA expressing HA coated on gold beads was developed [73]. Following three doses administered at 2-month intervals by injection into skin (inguinal skin and perineum) and mucosal (ventrum of tongue, conjunctiva, and third eyelid) sites of ponies, with a total of 12–37.5 μg of DNA per dose, this DNA vaccine was

shown to be immunogenic with induction of IgGa and IgGb antibody isotypes, and it provided partial virological protection and almost complete clinical protection against challenge [73]. In some ponies, responses were seen after the second dose. Lymphoproliferative and IFN-γ responses indicative of CMI were also observed. Co-administration of DNA for IL-6 did not affect local mucosal immune responses, although it promoted IgG(T) production, suggesting an elevation of Th2 responses [116]. Nasal IgA was detected at very low levels. To promote nasal IgA, a different strategy was followed which included two doses of the vaccine DNA mixed with 1 mg of cholera toxin B and administered IN, followed by two doses of the vaccine DNA administered on particles to the multiple mucosal sites as described above. This did indeed stimulate nasal IgA and also nasal IgGb, although only the final two doses stimulated serum antibodies [118].

These initial experimental DNA vaccinations of ponies used the PowderJect-XR Gene Gun, a helium-powered device that drives gold micropellets coated with the vaccine DNA into the skin and mucosal tissues. As described above [73], for each dose 24–60 such inoculations were delivered at multiple sites, and sedation of the ponies was necessary. This is too costly and impractical for routine field use. As an alternative, Landolt and colleagues [66] tested the injection of vaccine DNA directly into the submandibular lymph nodes, which are easily accessible in horses. Using three doses of 50 μg each (25 μg in each lymph node), they obtained serum IgGa and IgGb titers similar to those obtained with the Gene Gun, although less than those achieved by conventional vaccination. Another recent study has used a needle-free spring-powered jet device to deliver DNA-containing vaccine solution subdermally [5]. Although sedation was not required, the injection site was shaved, and transient swelling was noted after inoculation. DNA vaccination by IM injection was compared with needle-free delivery, and yielded both serological and clinically protective responses, although the serum antibody titers were less than those obtained with the needle-free device [5]. The relative disadvantage of both the IM and needle-free methods, compared with the Gene Gun, was that far more DNA was used (up to 4 mg). In the past, the preparation of so much DNA was impractical, but this should not be the case in the future.

Modified-live-virus vaccines

MLV vaccines for influenza are predicted to mimic the process of natural influenza infection and so induce host immune responses, including mucosal and CMI responses, that are arguably superior to those obtained from inactivated virus vaccines. A set of experimental temperature-sensitive (*ts*) MLV was produced by 6+2 reassortment between a *wt* H7N7 EIV and a human H3N2 virus made *ts* in PB2 and NP by chemical mutagenesis, the result bearing the EIV H7 HA and N7 NA [16]. A subsequent 6+2 reassortment between this and a *wt* H3N8 EIV yielded a *ts* H3N8 virus. In aerosolized ponies, both the H7N7 and H3N8 *ts* viruses produced virus shedding and seroconversion, but no remarkable clinical signs beyond mild hyperemia of the nasal mucosa or serous nasal discharge, and the ponies were protected against challenge 1 month later [56, 57]. Other 6+2 reassortant viruses were produced from *wt* H3N8 EIV and an avian influenza virus, A/Duck/New York/6750/78 (H2N2). The viruses were replication competent but attenuated in ponies exposed by the aerosol route, were immunogenic, and provided partial clinical and virological protection in ponies challenged at 5.5 months post vaccination [85]. However, the possibility that these viruses could cause infections in humans or poultry blocked their further development as vaccine candidates.

An MLV vaccine entirely derived from EIV is currently marketed in the USA. This vaccine was developed by cold adaptation from the parent strain, *wt* influenza A/equine/Kentucky/91(H3N8), through a process involving multiple passages in embryonating eggs at successively lower temperatures [139]. The resulting virus is capable of replicating at 33°C, which approximates the temperature of the equine upper respiratory tract, but has greatly reduced capability to replicate at 37°C or higher temperatures. The vaccine is administered as a spray through an intranasal catheter. In experimental vaccinations it was non-pathogenic even under conditions of exercise-induced immunosuppression [72], and had low spontaneous transmissibility [20]. At 6

months following administration of a single vaccine dose to influenza-naive horses it was shown to provide complete clinical protection, although not complete virological protection, with partial protection still evident at 12 months [20, 123]. However, post-vaccination serum antibody titers were low to undetectable. This suggests that the mechanism of protection involved either mucosal antibodies in the respiratory tract, or CMI responses, but these have not yet been demonstrated. The specific mutations responsible for cold adaptation of this virus have not been described.

Another MLV equine influenza vaccine is being developed [120], namely A/Hong Kong/Otar/6:2/2010. This is a 6+2 reassortant bearing the surface antigens (HA, NA) of the *wt* strain influenza A/equine/Otar/764/2007 (H3N8) on a backbone from the human-derived, cold-adapted reassortant donor strain, influenza A/Hong Kong/1/68/162/35CA. This vaccine is also administered by the IN route, and like the US MLV it is a poor inducer of serum HI antibodies. Experimentally, following two doses of vaccine administered at an interval of 6 weeks, there was complete clinical and virological protection for 3 months and robust clinical protection for 12 months against the antigenically homologous challenge virus. Against a heterologous challenge virus (A/equine/Sydney/2007) there was still partial protection at 12 months. The authors argue that the vaccine provides DIVA capability based on the absence of serum HI antibodies post vaccination and heightened responses post challenge compared with controls, but this seems problematic for field use, where the length of time between potential exposure and collection of sera for analysis may be unknown. The question of whether this MLV bearing genes derived from human influenza virus could infect humans does not appear to have been examined.

A set of experimental MLVs has been described, whose principle of attenuation is not cold adaptation but instead the progressive truncation of NS1 [111] by reverse genetics. These MLVs express the first 73, 99, or 126 amino acids of the 219-amino-acid NS1. The functions of viral NS1 include antagonism of the host type 1 interferon response [32, 65]. Mutant viruses with impaired NS1 functionality are unable to replicate in interferon-competent hosts, and have restricted growth in MDCK cells and mice as well as in embryonating eggs more than 9 days old [111]. Contrary to findings with truncated NS1 of human influenza viruses, the mutant EIV with the least truncation of NS1 (126 amino acids) was the most attenuated. When tested by two-dose IN administration to horses, that mutant EIV was safe, induced seroconversion, and following challenge it resulted in significantly reduced, although not completely abolished, clinical signs and virus shedding [21]. Reverse genetics of influenza viruses has the advantage that antigenic updating can be readily accomplished by mutating specific codons in an otherwise stable backbone genome. Attenuation residing in the viral NS1 frees the HA and NA genes for antigenic alteration.

Intranasal inoculation has been used to administer live influenza virus vaccines. In the horse, the method used is insertion of a nasal catheter into the nares, through which the vaccine solution is aspirated. This method has some drawbacks. Without drug-induced tranquilization or forcible restraint (e.g. use of a twitch), unaccustomed horses may react to the introduction of the catheter with strong avoidance behavior. The nasal meatus of a horse is straight for a length of around 25 cm, so with the 10-cm catheters that are in use there is potential for some vaccine fluid to drip out. Finally, such catheters are not standard veterinary equipment. For these reasons, IN vaccination has never been favored among equine practitioners in the USA. Specially designed inhalation devices, positioned tightly against the external nares, that aerosolize the vaccine fluid and also deliver it to the upper respiratory tract by forced air pressure without the need to insert a tube up the meatus, would solve this problem. Such a device was once used experimentally, but it was never marketed as a delivery system for any commercial vaccine, and may no longer be available from the manufacturer.

Recombinant virus vaccines

The principal concerns about the MLV vaccines are reversion to virulence, and safety in pregnant or immunocompromised animals. Reversion to virulence might occur either through mutations or through reassortment with a circulating *wt* virus. There is no evidence that this has yet happened (the EIV MLV has been marketed in the USA for

over a decade), but the issue has motivated the development of other vaccine technologies. To retain the advantageous immune responses to live virus vaccines while avoiding the risks of reversion to virulence, one approach is the development of recombinant virus vaccines whose parent viruses are not pathogenic or contagious in equids.

Poxvirus-based vaccines

Work with modified poxviruses [13, 14, 27] had shown that recombinant vaccinia viruses expressing EIV HA were immunogenic and clinically protective in ponies when used either alone or as boosters subsequent to priming with HA-DNA vaccine. Prime-boost with NP-DNA and vaccinia-NP was less effective. Virus-specific antibodies consisting primarily of IgGa, IgGb, and IgA were produced in both serum and nasal secretions, and even more strongly when the vaccinia-HA was used alone (two or three doses) compared with the DNA prime/vaccine boost regimen. CMI responses were evident based on stimulation of IFN-γ. However, the safety of vaccinia virus-based vaccines is a concern.

Recombinant canarypox is a poxvirus vector that is considered safe for field use because in mammalian cells its replication is abortive, but it is still immunogenic because early gene products are expressed [e.g. 107]. A recombinant canarypox live virus vaccine for EIV has been widely commercially available since 2003. This vaccine utilizes Carbomer (polyacrylic acid) adjuvant and is administered by IM injection. The recombinant virus expresses HA of two EIV H3N8 strains (originally representing the American and Eurasian lineages, since updated with a Florida clade 1 strain replacing the American lineage strain, and likely to be updated with a Florida clade 2 strain replacing the Eurasian lineage strain). Experimentally, even one dose induced SRH-detectable serum antibodies [35, 36, 99] and a significant level of clinical and virological protection against heterologous virus challenge at 2 weeks post vaccination [35, 117]. This rapid response is particularly useful when an influenza-naive population is at near-term risk of exposure, as occurred in the 2007 outbreak in Australia, and a study there showed that the typical 4- to 6-week interval between the two doses of the primary course could be successfully compressed to 2 weeks [36]. Following the two-dose primary course the duration of significant, although incomplete, clinical and virological immunity extended to 6 months [117], although serum antibody titers in some ponies had decayed below the benchmark of 85 mm^2 by 5 months [75]. Thus with the usual booster (third dose) at 5–6 months there is only a small risk of an immunity gap. By 12 months after the booster the levels of clinical and virological protection were virtually unchanged, and serum antibody titers were also relatively stable, with a mean value of 110 mm^2 [75]. Serum antibodies of the IgGa and IgGb isotypes were detectable after the two doses of the primary course of vaccination [117]. Following vaccination, IFN-γ production by *in-vitro*-stimulated PBMCs was no different from unvaccinated controls. However, it was significantly elevated in vaccinates compared with controls at 1 and 2 weeks after experimental challenge [99], which suggests that vaccination had primed T cells for an EIV-specific CMI response. Similar results were obtained in aged horses (20–28 years old) [1], which have reduced antibody responses [42, 59] and also reduced anamnestic responses to conventional EIV vaccines compared with young horses, even when pre-vaccination titers are comparable [81]. Since the canarypox vaccine expresses only HA and not the more important CTL targets NP or M, the effectiveness of the CMI response considered separately from the humoral response is unknown.

Studies have explored the impact of influenza maternal antibodies on canarypox-HA vaccination of foals [40, 76]. When administered in the presence of maternal antibodies at 10–20 weeks of age, there was no seroconversion. However, there was evidence of a priming effect, as the same foals, when revaccinated approximately 6 months later (when maternal antibodies should be absent), showed a somewhat stronger serum antibody response than age-matched controls that were being vaccinated for the first time. It is possible that this could satisfy the management needs to vaccinate foals and/or weanlings at an age when maternal antibodies may interfere with conventional vaccines.

The growing use of canarypox-vectored EIV vaccine together with the development of canarypox-vectored vaccines for other equine diseases raises the possibility that pre-existing immunity to the canarypox vector may limit the

effectiveness of any canarypox vaccine booster. This was studied by using a canarypox-West Nile virus vaccine [37], where horses received a two-dose primary course 28 days apart followed by boosters on days +300 and +454 after the first vaccine dose. Canarypox-specific antibodies and IFN-γ-producing PBMCs were detectable following the primary course, and anamnestic antibody responses were observed following both boosters, but recall responses to the West Nile virus antigen were also produced, and thus pre-existing immunity to the vector did not appear to inhibit the immunogenicity of the vaccine.

An experimental MLV has been described that expresses the EIV HA (Ohio/03 strain) from a recombinant equine herpesvirus type 1 (EHV-1) [124]. This recombinant vector, rescued from a mutated bacterial artificial chromosome, was attenuated by deletion of the EHV-1 IR-6 early gene [94] as well as open reading frame-1 (ORF-1), and the codon-optimized EIV HA was inserted in place of ORF-1 downstream from a human cytomegalovirus immediate-early promoter. The resulting EHV-HA recombinant virus, administered by a combination of subcutaneous and IM routes, induced HI antibodies in adult horse sera by 2 weeks after the initial vaccination. Two doses produced titers that might be sufficient to confer clinical protection, although this has apparently not been tested. Previous research using a different EHV-HA MLV construct had shown serological responses and partial protection in dogs experimentally challenged with canine influenza [113]. EHV-1 is a common pathogen of horses worldwide and a commercial MLV exists, so EHV-HA may serve in effect as a combination vaccine. Since many horses will have high levels of pre-existing immunity to EHV-1, it remains to be seen whether this or maternal antibodies to EHV-1 in foals could confound HA expression and vaccine performance. EHV-1 in horses produces a latent carrier state from which viral recrudescence is possible, and the impact of this on anti-HA immune responses is not known.

Other vaccine technologies

Other vaccine technologies have been considered. A recombinant baculovirus-HA EIV vaccine was tested in mice, by IN administration of infected cell lysates [93]. This was weakly immunogenic after two doses, inducing serum antibodies that were detectable by ELISA but not by neutralization assay, and vaccinated mice were only partially protected from challenge. Co-administration of cholera toxin did not improve the baculovirus-HA performance. Oral vaccination (e.g. vectored by recombinant attenuated *Salmonella*) has been considered, but to date there are no descriptions of its use for EIV vaccination.

Improving EIV vaccines and vaccine coverage

The EIV vaccines available today are superior to those which were available 20 years ago, in particular because of:

1. the development and wide use of a reliable challenge model in the target species [83]
2. the correlation of post-vaccination antibody titers with clinical and virological protection in the target species [87]
3. a determined effort to track antigenic drift in circulating viruses and make evidence-based recommendations for updating virus strains in vaccines [82].

Relatedly, quality control of EIV vaccine production has been improved by the introduction of single radial diffusion methodology to reproducibly measure antigen content [134].

Regulatory agencies, including the USDA and EMA, have accepted experimental vaccination and challenge of horses as critical evidence of vaccine efficacy, and have also accepted in principle the necessity for streamlined updating of virus strains in vaccines, although in practice further streamlining is needed. A common complaint made by EIV vaccine manufacturers is that the costs of updating are not justified by the market value of the products. Simplification of the updating requirements helps to address this situation. As vaccines developed by recombinant DNA technology come on the market and can in principle be updated by mutation of a few nucleotides in an otherwise stable vector platform, with no change in manufacturing procedures, one can envisage that licensure of such updated products might in the future become routine.

Effective updating of vaccine virus strains requires as comprehensive a knowledge as possible

of the antigenic variants currently in circulation. This in turn requires effective surveillance and diagnosis. Current diagnostic methods for EIV are effective, but surveillance is very far from comprehensive in much of the world, especially where equine influenza is enzootic. It is not a notifiable disease in the USA, and the author's view is that most equine influenza cases are never properly diagnosed. Fortunately, perhaps, new antigenic variants are more likely to trigger large-scale disease outbreaks somewhere in the world, and these outbreaks then become a major focus of attention.

Another impediment to vaccine updating is that in some major markets, including the USA, there is no efficient mechanism for the removal from the market of vaccines containing obsolete virus strains. Those vaccines are still licensed for sale, and are still sold. Mathematical modeling has indicated that although outdated EIV strains in vaccines can have a relatively small impact on individual horses, at the population level they significantly increase the risk of an outbreak [103].

Only large-scale information campaigns (e.g. local, national, and international continuing education) can make horse owners and equine practitioners aware of the importance of purchasing updated vaccines. Current levels of continuing education are inadequate. In many countries, including the USA, EIV vaccination is optional except in special circumstances (e.g. if the horse is traveling abroad to a country where vaccination is a requirement for importation). Survey information from the US National Animal Health Monitoring System (Equine 1998 and Equine 2005 surveys) showed that influenza vaccination was one of the more common vaccinations in the survey region, but even so only 63% of horses (yearlings or older) received it. Meanwhile, 24% of horse operations had not administered any vaccinations in the previous 12 months, and 85% of events (races, shows, trials, polo, and other events) had no vaccination requirement (www.aphis.usda.gov/wps/portal/banner/help?1dmy&urile=wcm%3apath%3a%2Faphis_content_library%2Fsa_our_focus%2Fsa_animal_health%2Fsa_monitoring_and_surveillance%2Fsa_nahms%2Fct_nahms_equine_studies). Survey data collected in the UK show that EIV vaccination coverage is about 80% [61], although it has been suggested that responder bias means that this figure is mainly representative of competition horses (the Jockey Club has a policy of mandatory EIV vaccination), and overall coverage may be as low as 45% [60].

With regard to the vaccines themselves, improvements in antigenic potency and duration of immunity are needed. Based on SRH, peak post-vaccination serum antibody titers in many horses are in a range (120–200 mm^2) that confers clinical protection but may be marginal for complete virological protection against challenge from heterologous virus strains, and in young horses in particular these peak titers are not long-lasting. One result, even in well-managed weanlings and yearlings, is the "immunity gap" discussed earlier in this chapter. Vaccines that induce higher and longer-lasting antibody titers will help to reduce the EIV vulnerabilities of young horses and of horses that are not regularly revaccinated. Higher antigenic potency should also help to overcome the problem of poor responders in horse populations, which sometimes act as index cases that shed and seed large amounts of EIV, which overcomes levels of partial protection in contact horses, setting off an outbreak. Lopez and colleagues [70] demonstrated one option for enhancement of conventional vaccine performance. Administration of a commercial adjuvanted whole-virus vaccine in combination with a synthetic unmethylated CpG-containing oligodeoxynucleotide (CpG ODN) induced significantly higher serum antibody titers than did the vaccine alone, and the critical IgGa and IgGb antibody isotypes, as well as IgG(T) subclasses, were also significantly elevated. However, CpG ODN as a secondary adjuvant did not yield longer-lasting antibody titers.

The importance of EIV neuraminidase as a vaccine antigen has not been explored. NA evolution has paralleled HA evolution, as shown by phylogenetic trees of similar structure, with divergent "American" and "Eurasian" lineages in the 1990s, and Florida clades 1 and 2 since 2003 [88, 136]. NA-specific antibody responses to conventional whole-virus EIV vaccines have not been reported, and might contribute significantly to a vaccinated horse's immune status. However, as the canarypox-HA vaccine and experimental DNA vaccines show, significant clinical and virological protection against EIV can be induced by HA alone.

Finally, there is much interest in development of a universal EIV vaccine which would not be

rendered obsolete by antigenic drift. For human influenza, universal vaccines directed at conserved epitopes in, for example, the HA stalk or M2 ectodomain are being studied (for a review, see Du *et al*. [33]). Developments for application to human influenza vaccines are closely followed by veterinary vaccinology researchers. If field testing of a universal vaccine is desired in animals before testing in humans, it is likely that swine or avian species will initially be preferred to the horse, as currently circulating EIV strains are neither so varied nor do they evolve so fast. However, the long lifespan of horses compared with food animals allows long-term field studies of the effectiveness of novel vaccines, such as the continuing effectiveness of repeated booster vaccinations, or use in combination with other vaccines, that may have an impact on human usage. The wide spectrum of vaccine technologies already commercialized for EIV vaccines indicates that a novel universal vaccine for EIV would find rapid acceptance.

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SUBSECTION IIIC

Sporadic and miscellaneous mammalian influenza

22 Canine influenza

Colin R. Parrish and Edward J. Dubovi

H3N8 canine influenza virus

Emergence

Equine influenza virus (EIV) H3N8 has been circulating in horses since around 1963, when it emerged in horses in South America, most probably due to the transfer of an avian influenza virus [29]. Infections of dogs by that virus were observed on a number of occasions, such as a small outbreak occurring among foxhounds in the UK in 2002, with some animals also becoming infected during 2003 [8], while single infections of dogs in close proximity to infected horses (10 of 40 dogs examined) were detected during a widespread outbreak of EIV in horses in Australia in 2007 [20]. However, in those cases the virus died out after a single infection or after a few months of transmission. In contrast, the emergence of a successful H3N8 canine influenza virus (CIV) was first recognized in 2004 when an outbreak of severe respiratory disease was seen among greyhounds in dog training facilities in the state of Florida, USA [7]. Before and during 2004 the virus was carried by infected greyhounds to racetracks in several other US states, as well as to other dogs, including those in animal shelters in some of the same states. Those CIV-infected dogs mainly suffered from a mild upper respiratory tract disease which spread rapidly within each facility, although some had a more severe disease that included hemorrhagic bronchopneumonia [7]. An H3N8 influenza A virus was isolated and shown to be closely related to the H3N8 EIV, and more specifically it was derived from the Florida clade 2 strain of EIV that was circulating in horses in Florida [7]. Analysis of sera collected from dogs in Florida in the 1990s and 2000s showed that the first positive sera were collected in 2000, indicating that the virus probably transferred to dogs in 1999 or 2000, and therefore had been circulating for 3 or 4 years before it was first identified as the cause of disease. Serological testing showed H3 antibodies in dog sera collected from the greyhounds involved in the different training facilities and racetracks, as well as in the affected animal shelters, with varying proportions (up to 100%) of the dogs in each facility being seropositive [2, 7, 30].

Analysis of viral sequences showed that the H3N8 CIV outbreak was initiated with a single virus that transferred from horses, and descendants of that virus have continued to spread among dogs since that time (Figures 22.1A and C), with no evidence of reassortment with any other influenza A virus [15, 32]. The lineage of CIV in dogs rapidly diverged from the EIV which continued to circulate in horses, and has acquired specific substitutions in each gene segment to form a CIV-specific clade for each gene segment, which are now distinct from the sequences from any of the viruses circulating in horses. Although specific properties of the canine viruses have not been clearly identified, some of the CIV-specific sequence changes may have been selected for canine adaptation [15]. Changes within and near the receptor-binding site of the HA1 protein were shown to alter binding to the sulfated glycans, and probably cause other changes to the sialic acid binding [6], which may favor the replication of the virus in the respiratory tract of the dog. It was shown that EIV strains may readily infect dogs [45, 46], as well as canine tracheal

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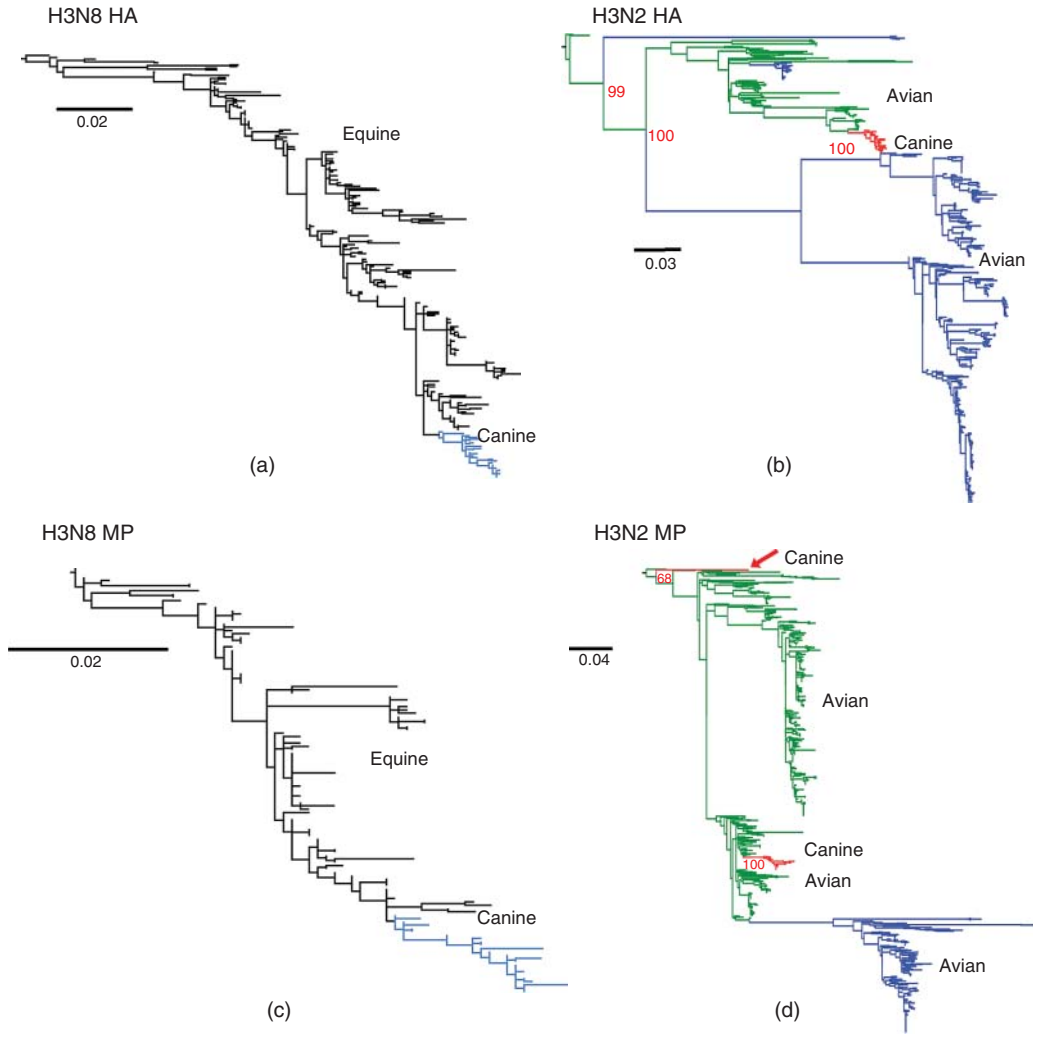

Figure 22.1 Phylogenies showing the origins of the widely circulating CIVs, namely the H3N8 virus that emerged in North America around 2000 as a variant of EIV, and the H3N2 virus that emerged in China and Korea around 2005 as a variant of an avian virus. (A) The H3N8 CIV HA and (C) the MP sequences, compared with the H3N8 EIV sequences from viruses collected from horses at various times after that virus emerged around 1963. Equine viruses are shown in black, and canine isolates in blue. (B) The H3N2 HA and (D) the MP sequences, compared with the sequences of different avian influenza viruses that are in the databases. The CIV sequences are shown in red, the Eurasian avian viruses are shown in green, and the American viruses are shown in blue. Modified from Figure 1 of Hayward, J. J., E. J. Dubovi, J. M. Scarlett, S. Janeczko, E. C. Holmes, and C. R. Parrish. 2010. Microevolution of canine influenza virus in shelters and its molecular epidemiology in the United States. *Journal of Virology* 84:12636–12645, and Figure 1 of Zhu, H., J. Hughes, and P. R. Murcia. 2015. Origins and evolutionary dynamics of H3N2 canine influenza virus. *Journal of Virology* 89:5406–5418.

explant cultures [13], so the amount of adaptation required for the H3N8 EIV to infect dogs appears to be low, and additional changes may favor the transmissibility of the virus among dogs. There is currently no evidence for the transfer of CIV back to horses in nature, and there may indeed be a barrier to such infections, as CIV isolates replicate inefficiently in experimentally challenged horses or horse tracheal cell cultures [31, 44, 46]. The H3N8 EIV has also been isolated from swine with clinical disease in China [42], as well as from Bactrian camels [47].

Epidemiology, spread, and control

By 2004, CIV had spread to at least 11 states in the USA, most frequently as a result of transport of infected racing greyhounds. At around the same time CIV also infected pet dogs, as well as dogs in animal shelters and boarding kennels in several regions of the USA [9, 30]. However, since about 2007, CIV has been primarily maintained in a small number of large animal shelters in large metropolitan areas which have sufficient susceptible animals arriving on a regular basis, as well as enough animals in residence, to allow continuous transmission and maintenance of the virus [9, 15]. Analysis of the viral sequences suggests that those populations can maintain the same viruses in continuous circulation, and infected dogs or virus are frequently transferred from those shelters to other populations of dogs, resulting in outbreaks [30]. These secondary outbreaks primarily occur in smaller shelters, boarding kennels, or among household dogs, and do not generally continue for very long, as the population sizes and turnover rates of susceptible dogs are not sufficient to support continuing transmission, so outbreaks fade out within a few weeks to months after entering the shelter [2, 9, 30].

Disease

The H3N8 CIV in dogs most often causes a respiratory disease with generally mild or subclinical signs, although severe disease is sometimes associated with the infection. Mild disease is associated with a cough that is typically moist, although it can be dry, and this may be associated with a nasal discharge. Symptoms may last for 7–10 days, and animals usually recover uneventfully. Dogs with more severe disease may have high fever (above 42°C) and develop signs very quickly. Virus is found in many of the respiratory tissues, and infection of the trachea, bronchi, and lungs is commonly seen (Figure 22.2) [4]. Pneumonia, including hemorrhagic pneumonia, can develop.

The severity of the disease may depend on the specific circumstances of the infection, and symptoms are exacerbated in particular by co-infections with other pathogens. Inoculation of dogs with EIV alone resulted in subclinical infections, which

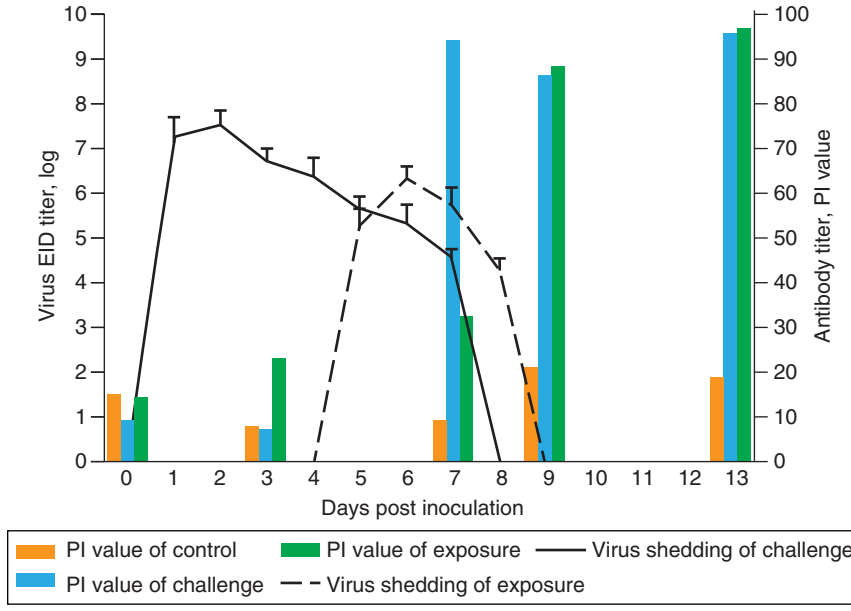

Figure 22.2 The time course of H3N2 canine virus replication in dogs. Virus shedding and the serological response of beagles after experimental contact transmission of H3N2 CIV in dogs. EID, egg infectious dose; PI, percentage inhibition. Source: Figure 1 from Song, D., C. Lee, B. Kang, K. Jung, T. Oh, H. Kim, B. Park, and J. Oh. 2009. Experimental infection of dogs with avian-origin canine influenza A virus (H3N2). *Emerging Infectious Diseases* 15:56–58, with permission.

may reflect the early stages of the CIV outbreak among greyhounds, which was not detected as a new disease over a period of a few years. Co-infection of dogs with CIV and other pathogens results in a more severe disease, and in one study co-infection with *Streptococcus equi* subspecies *zooepidemicus* resulted in much more severe disease and lung involvement than did infection with either pathogen alone [21].

Vaccination

A number of vaccines have been developed against H3N8 CIV. These include a number of different inactivated (killed) virus vaccines, which reduced symptoms of the infection and clinical disease when two doses were given 3 weeks apart [10, 21]. These vaccines are generally recommended for dogs at high risk for respiratory infection, but are not currently given routinely to most household dogs. Experimental canarypox-vectored vaccines that express the HA proteins of EIV or CIV were shown to be effective in reducing virus replication and severity of disease [18].

H3N2 canine influenza

Emergence and subsequent spread

A respiratory disease of dogs in China and Korea was recognized as being caused by an H3N2 influenza virus during 2006 and 2007, and probably arose through the direct transfer of an avian influenza virus, possibly from viruses circulating in live bird markets in Korea or China [35]. That virus spread widely among dogs in South Korea and in several regions of China [25, 26], and was also associated with an outbreak of respiratory disease in Thailand in 2012 [3]. A retrospective serological study demonstrated the presence of anti-CIV antibodies in dogs from Korea in 2005 [23]. The exact origin of the H3N2 CIV virus is not yet known, and phylogenetic analysis of the viral sequences shows that viruses from both China and Korea are close to the ancestor of the canine lineage (Figures 22.1B and D), indicating that there was rapid transfer of the virus between China and Korea, and over long distances within China [39, 43, 51], and the same virus was also associated with an outbreak of respiratory disease in Thailand in 2012 [3].

Infection of other hosts and re-assortments

H3N2 CIV was able to infect cats, and has caused natural outbreaks in cats under some circumstances [16]. When tested for its ability to replicate in other animals, it was found that inoculated chickens, pigs, mice, guinea pigs, and ferrets showed no clear disease, although seroconversion was seen in ferrets, guinea pigs, and chickens, but not in pigs or mice [27]. Virus shedding and lung lesions were seen in guinea pigs and ferrets [27]. Experimental transmission from infected dogs to co-housed cats was reported, while ferrets became infected after experimental inoculation [19], and some limited natural spread between ferrets was detected [24].

Reassortant viruses containing segments of the H3N2 virus and other segments from human viruses have been described, including one virus that contained 7 segments from the H1N1pandemic virus, and the HA segment from the canine virus [38].

Disease

The H3N2 virus appears to generally cause a mild upper respiratory tract disease, although the severity of disease may be greater than that caused by H3N8. In experimental infections, clinical signs were seen after day 1 post inoculation, with the highest clinical score between days 4 and 6, with most replication in the respiratory tract, although some virus was detected in other organs [36, 37, 48] (Figure 22.3). Some more severe infections have been reported, possibly associated with infections of CIV with other respiratory pathogens, or with the expression of genes that induce inflammation and apoptosis [17].

Epidemiology

The spread of H3N2 CIV appears to differ from that seen for the H3N8 virus in the USA, as higher levels of infections were reported in the serological sampling that has been undertaken, with higher levels of antibodies in household dogs, as well as among dogs in some shelters and kennels with large populations and high turnover rates. Most studies that have examined H3N2 antibodies in dogs were based on convenience samples, but the percentage of seropositive dogs ranged from less than 5% up to 30% or more in dogs from several

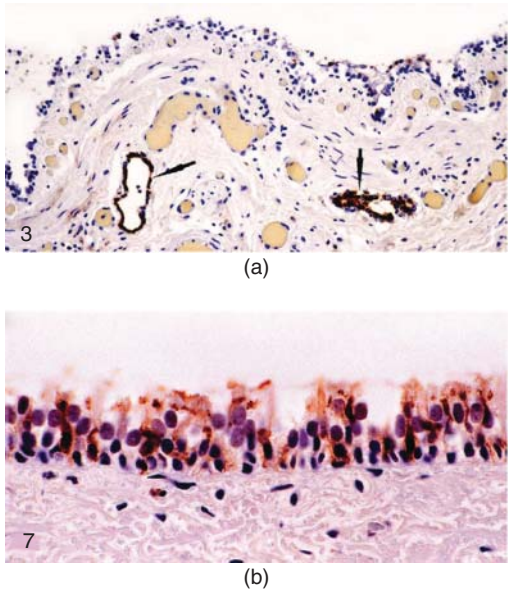

Figure 22.3 Infection and replication of the H3N8 CIV in dogs after natural or experimental infection. (A) Bronchus from greyhound spontaneously infected with canine influenza virus. There is focal epithelial erosion of the surface epithelium. The epithelial cell cytoplasm and luminal macrophage cytoplasm are positive for hemagglutin antigen (arrows). Immunohistochemistry staining is for H3 viral antigen. (B) Trachea from a dog 5 days after inoculation with canine influenza virus. Viral hemagglutinin antigen is present in the cytoplasm of ciliated and non-ciliated cells as well as basal cells. Immunohistochemistry for H3 viral antigen. Derived from Figures 3 and 7 of Castleman, W. L., J. R. Powe, P. C. Crawford, E. P. Gibbs, E. J. Dubovi, R. O. Donis, and D. Hanshaw. 2010. Canine H3N8 influenza virus infection in dogs and mice. *Veterinary Pathology* 47:507–517, with permission of Sage.

areas [50], and high levels of infection have been reported in farmed dogs in China [40].

Vaccination

Commercial inactivated vaccines have been developed for use in dogs in the USA. Their properties and use are similar to the H3N8 virus vaccines described above. Experimental vaccines have been described [22].

Other influenza infections of dogs (and cats)

A number of infections of dogs by other influenza viruses circulating in humans or birds have been reported, with antibodies being detected in most cases, and with disease also being observed in some circumstances. Numerous studies have detected the presence of H1N1pandemic influenza (H1N1p) or other human influenza viruses in dogs and cats. About 0.7% positive sera for H1N1p were reported from dogs in Italy [11], and this virus was also associated with an outbreak of clinical disease in cats, with 55% seropositivity [12]. Testing of cats and dogs in Japan using serological assays showed that 3.8% of cats and 2.1% of dogs were positive for human H3 antibodies [33], whereas higher numbers (20–50% seropositive by HAI assay) were reported for the human influenza viruses in cats in the USA [1], and similar percentages of positive sera were reported from dogs in the USA [28, 34]. The reasons for the high proportions of seropositive animals in the USA compared with other countries are not known, but may be related to the assays used to detect the specific antibodies. An H5N2 avian influenza virus was isolated from a dog in China in 2009 [49]. Serological studies of feral dogs that were frequenting live poultry markets in China showed low but consistent percentages with antibodies to H9N2, H3N2, and H5N1 infection [41]. Dogs and cats may also both be infected by the H5N1 avian influenza virus after experimental challenge [5], and infections may also occur after eating meat from infected animals.

Summary

Infections of mammals by the influenza A virus have been well documented for at least the past 100 years, and extended outbreaks and sustained epidemics have been observed in horses, seals, swine, mink, and humans, but until recently influenza infections of dogs were not widely recognized [14]. However, during the past 16 years, H3N2 and H3N8 influenza A viruses have emerged in separate events, and have spread in epidemic fashion among different dog populations. In addition, other more limited outbreaks have been reported in dogs, and there have also been spillover infections by the canine viruses to other hosts, including cats. Furthermore, there is increasing evidence of low but consistent levels of infection of dogs by some of the circulating human seasonal influenza viruses, and by avian viruses, although probably with little or no disease. Because dogs are frequently exposed

to viruses from other animals, there appear to be significant barriers to cross-species infection, but these barriers can be overcome by different types of influenza viruses under some circumstances.

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23 Sporadic influenza A virus infections of miscellaneous mammal species

Edwin J. B. Veldhuis Kroeze and Thijs Kuiken

Introduction

From the original wild bird reservoir, influenza A viruses (IAVs) have crossed the species barrier at some time or other in the past and established endemic IAV infections in humans, domestic pigs (see Chapters 16–19), horses (see Chapters 20 and 21), and, most recently, domestic dogs (see Chapter 22). However, there seem to be few limits with regard to the range of mammalian species that IAVs can infect. This may be in part due their use of ubiquitous sialosaccharides as the receptor for virus attachment, and their ability to efficiently suppress the host innate immune response. This chapter provides an overview of the many mammals for which there is evidence of sporadic infections by diverse IAVs, namely carnivores, cetaceans, non-human primates, bats, uneven-toed and even-toed ungulates, rodents, lagomorphs, and anteaters.

A game changer in recent years has been the discovery of IAV infection in New World bats. Not only do these IAVs appear to be endemic in these bat populations, but also they are subtypes that are not represented in the wild bird reservoir. Therefore they appear to represent an additional original reservoir of IAVs, and are only included in this chapter because of their recent discovery.

The characteristics that are shared by mammalian species in which IAV has become endemic are large population numbers and aggregation in enclosed spaces (public buildings for human beings, barns for domestic pigs, stables for horses, and kennels for domestic dogs). In these species, IAV infection is present in the population continuously, and the virus has adapted to its host species. At the other end of the scale are mammalian species in which IAV infections are limited to sporadic cases in individual animals due to cross-species transfer, exemplified by the spread of pandemic H1N1 IAV from humans to their pet cats and ferrets. Intermediate between these two extremes are mammalian species in which efficient IAV transmission appears to be possible, but for some reason does not result in persistence of the virus in the population. The multiple reports of avian IAV epidemics in harbor seals (*Phoca vitulina*) are a clear example of this.

However, the situation can change rapidly. Who would have thought 20 years ago that an avian IAV-like H5N1 would have wreaked such havoc among such a wide range of mammals, or that domestic dogs would harbor their own canine-adapted IAV? The scale of global change in animal populations and the ecosystems that they inhabit, together with the plastic nature of IAV, has resulted in a dynamic situation. Therefore the information presented in this chapter should be viewed as a snapshot of the current situation. Today's sporadic infection may be tomorrow's endemic situation.

H5N1 HPAIV infections in miscellaneous mammal species

The H5N1 highly pathogenic avian influenza virus (HPAIV) that emerged in Asia in 1996 in poultry has shown the capacity to infect a wide range of mammalian species, including humans.

Animal Influenza, Second Edition. Edited by David E. Swayne.

In these species, the virus may spread to multiple organs beyond the respiratory tract, resulting in severe disease and death. Natural infections have been reported in multiple species of wild and domestic carnivores [48, 100, 103, 106, 107, 117, 147, 166, 180, 188, 210, 211, 222, 245, 256] (Tables 23.1 and 23.2), domestic pigs [119], black-lipped pikas (*Ochotona curzoniae*) [252], and donkeys [1] (Table 23.3). Serological evidence of natural infection or exposure to H5N1 HPAIV has been recorded in brown rats (*Rattus norvegicus*) [55, 197], raccoons (*Procyon lotor*) [84], and horses [55]. Furthermore, experimental H5N1 HPAIV infections (not extensively discussed here) have been performed in laboratory mice, laboratory rats, laboratory hamsters [73, 128, 133, 195, 198], ferrets (*Mustela putorius furo*) [69, 133, 255], cynomolgus macaques (*Macaca fascicularis*) [112, 184, 185], red foxes (*Vulpes vulpes*) [183], cattle (*Bos taurus*) [98], and laboratory rabbits [252].

Two concerns about the many sporadic cases of mammalian H5N1 HPAIV infection are that they form a source of infection for humans, and they provide the opportunity for the virus to adapt to allow efficient mammal-to-mammal transmission. Until now there has been no concrete evidence of H5N1 HPAIV spreading from infected wild or domestic mammals to humans. With regard to efficient mammal-to-mammal transmission, the only strong evidence has been the probable tiger-to-tiger spread of H5N1 HPAIV at a zoo in Sri Racha, Thailand, in 2004 [222]. Therefore the main source of H5N1 HPAIV infection for humans continues to be poultry, in which the virus continues to circulate in eastern Asia and northern Africa [240].

The first indication that H5N1 HPAIV could spread from birds to mammals other than humans was in December 2003, when fatal H5N1 HPAIV infection was reported in two tigers (*Panthera tigris*) and two leopards (*Panthera pardus*) from a zoo in Suphanburi, Thailand [100]. This was followed by a second outbreak in October 2004 in Sri Racha, Thailand, which involved the death or euthanasia of 147 tigers [222]. Affected felids had high fever, respiratory distress, and (in some cases) nervous signs, and died with serosanguinous nasal discharge 3 days after the onset of clinical signs (Figure 23.1). Autopsy revealed severely congested and hemorrhagic lungs, which corresponded microscopically with bronchointerstitial pneumonia and co-localization of influenza virus antigen expression in pneumocytes. Extra-respiratory spread of the virus was demonstrated by meningoencephalitis and hepatitis, co-localized with influenza virus antigen expression in neurons and hepatocytes, respectively [100, 222]. The felids were initially infected as a result of feeding on fresh poultry carcasses – the H5N1 HPAIV isolates from felids at both zoos were very similar to H5N1 HPAIV strains circulating in poultry at the time [6]. It is likely that tiger-to-tiger transmission of H5N1 HPAIV also occurred at Sri Racha, because the outbreak continued after the feeding of fresh poultry carcasses had been stopped. There was limited evidence of H5N1 HPAIV spread to humans. Five zookeepers at Sri Racha were placed under surveillance after showing influenza-like signs [224]. However, only 2 of 58 zookeepers and veterinarians, neither of whom had shown clinical signs, had anti-H5N1 HPAIV antibodies in their serum 6 weeks after the outbreak [222].

H5N1 HPAIV infections were reported not only in Thailand, but also in Cambodia and China. In December 2003 there was an outbreak of H5N1 HPAIV in 26 species of birds, including birds of prey, in Phnom Tamao Wildlife Rescue Centre, Cambodia. During this outbreak, two lions (*Panthera leo*), two tigers, two Asiatic golden cats (*Catopuma temminckii*), three leopards, and one clouded leopard (*Neofelis nebulosa*) exhibited anorexia and lethargy for 5–7 days, but neither respiratory illness nor mortality. Serum samples were collected from one tiger, one leopard, one Asiatic golden cat, and one clouded leopard, and had neutralizing antibody titers of 10–40 against H5N1 HPAIV. The H5N1 HPAIV isolates from the zoo birds were phylogenetically highly similar to those from poultry in Cambodia, and it was assumed that infected poultry carcasses used as a food source both for birds of prey and for felids were the source of infection [48]. In 2005, a tiger at a zoo in Shanghai, China, died with similar clinical and pathological findings to those in tigers from Thailand. The H5N1 HPAIV isolate from the tiger's lung belonged to clade 2.2, and was phylogenetically almost identical to that isolated in the same year from a migratory duck at Poyang Lake, China. However, it was not reported whether the tiger had consumed chickens or wild birds [147].

Table 23.1 Virological evidence of natural influenza A virus infection in mammals of the suborder Caniformia (dog-like carnivores). Only reports where the virus was detected by virus isolation or RT-PCR are listed.

		Virus				Tissue tropism[a]					
Family	Species	Origin	Subtype	Morbidity	Mortality	Respiratory	Extra-respiratory	Sustained intraspecies transmission	Region[b]	Period	References
Canidae	Domestic dog	Human	H3N2	Yes[a]	No	Yes	No	Yes[a]	AS, EU	1970–71	[35, 150, 161, 189]
		Avian	H5N1	Yes	Yes	Yes	Yes	No	AS	2004	[211]
		Human/canine	H3N1	Yes	No	Yes	No	No	AS	2009–10	[207]
		Human	pH1N1	Yes	No	Yes	Yes[d]	No	AS, NA	2009	[123, 175, 177]
		Swine/avian	H5N2	Yes	No	Yes	Yes[e]	Yes[a]	AS	2009	[75, 209, 248]
		Avian	H9N2	Yes	No	Yes	No	No	AS	2010–12	[220]
		Human/canine	H3N2	Yes	No	Yes	No	Yes[a]	AS	2013	[144]
	Raccoon dog	Avian	H5N1	Yes	Yes	Yes	–[c]	–[c]	AS	2005	[180]
	Red fox	Avian	H5N1	–[c]	–[c]	Yes	Yes	–[c]	EU	2006	[58, 183]
Ursidae	Giant panda	Human	pH1N1	Yes	No	Yes	Yes[e]	No	AS	2009	[118]
Mephetidae	Striped skunk	Human	pH1N1	Yes	Yes	Yes	No	No	NA	2009–10	[23]
Mustelidae	American badger	Human	pH1N1	Yes	No	Yes	No	No	NA	2009	[193]
	American mink	Avian	H10N4	Yes	Yes	Yes	Yes[e]	Yes	EU	1984	[105]
		Avian	H5N1	Yes	No	–[c]	Yes	No	EU	2006	[103, 166, 256]
		Swine	H3N2	Yes	Yes	Yes	No	–[c]	NA	2007	[64]
		Human/swine	H3N2	Yes	Yes	Yes	No	Yes	EU	2009	[38, 115]
		Swine	H1N2	Yes	Yes	Yes	No	Yes	NA	2010	[247]
		Human	pH1N1	Yes	Yes	Yes	No	Yes	EU	2010–11	[3, 38, 104]
	Black-footed ferret	Human	pH1N1	Yes	No	Yes	No	No	NA	2009	[193]
	Domestic ferret	Human	Unspecified IAV	Yes	Yes	Yes	Yes[e]	Yes	EU, NA	1940s	[17, 61]
		Swine	H1N1	Yes	Yes	Yes	Yes[e]	Yes	NA	2008	[163]
		Human	pH1N1	Yes	Yes	Yes	No	No	NA	2009	[172, 173, 178, 179, 233]
	Stone marten	Avian	H5N1	Yes	Yes	Yes	Yes	No	EU	2006	[107]
Phocidae	Harbor seal	Avian	H7N7	Yes	Yes	Yes	Yes	Yes	NA	1979–80	[65, 114, 238, 239]
		Avian	H4N5	Yes	Yes	Yes	Yes	Yes	NA	1982–83	[82]
		Avian	H4N6	Yes	Yes	Yes	No	Yes	NA	1991	[30]
		Avian	H3N3	Yes	Yes	Yes	No	Yes	NA	1992	[30]
		Avian	H3N8	Yes	Yes	Yes	Yes	Yes	NA	2011	[9]
		Avian	H10N7	Yes	Yes	Yes	Yes[f]	Yes	EU	2014	[21, 110, 258]
	Harp seal	Avian	H3N8	–[c]	–[c]	Yes	–[c]	–[c]	NA	2005–07	[22]
	Northern elephant seal	Human	pH1N1	No	No	Yes	No	Yes, restricted	NA	2010	[67]

[a] Also based on results of experimental infections.
[b] AS, Asia; EU, Europe; NA, North America.
[c] –, not determined or not recorded.
[d] Tonsil was reported positive by IHC.
[e] Conjunctivitis and/or ocular discharge was reported.
[f] Spleen was reported PCR positive by Krog and colleagues [10].

Table 23.2 Virological evidence of natural influenza A virus infection in mammals of the suborder Feliformia (cat-like carnivores). Only reports where the virus was detected by virus isolation or RT-PCR are listed.

		Virus				Tissue tropism[a]					
Family	Species	Origin	Subtype	Morbidity	Mortality	Respiratory	Extra-respiratory	Sustained intraspecies transmission	Region[b]	Period	References
Felidae	Domestic cat	Avian	H5N1	Yes	Yes	Yes	Yes	Yes	AS, EU, ME	2004–12	[106, 117, 155, 168, 169, 210, 245]
		Human	pH1N1	Yes	Yes	Yes	No	No	NA,EU	2009	[60, 125, 213]
		Canine	H3N2	Yes	Yes	Yes	–[c]	Yes	AS	2010	[92, 208]
	Cheetah	Human	pH1N1	Yes	No	Yes	No	No	NA	2009	[41]
	Leopard	Avian	H5N1	Yes	Yes	Yes	Yes	Yes	AS	2003	[100]
	Tiger	Avian	H5N1	Yes	Yes	Yes	Yes	Yes	AS	2003–05	[100, 147, 222]
Viverridae	Owsten's palm civet	Avian	H5N1	Yes	No	Yes	Yes	No	AS	2005	[188]
	Bornean binturong	Human	pH1N1	Yes	No	Yes	No	No	NA	2009	[193]

[a] Also based on results of experimental infections.
[b] AS, Asia; EU, Europe; NA, North America; ME, Middle East.
[c] –, not determined or not recorded.

Starting in 2004, there were several reports from all around the world of domestic cats with natural H5N1 HPAIV infection – from Thailand in 2004 [169, 210], Germany and Austria in 2006 [106, 117, 237], Iraq in 2006 [245], Indonesia in 2006 [168], and Israel in 2012 [155]. Most of these reports indicated that contact with or feeding on infected birds was the route of infection, and described severe clinical disease or death in the cats. Five days after eating a pigeon, a cat in Thailand developed high fever, dyspnea, and depression, and it died 2 days later [210]. Several cats on the German island of Rügen were infected by an H5N1 HPAIV that belonged to clade 2.2 [214] and was genetically very similar to an isolate from a dead whooper swan (*Cygnus cygnus*) from the same area [106, 237]. Several cats in Israel showed respiratory signs, weakness, and subsequently died after feeding on turkey carcasses. The H5N1 HPAIV found in the cats was similar to that from the turkeys [155]. In contrast, no overt clinical disease was observed in several cats that had pharyngeal swabs positive for H5N1 HPAIV by PCR after contact with infected birds at an animal shelter in Austria [117].

Experimental H5N1 HPAIV infection, either by intratracheal inoculation or by feeding on infected chicks, showed that cats were susceptible to both severe respiratory disease and widespread extra-respiratory complications. Cats developed not only a severe bronchointerstitial pneumonia, but also severe necrosis and inflammation in the brain, heart, liver, kidney, spleen, adrenal glands, and intestine, co-localized with influenza virus antigen expression in epithelial and mesenchymal cells of these tissues (Figure 23.2) [111, 186, 231]. In addition, hemorrhagic pancreatitis was observed in naturally infected cats [245].

Serological evidence that cats are exposed to or infected with H5N1 HPAIV depends on the situation in poultry. In geographical regions where

Table 23.3 Virological evidence of natural influenza A virus infection in mammals of the orders Perissodactyla (Equidae), Artiodactyla (Bovidae, Camelidae, and Cervidae), and Cetacea (Balaenopteridae and Delphinidae). Only reports where the virus was detected by virus isolation or RT-PCR are listed.

		Virus				Tissue tropism[a]					
Family	Species	Origin	Subtype	Morbidity	Mortality	Respiratory	Extra-respiratory	Sustained intraspecies transmission	Region[b]	Period	References
Equidae	Domestic donkey	Avian	H5N1	Yes	No	Yes	No	No	ME	2009	[1]
Bovidae	Domestic cattle	Swine	H1N1	Yes	No	Yes	No	Suspect	EU	1959	[127, 190]
		Human	H3N2	Yes	No	Yes	No	Suspect	EU, AS	1968, 1971	[33, 127]
	Domestic sheep	Human	H2N2	Yes	No	Yes	Yes[d]	Suspect	EU	1959–60	[127, 190]
Camelidae	Bactrian camel	Human	H1N1	Yes	Yes	Yes	Yes[e]	Yes	AS	1978–88	[244]
		Equine	H3N8	No	No	Yes	No	–[c]	AS	2012–13	[246]
Cervidae	Reindeer	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]	AS	1970s	[127]
Balaenopteridae	Common minke whale	Avian	H1N3	–[c]	No	Yes	Yes	–[c]	South Pacific Ocean	1975–76	[129, 232]
Delphinidae	Long-finned pilot whale	Avian	H13N2, H13N9	Yes	No	Yes	Yes[f]	No	NA coastal waters	1984	[72, 81]

[a] Also based on results of experimental infections.
[b] AS, Asia; EU, Europe; NA, North America; ME, Middle East.
[c] –, not determined or not recorded.
[d] Virus was also isolated from fetus.
[e] Conjunctivitis and/or ocular discharge was reported.
[f] Virus was also isolated from hilar lymph node.

H5N1 HPAIV was endemic in poultry, the following proportions of cats were found to be seropositive: 8 of 111 (7%) in central Thailand [28], 100 of 500 (20%) on Java and Sumatra [167], and 9 of 25 (36%) in endemic areas of Egypt [55]. In contrast, no cats were found to be seropositive in areas of Europe where H5N1 HPAIV had occurred in birds as an epidemic [138, 160].

Guidelines for prevention and management of H5N1 HPAIV infections in pet cats were published by Kuiken and colleagues and by the European Advisory Board on Cat Diseases [223]. In areas where H5N1 HPAIV has been detected in poultry or wild birds, cat owners should avoid feeding uncooked poultry meats, and keep cats indoors to prevent contact between their pets and infected birds or their droppings. In suspected cases of H5N1 HPAIV infection in cats, veterinarians and cat owners should maintain stringent hygienic measures with regard to animal handling, and quarantine and test the affected cat(s). An inactivated, adjuvanted heterologous H5N6 avian influenza virus vaccine has been shown to protect cats against fatal disease from H5N1 HPAIV infection [231].

H5N1 HPAIV infection has also been reported in domestic dogs, but the associated disease appears to be milder than in cats. There is only one case report of natural H5N1 HPAIV infection in a dog

Figure 23.1 Natural infection of tigers with H5N1 highly pathogenic avian influenza virus in Sri Racha, Thailand, in 2004. Affected animals had high fever, respiratory distress, and (in some cases) nervous signs, and died with serosanguinous nasal discharge. Photograph courtesy of Dr. Roongroje Thanawongnuwech, Chulalongkorn University, Thailand. *See Plate section for color representation of this figure*.

[211]. Like cats, the dog was probably infected by feeding on infected birds. It developed high fever, dyspnea, and lethargy 5 days later, and died the following day. Autopsy revealed severe pulmonary congestion and edema, which correlated histologically with interstitial pneumonia and influenza virus antigen expression in pulmonary alveolar cells. Extra-respiratory spread of virus was demonstrated histologically by multifocal hepatic necrosis and tubulonephritis, which co-localized with influenza virus antigen expression in hepatocytes and epithelial cells of the glomeruli and renal tubules, respectively. The H5N1 HPAIV isolated from lung, liver, kidneys, and urine was genetically similar to that recovered earlier from a tiger in Thailand [211]. In experimental H5N1 HPAIV infections in dogs, clinical signs ranged from transient fever and conjunctivitis [130] to anorexia, fever, conjunctivitis, labored breathing, cough, and death in one of six dogs [37]. In contrast to the fatal case reported by Songserm and colleagues [211], virus replication and associated lesions in experimentally infected dogs were restricted to the respiratory tract. The high percentage of dogs with specific antibodies to H5N1 influenza virus suggests that dogs are commonly infected with or exposed to the H5N1 HPAIV in areas where the virus is endemic in poultry (160 of 629 dogs (25%) in central Thailand [28], and 4 of 25 dogs (16%) in endemic areas of Egypt [55].

There is one report of H5N1 HPAIV infection associated with die-off in raccoon dogs (*Nyctereutes procyonoides*), which belong to the family Canidae [180]. About 100 of a total of 1000 raccoon dogs from a fur farm in China died with respiratory disease, diarrhea, or both in 2005. Genetic and molecular characterization identified the viruses, which were isolated from the lungs of two of the dead raccoon dogs, as H5N1 HPAIV. It was assumed that chicken carcasses fed to the raccoon dogs were the source of infection [180]. None (0%) of 102 free-living raccoon dogs sampled in South Korea in 2011 had antibodies against IAVs [34].

Red foxes (*Vulpes vulpes*), which belong to the family Canidae, are an important predator on and scavenger of wild and domestic birds, and may potentially be exposed to H5N1 HPAIV by this route. An H5N1 HPAIV (A/fox/Azerbaijan/1413/2006) was isolated from a fox in Azerbaijan in 2006 [58]. Experimental infections show that red foxes excrete virus from the throat for up to 7 days after inoculation. Ingestion of infected chicks causes subclinical infection or mild pneumonia, whereas intratracheal inoculation causes severe pneumonia, myocarditis, and encephalitis. Together these results demonstrate that red foxes might play a role in virus dispersal [183].

There is one report of fatal H5N1 HPAIV infection in Owston's palm civets (*Chrotogale owstoni*), a globally threatened species belonging to the family Viverridae. It involved three Owston's palm civets that were kept together in captivity at a national park in Vietnam in 2005. They showed anorexia and neurological signs, including hind limb paralysis, for 1 or 2 days before death. Pathological examination revealed interstitial pneumonia, meningitis, cerebral edema, and multifocal hepatic necrosis. H5N1 HPAIV was detected by virus isolation, RT-PCR, and immunohistochemistry in all of these tissues, as well as in kidney and intestine, demonstrating systemic viral infection. Although the H5N1 HPAIV from the Owston's palm civets was similar to that in poultry, and undiagnosed poultry deaths were reported in the surroundings of the park, the civets were not fed on bird carcasses, so the source of infection remains unknown [165, 188].

There are reports of single cases of H5N1 HPAIV infection in a stone marten (*Martes foina*) [107] and an American mink (*Mustela vison*) [166], both of

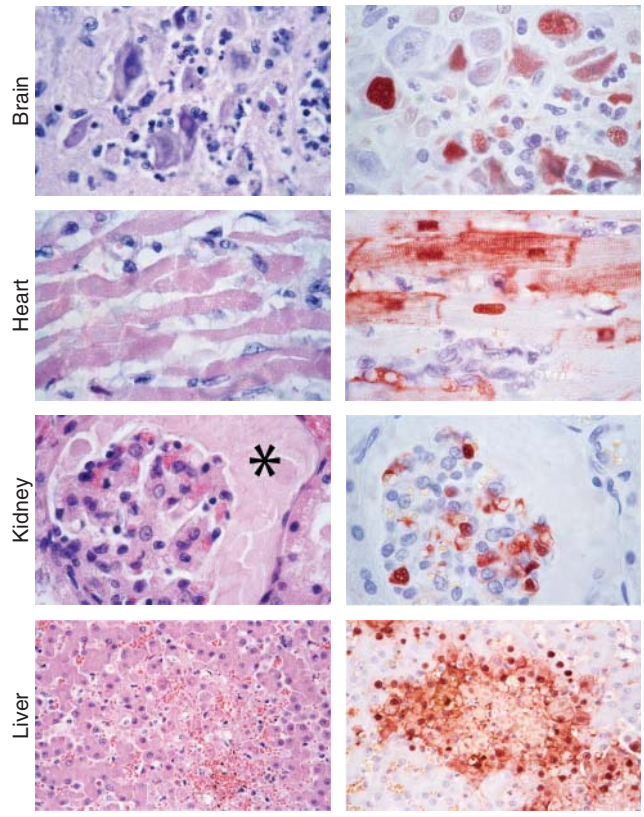

Figure 23.2 Systemic histological lesions in domestic cats after experimental HPAIV H5N1 infection. The left-hand column shows necrotizing inflammatory foci present in multiple tissues stained with hematoxylin and eosin. The right-hand column shows influenza virus antigen (red-brown staining) present in serial sections of the same tissues, stained for nucleoprotein by immunohistochemistry. Reprinted from *The American Journal of Pathology*, January 2006, Vol. 168, No. 1, pp. 176–183, Rimmelzwaan G. F., van Riel D., Baars M., Bestebroer T. M., van Amerongen G., Fouchier R.A., Osterhaus, A. D., Kuiken, T. Influenza A virus (H5N1) infection in cats causes systemic disease with potential novel routes of virus spread within and between hosts, with permission from Elsevier. *See Plate section for color representation of this figure*.

which belong to the family Mustelidae. The stone marten was from the Isle of Rügen, north Germany, and the American mink was from south Sweden. Both animals were free-living, had neurological signs, and were identified in 2006. They were probably infected as a result of feeding on infected wild birds. Histopathological examination of the stone marten revealed encephalitis and pancreatic necrosis, co-localized with influenza virus antigen expression in neurons and pancreatic acinar cells, respectively [107]. Surprisingly, neither pneumonia nor influenza viral antigen were observed in the lungs, which contrasts with the pneumotropism of H5N1 HPAIV in most other mammals. Molecular characterization of the Swedish mink isolate (A/Sweden/mink/V907/2006) revealed no specific adaptation to mammals [103, 256].

There is only serological evidence of H5N1 HPAIV infection in raccoons (*Procyon lotor*), which belong to the family Procyonidae. In total, 10 (0.9%) of 1088 healthy free-living raccoons that were sampled in Japan between 2005 and 2009 had virus-neutralizing antibodies to H5N1 IAV, but not to viruses of other hemagglutinin (HA) subtypes, including H1, H3, H7, and H9. During that period, Japan experienced two outbreaks of H5N1 HPAIV on poultry farms and one in free-living swans. Therefore it is likely that the raccoons became infected or exposed by feeding on infected bird carcasses [84].

There is also only serological evidence of H5N1 HPAIV infection in brown rats (*Rattus norvegicus*), belonging to the family Muridae. Hemagglutination-inhibiting antibodies to H5N1 IAV were found in some brown rats sampled at live poultry markets in Hong Kong during the 1997 H5N1 HPAIV outbreak [197], and in 1 (1.4%) of 72 brown rats sampled in Cairo, Egypt, and the surrounding area after H5N1 HPAIV became endemic in poultry there in 2006 [55]. Experimentally, not only laboratory rats, but also laboratory mice (*Mus musculus*), of the family Muridae, and hamsters (*Mesocricetus auratus*), of the family Cricetidae, develop both a productive infection and associated lesions upon H5N1 HPAIV inoculation [133, 195, 198].

There is one report of H5N1 HPAIV infection in free-living black-lipped pikas (*Ochotona curzoniae*), of the family Ochotonidae, which together with rabbits and hares belong to the order Lagomorpha. Evidence of H5N1 HPAIV infection was found in black-lipped pikas sampled between August 2006 and December 2007 in their natural habitat around Qinghai Lake, China [252], where there had been a large-scale outbreak of H5N1 HPAIV infection in migratory birds [36, 124, 253]. Initially, hemagglutination-inhibiting antibodies to H5N1 IAV were detected in 11 (13%) of 82 pikas. Subsequently, H5N1 HPAIV was isolated from brain, lung, and rectum samples from 5 (3%) of 147 newly caught pikas. Phylogenetically, these isolates could be divided into a mixed/Vietnam H5N1 lineage and a wild bird Qinghai-like H5N1 lineage. Presumably the black-lipped pikas contracted these viruses from wild birds at common weed-foraging sites. Experimental infection of rabbits (*Oryctolagus cuniculus*), of the family Leporidae, resulted in a productive infection and interstitial pneumonia, with influenza virus antigen expression in epithelial cells of nasal turbinates, trachea, and lungs [252].

Recently, the host range of H5N1 HPAIV has been extended to include donkeys (*Equus africanus asinus*), which together with horses (*Equus ferus caballus*) belong to the family Equidae. In an Egyptian village in 2009, H5N1 HPAIV was isolated from pooled nasal swabs from three donkeys with mild respiratory disease. These donkeys showed coughing, fever, and serous nasal discharge for 72 hours. Onset of these respiratory signs was 1 week after an outbreak of H5N1 HPAIV infection in poultry in the same village. Phylogenetic analysis of the isolate from the donkeys showed close homology to the lineage of Egyptian H5N1 HPAIV viruses circulating in poultry and humans. Subsequently, antibodies against H5N1 IAV were found in 27 (26%) of 105 donkeys from areas where H5N1 HPAIV was endemic in poultry. Possible routes of infection included aerosol exposure to bird feces, feed or water contaminated with bird feces, or direct contact with infected birds. Concerns were raised that donkeys commonly housed with poultry might spread a mammal-adapted H5N1 HPAIV to humans [1]. In a later serological survey, El-Sayed and colleagues [55] found antibodies against H5N1 not only in donkeys but also in horses from H5N1-endemic areas in and around Cairo.

Although cattle (*Bos taurus*), of the family Bovidae, may be naturally infected with IAV [33, 127], there are no reports of natural H5N1 HPAIV infection in cattle. Experimentally, four calves that were inoculated intranasally with H5N1 HPAIV from a naturally infected cat had a subclinical infection with low virus excretion from the nose. There was no firm evidence of calf-to-calf transmission; although one of two sentinel calves housed together with the inoculated calves seroconverted, the nasal swabs of both sentinel calves remained negative for H5N1 HPAIV RNA throughout the experiment [98].

Other influenza A virus infections in miscellaneous mammal species

Influenza A viruses in the order Carnivora, suborder Caniformia

Various species belonging to the order Carnivora, suborder Caniformia, have been infected with IAV (Table 23.1), and are described in detail in the following sections.

Influenza A viruses in the family Canidae

Sustained circulation of canine-adapted IAVs is a recent phenomenon. In 2002, an H3N8 IAV originating from horses caused an outbreak of respiratory disease in a pack of 92 English foxhounds in the UK. Although the route of transmission is not known, the dogs were housed adjacent to horse stables, and had recently been fed the

meat of two euthanized horses [43]. Two years later, another horse-origin H3N8 IAV caused an outbreak of respiratory disease in greyhound dogs in Florida, USA [39]. Starting in 2007, an H3N2 low-pathogenicity avian influenza virus (LPAIV) originating from birds caused respiratory disease outbreaks in dogs in South Korea [206], China [121], and Thailand [27]. These H3N8 and H3N2 viruses have now adapted to their new hosts, are able to spread efficiently in domestic dog populations, and are the recognized etiological agents of this new disease – canine influenza (for a detailed review, see Chapter 22).

In contrast to these dog-adapted IAVs, it has been recognized for years that human-origin IAVs may sporadically jump the species barrier and infect domestic dogs. Experimentally, the susceptibility of dogs to human H1N1 IAV infection was demonstrated as early as 1959 [2], and natural infection was first demonstrated in 1975, when human H3N2 IAV was isolated from affected dogs [35, 189]. Studies showing serological responses in dogs to human H3N2 IAV [63, 101, 189] also suggested that there was transmission of virus from humans to dogs. Both natural and experimental human H3N2 IAV infections in dogs are usually subclinical [35, 85, 101, 150, 161, 225], although they may cause transient fever [150]. Human H3N2 IAV was transmitted to sentinel dogs housed together with experimentally inoculated dogs [150].

When the most recent influenza pandemic, caused by pandemic H1N1 IAV (pH1N1), occurred in humans, it was also reported in domestic dogs in China [175] and the USA [177]. The pH1N1-positive dog from the USA had clinical evidence of pneumonia, with fever, coughing, and anorexia. pH1N1 had been confirmed in the dog's owner 1 week previously. Experimental inoculation of the canine isolate from China into dogs resulted in mild clinical signs and inefficient dog-to-dog transmission [123]. In contrast, inoculation of a human isolate of pH1N1 into dogs did not cause infection [11].

Recently, two reassortants of pH1N1 and H3N2 canine influenza virus (CIV) have been isolated in South Korea from nasal swabs from domestic dogs with respiratory signs. The first reassortant, H3N1, had the HA gene segment of H3N2 CIV and the remaining seven gene segments of pH1N1. Experimental inoculation into dogs resulted in a subclinical infection with virus shedding from the nose. At autopsy, the severity of pneumonia was intermediate between the mild lesions of pH1N1 IAV infection and the marked lesions of H3N2 CIV infection [207]. The second reassortant, H3N2, had the M gene segment of pH1N1 and the remaining seven gene segments of H3N2 CIV. Experimental infection of dogs resulted in similar virus shedding, dog-to-dog transmission, and severity of pneumonia as classic H3N2 CIV infection [144].

In 2009, an H5N2 LPAIV was isolated in China from nasal swabs from domestic dogs with respiratory signs [209, 248]. Experimentally infected dogs shed virus, had transient fever, and developed mild respiratory signs [209]. The virus was transmitted from infected dogs both to sentinel dogs [209] and to a cat and chickens [75].

An H9N2 LPAIV was detected by culture and PCR in 13 (2.2%) of 588 juvenile to young adult domestic dogs with clinical signs (coughing, vomiting, fever) in Guangxi, China, in 2010 and 2011. Serologically, up to 45% of dogs tested positive. The virus, known as A/canine/Guangxi/1/2011 (H9N2), showed more than 98.5% genetic homology with Eurasian-lineage H9N2 LPAIV [220]. Dogs could be infected by intranasal inoculation [250], but not by feeding on infected chickens [5]. Although virus was recovered from nasal turbinates, trachea, and lung in association with a mild pneumonia, infection was subclinical and no virus was shed from the upper respiratory tract. In contrast, intranasally inoculated dogs in another experiment [5] had mild respiratory signs, shed virus from the nose, and infected sentinel dogs. Concerns were raised that dogs may contribute to further spread, and adaptation to mammals, of this widely circulating Eurasian LPAIV.

Control of influenza in domestic dogs should include routine hygiene measures, such as isolation of infected dogs to prevent virus spread to other animals [230]. Because dogs are susceptible to avian IAV infection, live in close proximity to humans, and may have access to poultry at live animal markets, especially in South-East Asia, they are potential intermediate hosts for virus spread to humans. Furthermore, because dogs are susceptible to both human and avian IAVs, they have the potential to serve as a "mixing vessel" in which new reassortants may arise, as has been

seen recently for reassortant H3N1 and H3N2 IAVs [144, 207].

Influenza A viruses in the family Ursidae

Bears are long-lived, wide-ranging, opportunistic animals that one would expect to be easily exposed to infectious agents from a wide range of animals and humans. However, reports of exposure to IAVs in bears are rare. There is one report of weak positive IAV (and influenza B virus) serum antibody titers in a juvenile captive Eurasian brown bear (*Ursus arctos arctos*) from Croatia, suggesting exposure to infected humans [132]. More compelling evidence of infection with IAVs was found in giant pandas (*Ailuropoda melanoleuca*) by Li and colleagues [122]. In 2009, during the human H1N1 pandemic, three captive giant pandas from a conservation center in Sichuan Province, China, showed clinical signs of respiratory disease. A nasal swab taken from one animal tested positive by PCR for the HA gene of pH1N1, and by culture for IAV. Phylogenetic analysis of the virus isolate suggested human-to-panda transmission without significant adaptation. All three pandas received 75 mg of oseltamivir twice daily for 5–6 days, recovered, and seroconverted to pH1N1 [118].

Influenza A viruses in the family Ailuridae

There is a report of weak positive IAV-nucleoprotein antibody titers by agar gel immunodiffusion in one of 73 captive red pandas (*Ailurus fulgens*) from China [181]. The source or type of influenza virus was not specified.

Influenza A viruses in the family Mustelidae

The American mink (*Mustela vison*) is a mustelid species that is kept in captivity in large numbers for its fur. Recently this species has been placed in a separate genus (*Neovison vison* or *Vison vison*) from the domestic ferret (*Mustela putorius furo*) and the European mink (*Mustela lutreola*), based on molecular phylogeny [78]. The susceptibility of American mink to IAV infection has been recognized for several decades. In the late 1970s and early 1980s, antibodies against human H3N2 and H1N1 IAVs were detected in farmed mink from Japan [157, 159]. Inoculation of mink with human H3N2 IAV resulted in a productive infection with respiratory signs, and transmission to contact mink. Productive infection of mink also resulted from inoculation of human H1N1, swine H1N1, equine H1N2, and avian H3N2 and H4N1 IAVs [139]. In similar experiments [156, 243], inoculation of different avian (H3N8, H5N3, H7N2, H7N7, H8N4, and H11N4) or mammalian (human and swine H1N1, and equine H2N2) IAVs also resulted in productive infection, with transmission to contact mink.

In 1984, H10N4 IAV, probably of avian origin, caused an outbreak of severe respiratory disease with 100% morbidity and 3% mortality in 100 000 mink on neighboring farms in Sweden [105]. Clinical signs included anorexia, sneezing, coughing, and nasal and ocular discharge. Pathological examination of fatal cases showed an acute interstitial pneumonia. Experimental infection in mink induced similar clinical signs and pathological changes, with transmission to sentinel mink separated by a wire fence. The presumed origin of the virus was wild birds (corvids, gulls, and ducks) that were attracted to the tops of the open wire cages by offal fed to the mink [105]. Interestingly, comparative infections of mink with either H10N4 IAV (A/mink/Sweden/3900/1984) or H10N7 IAV (A/chicken/Germany/N/1949) revealed that only H10N4 IAV was transmitted to sentinels, and that it caused more severe pneumonia than H10N7 IAV [56, 57]. Recent full-genome analysis of the viruses showed that the non-structural (NS) gene of H10N4 IAV may have contributed to its virulence for mink by helping the virus to evade the innate immune response [257].

In 2006 and 2007, swine H3N2 IAV was associated with increased respiratory disease and mortality in mink farmed in Nova Scotia, Canada. Clinical signs included dry cough, and pathological examination of fatal cases revealed interstitial pneumonia and bronchiolitis. The virus isolated from affected mink was related to a triple reassortant swine IAV that had emerged in 2005. The presumed route of transmission was the feeding of uncooked meat by-products, including ground swine lung from parts of Canada where swine IAV H3N2 was known to occur [64].

In 2009 and 2010, a human/swine reassortant H3N2 IAV caused an outbreak of respiratory disease in mink on 18 farms in Denmark. Clinical signs included sneezing, coughing, and hemorrhaging from the nose, and the average mortality rate was 1.2%. The HA and (neuraminidase) NA genes of the isolated virus were homologous with human

H3N2 IAV, and the six remaining genes were homologous with a circulating swine H1N2 IAV. These findings suggest that mink are susceptible to infection by both swine and human IAVs, and may act as a "mixing vessel" [115]. The probable source of infection was feeding of raw offal, including swine tracheas and lungs. All of the affected mink farms received this offal from the same slaughterhouse. The outbreak, which lasted for 10 weeks, may have been sustained by continued feeding of infected offal, by horizontal transmission, or both [38].

In 2010 and 2011, human pH1N1 IAV caused respiratory disease outbreaks on several mink farms in Denmark, Norway, and the Netherlands [3, 38, 104]. Clinical signs included nasal discharge, coughing, and sneezing in vixens, and dyspnea in kits. Mortality rates in kits ranged from 14% in Norway to 30% in the Netherlands. Pathological examination of dead kits showed severe acute interstitial pneumonia. Phylogenetic analysis revealed that the virus isolated from Norwegian mink closely resembled human pH1N1 IAV from 2009 that circulated among people in Norway during the winter of 2010–2011. However, respiratory symptoms were not reported for the Norwegian mink farmers at the time of the outbreaks, and feeding of pig offal was considered the most likely source of infection. Dutch mink farmers were suffering from an influenza-like illness at the time of the outbreaks. It was not reported whether mink on Dutch farms were directly exposed to swine or fed on raw swine offal [3, 104].

In 2010, avian/swine reassortant H1N2 IAV caused respiratory disease on a farm in the Midwestern USA that had 15 000 mink. Clinical signs included persistent severe respiratory distress, and hemorrhaging from the nasal and oral orifices, and mortality rates were approximately 3%. Pathological examination of fatal cases revealed a hemorrhagic bronchointerstitial pneumonia associated with H3N2 IAV and hemolytic *Escherichia coli*. Phylogenetic analysis revealed that the virus had a matrix gene and a nucleoprotein gene that showed genetic relatedness to the swine lineage of IAV. The source of the infection appeared to be feeding of raw turkey meat; no swine offal was fed, and there were no swine herds nearby [247].

Conclusions from the above reports are that American mink are highly susceptible not only to infection, but also to severe disease caused by human, avian, and swine IAVs, and that efficient mink-to-mink transmission is possible. Thus mink may serve as "mixing vessels" that facilitate the reassortment of IAVs from different host species [115]. In addition, commonly recurring sources of infection include open housing, allowing contact with wild birds, and feeding of raw products from IAV-infected animals, such as swine and poultry. Consequently, the use of housing that prevents contact with wild birds, and the cooking of animal products prior to feeding [247] are important measures for prevention of influenza in American mink.

The domestic ferret (*Mustela putorius furo*) originates from the European polecat (*Mustela putorius*), and has been used since the 1930s in animal models for IAV infection in humans. In part this can be explained by the similarity in the pattern of IAV attachment to different parts of the ferret and human respiratory tracts [235]. The ferret proved highly susceptible to infection with both human [202] and swine IAVs [196]. Furthermore, human IAV infection in ferrets induced similar clinical signs to those observed in humans, namely fever, lethargy, anorexia, and nasal catarrh. In contrast, swine IAVs induced more severe disease, and death. In 1934, Shope provided detailed, accurate, and well-illustrated descriptions of the associated lesions in affected ferrets, both grossly and microscopically [196]. IAV transmission was demonstrated from ferrets to humans [203], and among ferrets, both by direct contact [216] and by air [8]. Numerous studies on vaccine efficacy, antiviral products, pathogenesis and transmission, and virus reassortment, all relating to IAVs, have been performed in ferrets [12, 14, 49, 83, 86, 89, 90, 102, 109, 201, 226]. In these studies, inoculation of many human and avian IAVs resulted in productive infection and disease.

Based on the above information, one would therefore expect natural IAV epidemics in ferrets to be common. However, even individual cases of natural IAV infection in ferrets, let alone epidemics, are rarely reported. Fisher and Scott reported natural IAV infection in ferrets in 1944 [61]. Subsequently, Bell and Dudgeon reported an outbreak of IAV infection in two ferret colonies in Sussex, in the UK, in February 1947 [17]. The affected ferrets exhibited nasal and ocular

discharge, blepharosynechia, sneezing, lethargy, and fever for about 7 days, and eight ferrets died. Remarkably, none of these animals exhibited gross lung lesions at autopsy, although their nasopharynges were congested. The animal attendants had influenza-like symptoms immediately before and during the outbreak, and were assumed to be the source of infection. The widespread seroconversion against IAV in these group-housed ferrets suggested that there was efficient ferret-to-ferret transmission of IAV [17].

There were multiple cases of pH1N1 IAV in pet ferrets in the USA in 2009. The affected ferrets displayed mild to severe respiratory disease, and some died. Clinical signs included fever, lethargy, sneezing, and coughing. In all cases, humans in the household were suffering from influenza, and were the probable source of infection [172, 173, 178, 179]. Ferrets that were infected experimentally with pH1N1 IAV showed similar clinical signs, and at autopsy exhibited multifocal necrotizing bronchointerstitial pneumonia [201, 233].

Natural infection of ferrets with swine IAVs was not reported until 2009, when there was an outbreak of contemporary reassortant swine H1N1 IAV in a ferret colony in the USA [163]. Ferrets showed typical respiratory signs, and at autopsy exhibited bronchointerstitial pneumonia with necrotizing bronchiolitis. The genetic characterization of the isolated virus suggested that swine was the source of infection.

In October and November 2009, pH1N1 IAV infection occurred in an American badger (*Taxidea taxus*) and a black-footed ferret (*Mustela nigripes*) that were housed separately in a zoo in California, USA. Clinical signs included lethargy, inappetence, dyspnea, nasal discharge, and coughing. The American badger was euthanized due to the severity of disease, and at autopsy exhibited bronchopneumonia with IAV antigen expression. pH1N1 IAV was identified in lung samples from the American badger and swabs from the black-footed ferret by PCR and sequencing. Humans were assumed to be the source of infection [193].

Serological evidence of pH1N1 IAV infection was found in free-ranging northern sea otters (*Enhydra lutris kenyoni*), with an estimated age range of 2–19 years, captured off the coast of Washington, USA, in August 2011. ELISA revealed that 21 (70%) of 30 sea otters had detectable IgG (>200 mg/dL) for rHA of pH1N1 (A/Texas/05/2009); 22 (73%) of these 30 animals had HI antibody titers of ≥ 40 against pH1N1 virus [122]. The source of infection remains unknown, although potential contact between pH1N1-IAV-infected northern elephant seals (*Mirounga angustirostris*) [67] and sea otters was considered to be one possibility, as their feeding ranges and breeding areas along the North-East Pacific coast overlap [122].

Influenza A viruses in the family Procyonidae

There is serological evidence of natural avian IAV infection in raccoons (*Procyon lotor*). Of 730 free-living raccoons sampled between 2004 and 2006 in several states of the USA (California, Texas, Louisiana, Maryland, Wyoming, and Colorado), 2.4% had antibody to avian IAVs of the subtypes H10N7, H4N6, H4N2, H3, and H1 [76]. Presumably they were infected by direct or indirect contact with infected wild waterbirds. Intranasal inoculation of avian H4N8 IAV (A/chicken/Alabama/1975) into raccoons resulted in subclinical infection with nasal shedding up to 14 days post inoculation (DPI), and transmission to sentinel raccoons [76]. In another experiment, exposure of raccoons to avian H4N6 IAV via drinking and washing water only led to a productive infection at a high dose, and exposure via infected eggs and waterfowl carcasses did not lead to infection [191]. These results, together with the peridomestic nature of raccoons, suggest that this species is capable of infecting poultry and swine [76].

Influenza A viruses in the family Mephetidae

Between December 2009 and January 2010, eight striped skunks (*Mephitis mephitis*) died on a mink farm near Vancouver, Canada. Autopsy of two of these animals showed splenomegaly and pneumonia on gross examination. Histopathological findings included rhinitis, bronchopneumonia with intralesional bacteria, multifocal interstitial pneumonia, and plasmacytosis of lymph nodes and spleen. Both pH1N1 IAV and Aleutian disease virus were identified in organ samples by PCR and sequencing. The cause of death was determined as primary influenza viral pneumonia with secondary bacterial infection. The presumed source of both viruses was the population of co-habiting farmed American mink, some of which had nasal discharge. However, the possibility of direct IAV

transmission from humans to striped skunks could not be excluded [23].

Influenza A viruses in the families Phocidae, Odobenidae, and Otariidae

Reports of natural IAV infection are more frequent in pinnipeds – a mammalian clade of the order of carnivores that includes the Odobenidae (walruses), the Phocidae (true seals), and the Otariidae (fur seals and sea lions).

There have been repeated avian influenza A virus (AIV) outbreaks in harbor seals (*Phoca vitulina*), with efficient seal-to-seal transmission and high mortality. The first recorded outbreak, involving H7N7 AIV, occurred on Cape Cod Peninsula, New England, USA, in the winter of 1979–1980 [65, 114, 239]. Clinical signs included dyspnea, lethargy, emphysema of the neck, and frothy white to red discharge from the nose and mouth. More than 400 harbor seals, mostly juveniles, died, with an estimated mortality rate of 20% [65]. This high number suggests efficient seal-to-seal transmission, yet apparently the virus was not able to persist in the harbor seal population. Autopsy showed pneumonia characterized by necrotizing bronchitis and bronchiolitis, and hemorrhagic alveolitis [65]. H7N7 AIV was isolated at high titers from the lung and at lower titers from the brain of diseased harbor seals. Experimentally infected harbor seals also developed pneumonia, but this was less severe than in natural cases [239]. Antibodies against this virus were found in sera of gray seals (*Halichoerus grypus*) from Nova Scotia, Canada, more than 500 miles from Cape Cod, but no mortality of gray seals was reported [65]. Although avian in origin, the virus replicated more efficiently in mammals (ferret, cat, and pig) than in birds (chicken and turkey), thus suggesting adaptation to mammals. This included accidental human infection during a seal autopsy, which resulted in conjunctivitis, but no human-to-human transmission [238]. Experimental conjunctival inoculation in squirrel monkeys also induced conjunctivitis, along with respiratory disease and systemic viral spread [145]. The source of the virus was not determined, but was suggested to be waterbirds such as terns (*Sterna* species), as they were known to harbor IAVs and to associate with harbor seals in water and on land. Possible factors contributing to the outbreak were abnormally high population densities and unseasonably high temperatures, which led harbor seals ashore [65].

In the winter of 1982–1983, there was an outbreak of avian-origin H4N5 IAV infection in harbor seals from the New England coast, USA. Approximately 60 harbor seals died, and the mortality rate was estimated to be 2–4%. Histopathological examination revealed a necrotizing bronchopneumonia, and H4N5 IAV was isolated from lungs, hilar lymph nodes, and brains of affected harbor seals. Interestingly, this virus did replicate in duck intestines upon intranasal inoculation, in contrast to earlier avian-origin IAV isolates from mammals [82].

In January 1991 and January 1992 there were outbreaks of avian-origin IAV infection of the subtypes H4N6 and H3N3, respectively, in harbor seals from Cape Cod, Massachusetts, USA. Autopsy showed subcutaneous emphysema and acute interstitial pneumonia, acute hemorrhagic pneumonia, or both [30].

From September to December 2011, there was an outbreak of avian-origin H3N8 IAV in harbor seals from New England, USA. A total of 162 harbor seals were found dead, and autopsy showed acute pneumonia. Based on genetic analysis, the virus isolated from the lungs was closely related to H3N8 IAV circulating in waterfowl [9]. Interestingly, an avian-origin H3N8 IAV had been detected by PCR in a harp seal (*Phoca groenlandica*) caught in coastal waters of the North-West Atlantic Ocean several years previously [22]. It was not reported whether this seal had respiratory disease. The harbor seal H3N8 IAV had a D701N amino acid substitution in the PB2 protein. This substitution was also found in H5N1 HPAIV infecting humans [42, 192], and indicates adaptation to virus replication in mammals. Based on agglutination assays, this virus had an affinity not only for avian-type sialic acid α2,3-galactose (SAα2,3)-linked receptors, but also to human-type sialic acid α-2,6 galactose (SAα-2,6)-linked receptors. These mammalian adaptations pose an increased risk of human infection [9].

More recently, between March and October 2014, an outbreak of avian-origin H10N7 IAV infection in harbor seals spread southward along the North-Western European coasts of Sweden, Denmark, Germany, and the Netherlands [21, 110, 258]. Unusually high numbers of dead stranded seals (around 2000 in total) were found. Similar

pulmonary lesions of acute bronchointerstitial pneumonia with emphysema to those reported earlier in the North American outbreaks were found. The virus was detected in the lungs [21, 110, 258] and spleen [110]. The HA and NA genes of this seal virus were genetically closely related to those of H10N7 IAVs recently found in migratory ducks from Georgia, Egypt, and the Netherlands [21].

In April 2010, human-origin pH1N1 IAV infection was detected in northern elephant seals (*Mirounga angustirostris*) from California, USA. The virus was isolated from nasal swabs from 2 of 42 apparently healthy adult females, which had just come ashore after months at sea. Genetic sequencing of the seal isolate revealed more than 99% homology with pH1N1 IAV that had emerged in humans in 2009. Humans were the most likely source of infection, although human exposure at sea was limited to shipping vessels. Possible adaptation of this isolate to elephant seals was assumed, as replication was normal in MDCK cell cultures, but inefficient in human tracheobronchial epithelial cells compared with human pH1N1 IAV reference strains. Specific antibodies to pH1N1 IAV were detected in sera collected from elephant seals after April, whereas sera collected earlier were all negative [67].

All of the above-mentioned pinniped species belong to the family Phocidae. In addition to virological evidence of IAV infection, there are many articles reporting the presence of antibody against IAV in sera of pinnipeds (Table 23.4). This serological evidence has been found not only in pinniped species belonging to the family Phocidae, but also in Pacific walruses (*Odobenus rosmarus divergens*), belonging to the family Odobenidae, and South American fur seals (*Arctocephalus australis*), belonging to the family Otariidae. These serological data indicate that the susceptibility of pinnipeds to IAV infection of both human and avian origin involves more species than those in which IAV infection has actually been detected.

The ability of avian IAVs to transmit efficiently among harbor seals and cause high mortality is unusual. To investigate this, Ramis and colleagues determined the pattern of IAV attachment to the respiratory tract of the harbor seal (Figure 23.3). They found abundant attachment of avian IAVs to tracheal and bronchial epithelial cells, which is consistent with efficient seal-to-seal transmission. In the same study, they also found scarce attachment of avian IAVs to bronchiolar and alveolar epithelial cells of harbor seals [182]. This was paralleled by rare expression of SAα-2,3 receptors in harbor seal lungs [9]. These findings do not fit with the reports of high mortality of harbor seals [65, 114, 239], although they are consistent with the low pathogenicity seen in experimental infections with AIV H7N7 in harbor seals [65]. One possible explanation is that the natural avian IAV epidemics in seals were aggravated by co-infecting agents, such as *Mycoplasma* species [65].

Influenza A viruses in the order Carnivora, suborder Feliformia

Various species of the order Carnivora, suborder Feliformia have been infected with IAV (Table 23.2), and are described in detail in the following sections.

Influenza A viruses in the family Felidae

Unlike canine influenza in dogs, there is no evidence of sustained transmission of a cat-adapted IAV among domestic cats. Historically, cats were not even considered susceptible to disease from IAV infection [77, 83]. However, like dogs, pet cats live in very close contact with humans. Indeed, following the human 1968 H3N2 IAV pandemic, naturally exposed cats had HI titers of > 40 against human H3N2 IAV, suggesting susceptibility to infection. Experimentally, cats were shown to develop a subclinical infection after inoculation not only with human H3N2 IAV, but also with avian H7N3, swine H1N1, and seal H7N7 IAVs, and with human influenza B virus [83, 161, 162, 189].

The idea that IAV infection does not cause disease in cats was proved to be incorrect with the emergence of H5N1 HPAIV in cats (see above). In addition to the pathogenicity of this avian virus infection for cats, human-origin pH1N1 IAV was also reported to cause severe respiratory disease in cats, both in the USA [32, 125, 164, 170, 174, 176, 213] and in France [116]. In most of these cases, the cat owners or their family members had been diagnosed with pH1N1 IAV and were considered to be the source of infection for the cats. Some cats died from the infection. At autopsy, they were found to have severe necrotizing bronchointerstitial

Table 23.4 Serological evidence of natural influenza A virus infection in marine mammals of the order Carnivora, clade Pinnipedia (Phocidae, Otariidae, and Odobenidae), and of the order Cetacea (Balaenopteridae and Delphinidae). Only reports where antibody to influenza A virus in serum was detected are listed.

		Virus									
Family	Species	Origin (likely)	Subtype	Morbidity	Mortality	Number positive/total number (%)	Serological assay[a]	Sustained intraspecies transmission	Region[b]	Period	References
Phocidae	Baikal seal	Avian	H3N2	–[c]	–[c]	2/7 (29)	ELISA and HI	No	AS	1998	[152]
	Caspian seal	Human	H3N2	–[c]	–[c]	28/77 (36)	ELISA and HI	Suspect	AS	1993, 1997–98, 2000	[154]
	Gray seal	Avian	H7N7	–[c]	–[c]	–[c]	–[c]	–[c]	NA	–[c]	[65]
	Harp seal	–[c]	Unspecified IAV	No	No	33/183 (18)	NP-ELISA	No	AS, Barents Sea	1991–92	[217]
	Hooded seal	–[c]	Unspecified IAV	No	No	8/100 (8)	NP-ELISA	No	AS, Barents Sea	1991–92	[217]
	Kuril harbor seal	Avian	H3, H6	–[c]	–[c]	15/211 (7)	ELISA and HI	No	AS	1998, 2003–05	[62]
	Northern elephant seal	Human	pH1N1	No	No	Adults: 20/44 (40) Pups: 14/71 (19)	HI	Yes, restricted	NA	2010	[67]
	Ringed seal	Avian	H3, H7	No	No	1/32 (3)	DID and HI	No	NA	1984	[45]
		–[c]	Unspecified IAV	–[c]	–[c]	23/903 (2.5)	NP-ELISA	–[c]	NA	1984–97	[149]
		Avian	H3N2, H7N7	–[c]	–[c]	H3N2: 5/6 (83) H7N7: 1/6 (17)	ELISA and HI	No	AS	2002	[152]
	Seal (unspecified species)	Avian	H1, H3, H4, H7, H12	–[c]	–[c]	10/338 (3)	HI	No	Bering Sea	1978–88	[47]
		Avian	H4	–[c]	–[c]	1/757 (0.1)	HI	No	EU	1988	[47]

(*continued*)

Table 23.4 (*Continued*)

		Virus									
Family	**Species**	**Origin (likely)**	**Subtype**	**Morbidity**	**Mortality**	**Number positive/total number (%)**	**Serological assay[a]**	**Sustained intraspecies transmission**	**Region[b]**	**Period**	**References**
Otariidae	South American fur seal	–[c]	H1N1	–[c]	–[c]	1/37 (3)	HI	No	SA	2004	[20]
Odobenidae	Pacific walrus	Avian	H10, N2, N3, N5, N6, N7	–[c]	–[c]	8/38 (21)	AGID	No	NA	1994–96	[31]
Balaenopteridae	Common Minke whale	–[c]	Unspecified IAV	–[c]	–[c]	7/140 (5)	ELISA	No	AS, Western North Pacific	2000–01	[153]
Delphinidae	Beluga whale	–[c]	Unspecified IAV	–[c]	–[c]	5/418 (1.2)	NP-ELISA	–[c]	NA	1991–92	[149]
	Dall's porpoise	–[c]	Unspecified IAV	–[c]	–[c]	2/34 (5)	ELISA	No	AS, Western North Pacific	2000–01	[153]

[a]NP-ELISA, nucleoprotein enzyme-linked immunosorbent assay; HI, hemagglutination inhibition; DID, double agar immunodiffusion; AGID, agar gel immunodiffusion.

[b]AS, Asia; EU, Europe; NA, North America; SA, South America.

[c]–, not determined or not recorded.

pneumonia associated with pH1N1 IAV [125, 213]. Experimentally inoculated cats showed similar lesions, and transmitted the virus to in-contact sentinel cats [234]. Although most cases involved single animals, there was one outbreak in Italy in which 25 of 90 cats in a colony died. The lungs of two cats that died of severe respiratory disease showed necrotizing bronchointerstitial pneumonia associated with pH1N1 IAV. Of the surviving cats, 21 animals had serum antibodies to pH1N1 IAV, and two had PCR-positive nasal swabs. Taken together, these findings were strongly indicative of cat-to-cat transmission of pH1N1 IAV [60].

Serological screening of cats for antibodies to pH1N1 IAV yielded variable results. Of sera collected from pet cats during the 2009–2010 influenza season, 22.5% from Ohio, USA ($n = 400$) [4] and 21.8% from the southern and Midwestern states of the USA ($n = 78$) [140] had hemagglutination-inhibiting (HI) antibodies to pH1N1 IAV, suggesting that cats are highly susceptible to pH1N1 IAV infection. In contrast, only 1.2% of sera collected from cats ($n = 1080$) during the same period in southern China had antibodies to pH1N1 IAV by nucleoprotein (NP)-specific ELISA [218], and only 1.93% of sera collected from cats ($n = 1150$) in Germany in 2010–2011 had antibodies to pH1N1 IAV by virus neutralization assay [44]. Feral cats appeared to be less likely to become infected with pH1N1 IAV than pet cats. Only

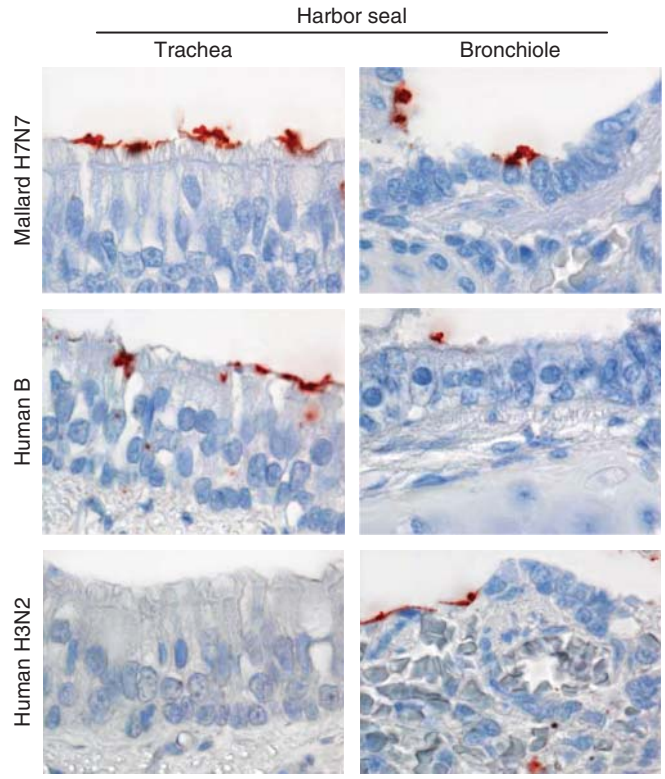

Figure 23.3 Low pathogenic avian influenza A virus (H7N7), human seasonal influenza A virus (H3N2), and human influenza B virus show different degrees of attachment to the trachea and bronchiole of a harbor seal (*Phoca vitulina*). Red staining indicates virus attachment to the epithelial cell surface. Reprinted from Ramis A. J., van Riel D., van de Bildt M. W. G., Osterhaus A., Kuiken T. Influenza A and B virus attachment to respiratory tract in marine mammals. *Emerging Infectious Diseases* [serial on the Internet]. 2012 May [date cited]. Available from 10.3201/eid1805.111828. With permission of EID. *See Plate section for color representation of this figure*.

0.43% of sera collected from feral cats ($n = 200$) in Florida between November 2008 and July 2010 had antibodies to pH1N1 IAV by ELISA [68], and in a survey of cats ($n = 1140$) in north-east China, only 11% of sera from feral cats had antibodies to pH1N1 IAV, compared with 30.6% of sera from pet cats [251].

Similar short-lived influenza epidemics in cats, but now from H3N2 CIV, occurred in South Korea in 2010 in two large animal shelters. These shelters housed dogs as well as cats, and both epidemics coincided with or were preceded by H3N2 CIV infections in dogs. It is likely that there was virus transmission from dogs to cats, followed by rapid cat-to-cat transmission. In one shelter, which had 60 cats, there was 47% morbidity and 22% mortality; in the other shelter, which had 50 cats, there was 100% morbidity and 44% mortality. Clinical signs in cats included high fever, lethargy, dyspnea, and coughing. At autopsy, the lungs showed severe bronchopneumonia, and the isolated virus was nearly identical to H3N2 CIV based on sequencing of all eight gene segments [92, 208]. Experimental H3N2 CIV infection of cats resulted in similar clinical signs and severe necrosuppurative bronchointerstitial pneumonia, co-localized with abundant influenza virus antigen in bronchial epithelial cells [208]. Both for pH1N1 IAV and for H3N2 CIV, housing many cats together appeared to be a risk factor for efficient cat-to-cat transmission of virus.

There is concern that cats, like dogs, might act as an intermediate host for AIV and either facilitate its adaptation to mammals or transmit the virus to humans [111, 140]. Given the recent reports of IAV transmission from birds, dogs, and humans to

cats, and the potential for efficient cat-to-cat transmission of such viruses, cats need to be included in influenza monitoring programs to protect public health [77].

Infection with pH1N1 IAV occurred in four captive cheetahs (*Acinonyx jubatus*) in an animal park in California, USA, in November 2009. Clinical signs included ptyalism, anorexia, and lethargy. An IAV isolated from swabs taken from one animal had 100% homology by sequence analysis with human isolates of pH1N1 IAV. The animals' keeper had an influenza-like illness and was considered likely to be the source of infection [41].

Serological evidence of infection with an unspecified IAV was detected in one of 16 wild Pallas's cats (*Felis [Otocolobus] manul*) on the Daurian Steppe of Russia in 2010–2011. The exact source of IAV exposure for these cats was not known, although they may have had contact with horses, dogs, cats, and house mice in remote human settlements. Furthermore, the Pallas's cats occupied fox burrows and preyed upon Daurian pikas (*Ochotona daurica*) and voles (*Microtus* species) [148]. Interestingly, both red foxes (*Vulpes vulpes*) [183] and black-lipped pikas (*Ochotona curzoniae*) [252] were found to be susceptible to H5N1 IAV infections.

Influenza A viruses in the family Viverridae

In autumn 2009, severe respiratory disease occurred in a Bornean binturong (*Arctictis binturong penicillatus*) in a zoo in California, USA. Clinical signs included lethargy, inappetence, dyspnea, nasal discharge, and coughing. The animal was euthanized because it had severe disease, and autopsy showed interstitial pneumonia. By PCR and sequencing, pH1N1 IAV was identified in lung samples from an American badger (*Taxidea taxus*) and in swabs from a black-footed ferret (*Mustela nigripes*) that were housed separately at the same zoo and were suffering from respiratory disease during the same time period. It was assumed that humans were the source of infection [193].

Influenza A viruses in non-swine species in the order Artiodactyla

Despite the fact that the order Artiodactyla (even-toed ungulates) contains about 220 species, many of which are important domestic or hunted animals, IAV infection has been reported only sporadically in a few species other than domestic and wild pigs (*Sus scrofa*) belonging to the family Suidae. Evidence of infection with or exposure to IAVs has been reported in cattle (*Bos taurus*), sheep (*Ovis aries*), goats (*Capra aegagrus hircus*), yak (*Bos grunniens*), and water buffalo (*Bubalus bubalis*), which belong to the family Bovidae, in alpaca (*Lama pacos*), which belongs to the family Giraffidae, and in reindeer (*Rangifer tarandus*), fallow deer (*Dama dama*), and European roe deer (*Capreolus capreolus*), which belong to the family Cervidae (Table 23.3) [53, 54, 63, 71, 127, 131, 187, 190]. The only epidemics of severe respiratory disease caused by IAV infection in non-swine artiodactyls were reported in Bactrian camels (*Camelus bactrianus*), which belong to the family Camelidae [244]. Influenza in domestic and wild pigs is discussed in detail in Chapters 16–19.

Possibly the first IAV to be isolated from ruminants was from a sheep in Hungary in 1960 [127, 190], followed by several IAV H3N2 isolations from cattle in Russia from the early 1970s to the 1980s [127]. These viruses were isolated during outbreaks of respiratory disease in sheep and cattle that coincided with pandemics and circulation of H2N2 IAV Asia/1957 and H3N2 Hong Kong/1968 in humans.

Romvary and colleagues reported the isolation of Asian H2N2 IAV (A/Borzsony/111/1960) from an adult sheep and her mature fetus suffering from respiratory disease in Hungary in 1960 [190]. This coincided with the H2N2 IAV Asia/1957 pandemic in humans. Respiratory disease was observed in several flocks of sheep. To confirm the susceptibility of sheep to human IAVs, lambs were intratracheally inoculated with egg-adapted strains of H2N2 and PR8 IAV. This resulted in fever, anorexia, coughing, dyspnea, and lassitude. Autopsy at 7 DPI revealed viral pneumonia both macroscopically and histologically, as well as marked immune responses to the inoculated strains [190]. However, it was not reported whether IAV was re-isolated from inoculated sheep. Re-isolation was attempted by McQueen and Davenport in 1963, when they infected several 3- to 10-week-old lambs intratracheally with the Hungarian sheep isolate H2N2 IAV (A/Borzsony/111/1960) and PR/8/1934. The lambs showed febrile responses but no respiratory signs, and no virus could be re-isolated from nasal swabs taken at 2 and 3 DPI, or from lungs and tracheas

at autopsy 3 DPI. Homologous antibody titers were detected in the sera from all inoculated lambs [141]. During a major H2N2 influenza epidemic in humans in Ireland in January 1961 [142], cattle sera taken between early 1960 and summer 1961 were screened for antibodies against H2N2 IAV. No compelling evidence of spread to cattle was found, as all of the sera were negative [143].

Naturally occurring antibodies against H3N2 IAV were detected in 16 of 28 cattle, 5 of 12 goats, two water buffalo, and one yak–zebu cross in Nepal and India, which were sampled between 1972 and 1973 [71]. This coincided with the circulation of H3N2 IAV (A/England/42/1972, closely related to A/Hong Kong/1/1968) among humans in India and Nepal [212]. The animals were not reported to exhibit any signs of disease. Experimental inoculation of H3N2 IAV into yak induced mild signs of respiratory disease, including coughing and malaise, for 6 DPI [71]. A more severe influenza-like illness was observed in 3- to 4-week-old calves that were experimentally inoculated with cattle strain H3N2 IAV (A/calf/Duschanbe/55/1971) isolated from a calf in Russia. For 4 DPI the calves had nasal discharge and coughed. The virus was shed from the nose for 7 DPI. Similar infections of calves with human H3N2 IAV isolates did not induce signs of respiratory disease [33].

IAV infections in cattle were reported to be associated with cases of acute reduction in milk production, known as "milk drop syndrome" [24, 40, 70, 74]. A case–control study of a dairy herd in Devon, UK, showed that rising antibody titers against human H1N1 IAV (A/England/333/1980) and H3N2 IAV (A/England/427/1988) were associated with sudden milk drop, signs of respiratory disease, and higher rectal temperatures compared with controls [40].

In contrast to sporadic reports of IAV in other ruminants, many outbreaks of severe respiratory disease associated with human H1N1 IAV infection were recorded in Bactrian camels on farms throughout Mongolia between 1978 and 1988. During a severe epidemic in the winter of 1979–1980, 4000 camels showed signs that included fever, coughing, bronchitis, and nasal and ocular discharge. Clinical signs typically lasted for 5–7 days. Some camels aborted, and the mortality rate was 9.1%. Isolates of H1N1 IAV from nasopharyngeal swabs from affected animals induced respiratory disease in experimentally inoculated serologically naive camels. Genetic sequence analysis of the isolates revealed that the PB1, HA, and NA genes were almost identical to a human H1N1 IAV isolate from 1977 that was closely related to a UV-light-inactivated reassortant (USSR/77 × PR/8/34) H1N1 vaccine strain used in Mongolian people in Leningrad, whereas the remaining genes originated from the H1N1 PR/8 laboratory strain. It was speculated that humans were the source of infection in camels, because the epidemic in camels coincided with a mild influenza H1N1 epidemic among vaccinated Mongolian children [244]. During that same time period and in the same region as the outbreaks in Mongolian camels in 1985, an H1N1 IAV was isolated from a child with respiratory disease. This isolate was genetically almost identical to the camel H1N1 IAV [7], suggesting that this H1N1 IAV reassortant was capable of crossing the species barrier.

Following the surge of the 2009 H1N1 pandemic, concerns were raised that pilgrims gathering at the Hajj might infect dromedary camels (*Camelus dromedarius*, belonging to the family Camelidae) in Saudi Arabia, and that returning pilgrims might infect dromedary camels in their countries of origin [171]. However, there were no subsequent reports that substantiated these concerns. Although parainfluenza-3 virus has been associated with respiratory disease in dromedary camels [87], antibodies against IAVs have not been reported to date in sera of dromedary camels.

Very recently, an H3N8 IAV was isolated from one of 460 nasal swabs collected from healthy Bactrian camels from Mongolia between January 2012 and January 2013. Phylogenetic analysis of the isolate indicated that it was a relatively recent horse-to-camel transmission of an IAV closely related to equine H3N8. In Mongolia, recurring equine H3N8 IAV epidemics arise in areas occupied by many free-ranging horses and Bactrian camels. Camel-to-camel transmission has not been reported to date [246].

Serological screening has been performed on other members of the family Camelidae. Antibodies against IAV were detected in more than 100 Peruvian alpacas (*Lama pacos*), with a prevalence of 4% [187]. More recent serological screenings for antibodies against IAV, including human H1N1 and equine H3N8, in wild vicuñas (*Vicugna vicugna*) and

llamas (*Lama glama*) from Argentina were negative [13, 136].

Influenza A viruses in the order Cetacea

Reports of natural exposure to IAVs are rare in cetaceans, the mammalian order that includes whales, dolphins, and porpoises (Table 23.3). Avian-origin H1N3 IAV was isolated from several lungs and one liver collected from live-caught minke whales (*Balaenoptera acutorostrata*) on a whaler in the South Pacific during 1975–1976. The virus was identified by electron microscopy and was cultured in eggs. The NA protein was antigenically most close to AIV. No associated signs of disease were reported [129, 232].

H13N2 and H13N9 AIVs were isolated from a long-finned pilot whale (*Globicephala melaena*, currently *G. melas*) in association with two mass stranding events along the coast of Cape Cod peninsula, USA, in 1984 [81]. One of the diseased and disorientated pilot whales was caught alive, euthanized, and examined. It was extremely emaciated and had sloughed skin. Gross autopsy revealed enlargement of the hilar lymph node, hemorrhagic lungs, and a small friable liver. Although AIVs of H13N2 and H13N9 subtypes were isolated from the hilar lymph node and lungs, there was no evidence that AIV infection had caused these lesions. Genetic and antigenic properties of the pilot whale AIV isolates suggested that they originated from gulls [81]. Indeed, 28 years after the original isolation, the gull origin of the pilot whale H13N2 AIV isolate was confirmed by genomic analysis [72]. In contrast to other duck-enterotropic H13 gull isolates, these viruses were apparently sensitive to low pH, as they did not replicate or induce disease in orally inoculated ducks. They did replicate in the lower intestine of ducks when rectally inoculated, thereby avoiding the acidic milieu of the proventriculus. The two isolates also replicated in the nose of intranasally inoculated ferrets. Fecal–oral transmission from shedding gulls to feeding whales was proposed as a possible route of transmission [81]. Such transmission may be facilitated by gulls and whales feeding concurrently on the same fish species during so-called "multi-species feeding frenzies." Accidental ingestion as a route of transmission is also a possibility, since it is not unusual for whole birds to be caught in the mouth of a baleen whale during such feeding frenzies, and case reports of birds being ingested by baleen whales have been published [79, 200].

Serological evidence of IAV infection in cetaceans has been reported for minke whales, Dall's porpoises (*Phocoenoides dalli*), and belugas (*Delphinapterus leucas*) (Table 23.4). Interestingly, the five positive beluga sera originated from a relatively small sample of 34 belugas from one population from the same area (Baffin Island, Nunavut, Canada), sampled between 1991 and 1992. No antibodies against IAV were detected in 76 narwhals (*Monodon monoceros*) or four bowhead whales (*Balaena mysticetus*) from the same survey [149].

Influenza A viruses in non-human primates

Only three published articles present virological evidence of natural IAV infection in non-human primates (NHPs) (Table 23.5). First, in 1971 Johnsen and colleagues [93] reported an H3N2 IAV (A/Hong Kong/1968) epidemic in a colony of white-handed gibbons (*Hylobates lar*) from Thailand. The virus was initially introduced into the colony by experimental inoculation of a few selected animals, but after 2–3 weeks it developed into an epidemic in the colony. The gibbons suffered from mild to fatal respiratory disease. Clinical signs consisted of fever, serous to purulent rhinitis, coughing, anorexia, depression, and gastrointestinal disturbances. Autopsy of the four fatal cases revealed dark red, edematous lungs, which corresponded to necrohemorrhagic pneumonia demonstrated by histological examination [93]. Second, in 1975, Malherbe and colleagues isolated an unspecified influenza-like virus from the throats of 3 of 20 healthy yellow baboons (*Papio cynocephalus*) that had been imported into the USA from Kenya [134]. Third, one of 48 oral swabs from pet and free-ranging urban macaques (*Macaca fascicularis* and *M. nemestrina*) from Cambodia was found to be PCR-positive for IAV [99].

Serological evidence of IAV infection in NHPs has been reported in several articles (Table 23.6). These data suggest that NHPs are commonly exposed to and infected with IAV, but are relatively resistant to development of disease. Possible sources of IAV for both captive and free-living NHPs are humans,

Table 23.5 Virological evidence of natural influenza A virus infection in mammals of the order Primates (Cercopithecidae and Hylobatidae) and Chiroptera (Vespertilionidae and Phyllostomidae). Only reports where the virus was detected by virus isolation or RT-PCR are listed.

		Virus				Tissue tropism[a]					
Family	**Species**	**Origin**	**Subtype**	**Morbidity**	**Mortality**	**Respiratory**	**Extra-respiratory**	**Sustained intraspecies transmission**	**Region[b]**	**Period**	**References**
Cercopithecidae	Yellow baboon	–[c]	Influenza-like "myxo"-virus	No	No	Yes	–[c]	–[c]	NA (African import)	1974	[134]
	Macaque	–[c]	Unspecified IAV	No	No	Yes	–[c]	No	AS	2011	[99]
Hylobatidae	White-handed gibbon	Human	H3N2	Yes	Yes	Yes	No	Yes	AS	1970?	[93]
Vespertilionidae	Common noctule bat	Human	H3N2	–[c]	–[c]	Yes	–[c]	No	AS	1977	[88, 113]
Phyllostomidae	Little yellow-shouldered bat	–[c]	H10N17	No	No	Yes	Yes	–[c] (suspect)	CA	2009–10	[227]
	Flat-faced fruit-eating bat	–[c]	H11N18	No	No	Yes	Yes	–[c] (suspect)	SA	2010	[228]

[a] Also based on results of experimental infections.

[b] AS, Asia; EU, Europe; NA, North America; SA, South America; CA, Central America.

[c] –, not determined or not recorded.

with whom NHPs often have close contact. However, other sources of IAV (e.g. birds) cannot be excluded.

Experimental inoculation of IAV has shown that multiple NHP species are susceptible to both IAV infection and associated disease. In the 1920s and 1930s, chimpanzees (*Pan troglodytes*) developed signs of influenza-like illness after inoculation with nasal washings from human patients with influenza [50, 126]. In 1969, Kalter and colleagues inoculated H3N2 IAV into baboons (*Papio* species), which transmitted the virus to sentinel baboons but did not develop overt respiratory disease [96]. Several other NHP species have been found to be susceptible to experimental IAV infection and to develop respiratory disease. The most commonly studied species are squirrel monkeys (*Saimiri sciureus*) [19, 146, 194, 204, 205, 215, 229] and cynomolgus, rhesus, pigtailed, and bonnet macaques (*Macaca* species) [15, 16, 18, 25, 66, 80, 89, 91, 95, 137, 161, 236, 249].

Influenza A viruses in the order Chiroptera

Traditionally, the original reservoir of all IAVs was considered to be wild waterbirds [158]. This dogma was recently overturned by the discovery of IAVs with new HA (H17 and H18) and NA (N10 and N11) subtypes in frugivorous bats from Central and South America [227, 228] (Table 23.5). This was very surprising, because previously there had only been a single published report of IAV infection in bats (which belong to the order Chiroptera), when an H3N2 IAV was cultured and isolated from the lungs of insectivorous common noctule bats (*Nyctalus noctula*) from Kazakhstan [113].

Table 23.6 Serological evidence of natural influenza A virus infection in mammals of the order Primates (Homidae, Hylobatidae, and Cercopithecidae). Only reports where antibody to influenza A virus in serum was detected are listed.

Family	Species	Virus		Morbidity	Mortality	Number positive/total number (%)	Serological assay[a]	Sustained intraspecies transmission	Region[b]	Period	References
		Origin (likely)	Subtype								
Homidae	Chimpanzee	Human	H1N1, H2N2	No	No	H1N1(PR8): 8/56 (14) H2N2: 23/56 (41)	HI	No	NA (captive)	1960s	[97]
		Human	pH1N1, H3N2	No	No	pH1N1: 218/305 (71.5 H3N2: 34/305 (11.2)	HI	No	EU (captive)	1986–2000	[26]
Homidae	Orangutan	Human	H1N1, H2N2	No	No	H1N1 (PR8): 4/22 (18) H2N2: 10/22 (45)	HI	No	NA (captive)	1960s	[97]
		Human	pH1N1, H3N2	No	No	pH1N1: 34/179 (19.0) H3N2: 10/179 (5.6)	HI	No	EU, AS (captive)	1994–98	[26]
Homidae	Gorilla	Human	pH1N1, H3N2	No	No	pH1N1 & H3N2: 3/77 (3.9)	HI	No	EU (captive zoo)	–[c]	[26]
Hylobatidae	Gibbon	Human	H1N1, H2N2	No	No	H1N1(PR8): 2/9 (22) H2N2: 3/9 (33)	HI	No	NA (captive)	1960s	[97]
Cercopithecidae	Baboon	Human	H1N1, H2N2	No	No	H1N1(PR8): 13/122 (11) H2N2: 1/122 (0.8)	HI	No	NA (captive) and AF (wild)	1963–64	[97]
		Human	Unspecified IAV	No	No	12/14 (86)	CF	–[c]	NA (captive)	1966	[10]
	Rhesus macaque	Human	H1N1, H2N2	No	No	H1N1 (PR8): 5/48 (10) H2N2: 1/48 (2)	HI	No	NA (captive)	1960s	[97]
		Human	Unspecified IAV	No	No	159/171 (93)	CF	–[c]	NA (captive)	1966	[10]
	African green monkey	Human	Unspecified IAV	No	No	94/141 (67)	CF	–[c]	NA (captive)	1966	[10]
	Tonkean macaque	Human	Unspecified IAV	No	No	Wild: 5/15 (33) Captive: 2/11 (18)	RDIA	No	AS (pet and wild free-ranging)	1999	[94]
	Macaque	Human/avian	H1N1, H3N2, H9N2	No	No	33/163 (20)	NP-ELISA and HI	No	AS (pet and urban free-ranging)	2011	[99]

[a]NP-ELISA, nucleoprotein enzyme-linked immunosorbent assay; HI, hemagglutination inhibition; CF, complement fixation; RDIA, rapid dot-immunobinding assay.

[b]AS, Asia; EU, Europe; NA, North America.

[c]–, not determined or not recorded.

The first IAV of previously unknown subtype, H17N10, was detected in little yellow-shouldered bats (*Sturnira lilium*) from Guatemala by next-generation sequencing of rectal swabs and internal organs, including lungs, liver, intestines, and kidneys. The second IAV of previously unknown subtype, H18N11, was detected in a flat-faced fruit bat (*Artibeus planirostris*) from Peru by next-generation sequencing of rectal swabs and intestines (liver and spleen were negative). The consistent detection of virus in intestinal and rectal swabs suggested that these new bat-origin IAVs replicated in the intestine. The viruses could not be propagated in cell cultures or eggs. No clinical signs were reported in these bats, which were caught alive [227, 228].

Serological analysis of several Peruvian bat species, including *Artibeus* species, yielded a high percentage (50%, 55 of 110) of sera that contained specific antibodies against recombinant H18 and N11 proteins by ELISA. Likewise, specific antibodies against recombinant H17 protein were detected by ELISA in 38% (86) of 228 sera from eight bat species from Guatemala collected during 2009–2010 [228]. Tong and colleagues interpreted these high seroprevalences of identical bat IAV infections in multiple species from distant geographic locations spanning several years as being indicative of widespread endemic infections with sustained bat-to-bat transmission in New World bats [228]. However, no virological evidence was found for such new IAVs from a large survey of 26 species of bats from Central Europe, in the Old World [59].

These bat IAVs contain newly discovered gene segments that encode the major surface envelope proteins HA (H17 and H18) and NA (N10 and N11). They differ in form and function from all previously known HAs (H1–H16) and NAs (N1–N9). The bat H17 showed on average 45% amino-acid-sequence similarity to HAs from known IAV subtypes. Sequence motifs of the sialic acid (SA) receptor-binding site were identified in bat IAV H17, although position changes in specificity for galactose–SA linkage indicated a ligand preference other than SA receptors [227]. Zhu and colleagues indeed showed that the presumed receptor-binding site of HA H17 was highly acidic, making it unfavorable for binding of the negatively charged SA receptors [254]. Unlike the HA gene and internal genes, the bat N10 was extraordinarily divergent from known NAs. It showed only 24% amino-acid-sequence similarity to other IAV NA subtypes. Interestingly, this sequence similarity was even lower than the similarities between NAs from IAV and influenza B virus [227]. Otherwise, the crystal structure of N10 resembled other IAV NA structures (e.g. the highly conserved N-glycosylation site N146 shared in all IAV NAs). However, enzymatic MUNANA assays showed a lack of typical neuraminidase activity [120].

Bat H18 showed 49.1% amino-acid-sequence similarity with other HA subtypes, and only 60.2% sequence similarity with H17. The bat N11 NA (more accurately referred to as "NA-like protein" or NAL) had only 29.6% identity with all other NAs [228]. Furthermore, the HAs H17 and H18 showed no specific binding to sialosides evaluated by sialoside microarray and glycan ELISA [228, 254], and it was also found that N10 and N11 NA-like proteins did not bind or cleave SAs [120, 228]. In contrast to non-bat IAVs, these results indicate that bat H18N11 and H17N10 IAVs do not mediate host cell attachment and release via SA receptors. The receptors or mechanisms that these bat IAVs use for host cell attachment, fusion, entry, and release have yet to be identified [219, 227, 228].

The amino acid sequences of the remaining internal genes of H17N10 and H18N11 IAVs showed most of the known functional sequence motifs of other IAVs. The polymerase complex proteins (PB2, PB1, PA, and NP) of both showed functional viral transcription by means of reporter minigenomes in human and primate cells. This transcription was abrogated when PB1 was removed from the minigenome [227, 228]. Furthermore, it was determined that the N-terminal domain of PA (PAn) from H17N10 has manganese or magnesium ion-dependent endonuclease activity producing small RNA primers essential for initiation of viral gene transcription like any other IAVs [221].

Phylogenetic analysis of the primary genetic sequences of the HA molecules of bat H17 and H18 indicated that they belong to group 1 HAs (together with H1, H2, H5, H6, H8, H9, H11, H12, H13, and H16), and not to group 2 HAs (H3, H4, H7, H10, H14, and H15). The more divergent NA-like molecules N10 and N11 did not belong to either of the existing NA groups 1 and 2, but were

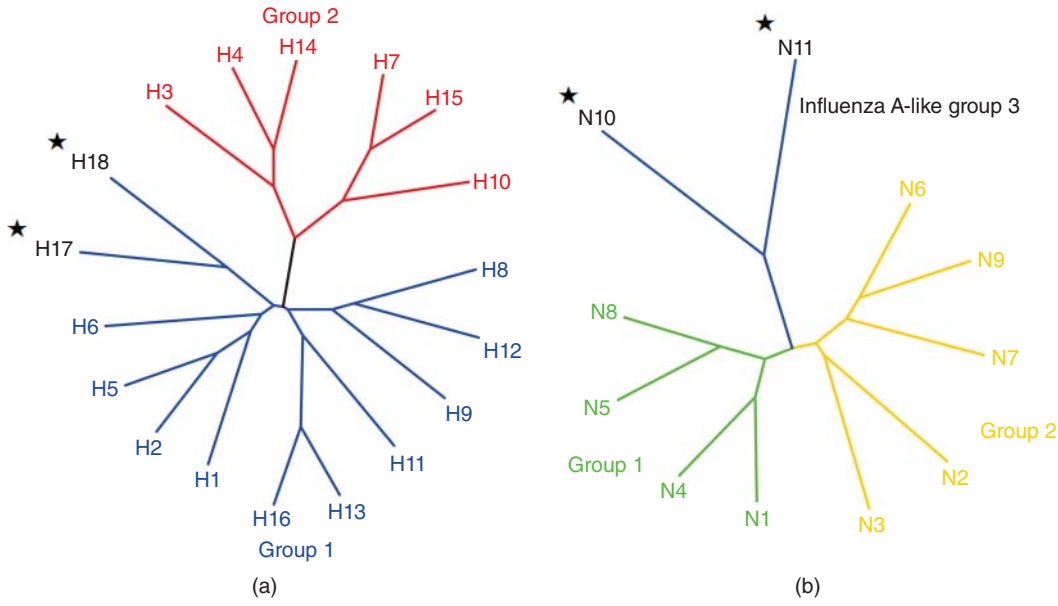

Figure 23.4 Phylogenetic trees displaying the hemagglutinin (HA) and neuraminidase (NA) genes of bat-derived H17N10 and H18N11 influenza viruses (denoted by asterisks) compared with the relative distance of HAs and NAs of all previously known influenza A virus subtypes. Reprinted from *Trends in Microbiology*, April 2014, Vol. 22, No. 4, pp. 183–191, Wu Y., Wu Y., Tefsen B., Shi Y., Gao G. F. Bat-derived influenza-like viruses H17N10 and H18N11, with permission from Elsevier. *See Plate section for color representation of this figure*.

categorized as a separate influenza A-like group 3 (Figure 23.4) [242]. Although their internal genes were almost the same as known IAVs, Wu and colleagues proposed that on the basis of the different NA and HA genes these viruses should be renamed as "influenza-like viruses" [242]. The origins of the NALs are not known, but they might be derived from an unknown influenza type other than influenza A, B, or C, either extinct or yet to be identified [120]. The positions of the six internal genes of bat H17N10 in the phylogenetic tree were between the IAV and influenza B virus split. However, they were more closely related to IAV-type genes [227]. Tong and colleagues concluded from their findings that these newly discovered IAVs needed to have evolved in bats for a long period of time [228]. Briefly, these findings included higher viral genetic diversity than was previously known to exist, divergence into multiple HA and NA subtypes with a presumed SA-independent alternative mechanism or receptor for host cell entry and release, and a widespread geographic distribution of these two monophyletic bat IAVs.

The two major surface proteins, HA and NA, of bat H17N10 and H18N11 IAVs lacked typical cell attachment and cleavage functions. However, their more conserved internal genes responsible for viral transcription were shown to be functional *in vitro*. These genes are considered to be potentially interchangeable with known IAVs that contain classical functional HAs and NAs. This raised serious scientific and public health questions about whether such genomic reassortments could occur, thereby possible generating an infectious influenza virus capable of causing disease in species other than the bat species in which they were detected [227, 228, 242]. Furthermore, bats are known to harbor many viruses with considerable zoonotic disease potential [29, 108], and are sometimes regarded as a "treasure trove" hosting many unknown viruses [29]. Indeed discoveries of new viruses in bats are ongoing [51, 52]. In general, bats possess specific characteristics that could favor the evolution and spread of novel viruses, including IAV. Belonging to the taxonomic order Chiroptera that contains approximately 1150 species worldwide, bats are long-lived globally abundant mammals, which migrate and inhabit urban, rural, and natural environments, with possible contacts with

humans, livestock, and other wildlife. Furthermore, they exhibit clustered roosting in extremely high densities in multi-species colonies, practically guaranteeing bat-to-bat transmission of viruses [29]. Bats have to be considered as a novel potentially important mammalian reservoir of influenza viruses.

Influenza A viruses in the orders Rodentia and Lagomorpha

Although there are about 2300 species in the order Rodentia and around 80 species in the order Lagomorpha [241], natural IAV infection has very rarely been reported in species belonging to these two orders [55, 63, 198, 199]. Specific antibodies against IAV indicating infection with human IAV were detected unequivocally by HI and complement fixation tests in one domestic rabbit (*Oryctolagus cuniculus*) and one chipmunk (*Tamias striatus*). This was part of a serological screening study for antibodies against IAV H3N2 (A/Hong Kong/1/1968) in 6 wild chipmunks, 25 groundhogs (*Marmota monax*), 13 cottontail rabbits (*Sylvilagus* species), 42 snowshoe hares (*Lepus americanus*), and 106 pet rabbits from the Ottawa area, Canada, sampled between 1966 and 1970 [63].

Six of six wild house mice (*Mus musculus*) caught on a gamebird farm in Idaho, USA, during an H5N8 LPAIV outbreak in 2008 were found to be positive for antibodies against IAV by indirect NP-ELISA [199]. Six brown rats (*Rattus norvegicus*), one harvest mouse (*Reithrodontomys megalotis*), and one deer mouse (*Peromyscus maniculatus*) that were caught and tested in the same study were all seronegative. Subsequent experimental intranasal inoculation of newly caught serologically naive house mice with AIV isolates from wild birds (H3N6, H3N8, and H4N6) or chickens (H6N2 and H4N8) resulted in virus replication in the nasal turbinate, trachea, and lungs. The virus isolates from the wild birds replicated to higher titers in the mouse tissues than did the chicken isolates. The results indicated that house mice might be a risk factor for transmission of IAV to poultry and gamebird farms [199].

Influenza A viruses in other species

An outbreak of human seasonal H1N1 IAV infection occurred in giant anteaters (*Myrmecophaga tridactyla*, belonging to the family Myrmecophagidae, in the order Pilosa) in Nashville Zoo, USA, in February 2007. All 11 animals in the group exhibited clinical signs of severe nasal discharge and congestion, inappetence, and lethargy. The isolated virus was identified as IAV and showed more than 99% nucleotide identity with a human seasonal IAV isolate Tennessee/UR06-0119/2007 (H1N1). The anteaters had no contacts except with their keepers, who were suffering from respiratory disease, and presumably were the source of infection [151].

The reports, albeit rare, of IAV infection in reptiles and amphibians emphasize the broad host range of this virus. In 2006, IAV was detected by PCR for IAV-matrix gene in blood samples from 4 of 37 captive crocodilians in Florida, USA, namely a Chinese alligator (*Alligator sinensis*), a Schneider's dwarf caiman (*Paleosuchus trigonatus*), a Nile crocodile (*Crocodylus niloticus*), and a broad-snouted caiman (*Caiman latirostris*). Antibodies to IAV were detected by agar gel immunodiffusion testing in sera of all these animals except for the broad-snouted caiman. These crocodilians were kept in open pens with exposure to wild birds, some of which were eaten, and it is likely that these were the source of infection. This is supported by sequence analysis of the non-structural protein 1 (NS1) gene of the PCR products, which revealed more than 99.7% homology with the NS1 gene from duck isolates [46]. One other study, by Mancini and colleagues [135], suggested that there was susceptibility to IAV (and influenza B virus) infection in poikilothermic animals. Antibodies against human H1N1 and H3N2 IAVs and equine H7N7 and H3N8 IAVs were detected by HI assay in sera collected from captive and free-ranging snakes and amphibians from Brazil, namely pit vipers (*Bothrops jararaca* and *B. jararacussu*), Cascavel rattlesnakes (*Crotalus durissus terrificus*), Rococo toads (*Bufo paracnemis*), and American bullfrogs (*Lithobates catesbeianus*, formerly *Rana catesbeiana*) [135].

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24 Mammalian experimental models and implications for understanding zoonotic potential

Jessica A. Belser and Terrence M. Tumpey

Introduction

The emergence in avian and non-human mammalian species of influenza A viruses that possess the capacity to spread to human hosts represents an ongoing public health concern. There is a pressing need to study the influenza viruses that are circulating in non-human hosts in order to better understand their pandemic potential should they acquire the ability to cause sustained human infection. Concurrently, an understanding of the inherent variability of human influenza viruses is critical for mitigating the severity of disease during annual epidemics. The use of mammalian models allows the ability of influenza viruses to cause severe disease and/or transmit to naive contacts to be studied in a safe and controlled environment. This information makes a vital contribution to the development of prevention and control measures. However, the results of such studies can be influenced by the choice of mammalian model to be used in the laboratory, the selection of virus(es) to be studied in a particular species, the method of inoculation used, and the dose of virus administered. Informed decisions about these and many other experimental variables are necessary in order to ensure that the data obtained are as relevant and scientifically reliable as possible.

There is an ever-growing awareness of the public health threat posed by influenza viruses [41]. Seasonal influenza viruses generally cause mild illness in humans, but have the potential to cause severe disease, especially in the elderly or in other immunocompromised populations. Aquatic wild birds, which serve as the natural reservoir for most influenza viruses, typically exhibit asymptomatic infection with low pathogenic avian influenza (LPAI) viruses. Sporadic infections with LPAI viruses in humans usually occur following exposure to infected poultry and, to a lesser extent, to infected aquatic wild birds, and with the exception of H7N9 viruses they are typically non-fatal. Highly pathogenic avian influenza (HPAI) viruses, in contrast, can exhibit a lethal phenotype in both avian and human hosts. Although the 2009 H1N1 pandemic virus is the most frequently studied virus of swine origin, sporadic infection of humans with other swine-origin viruses continues to occur. All of these viruses warrant detailed examination to determine their pandemic potential and the threat that they pose to human health. The high level of heterogeneity among these viruses necessitates an equally heterogeneous array of mammalian models for their study.

In this chapter, we discuss the main considerations that must be addressed when designing, undertaking, and interpreting research conducted in mammalian models. Several examples of the role that these studies play in public health are discussed. Performed in concert with *in-vitro*, *ex-vivo*, and other experimental modeling approaches (Figure 24.1), mammalian *in-vivo* studies offer an unparalleled opportunity to measure complex virus–host interactions, which is essential for understanding the pathogenesis, pathobiology, and transmissibility of avian and human influenza viruses.

Animal Influenza, Second Edition. Edited by David E. Swayne.
 Published 2017 by John Wiley & Sons, Inc.

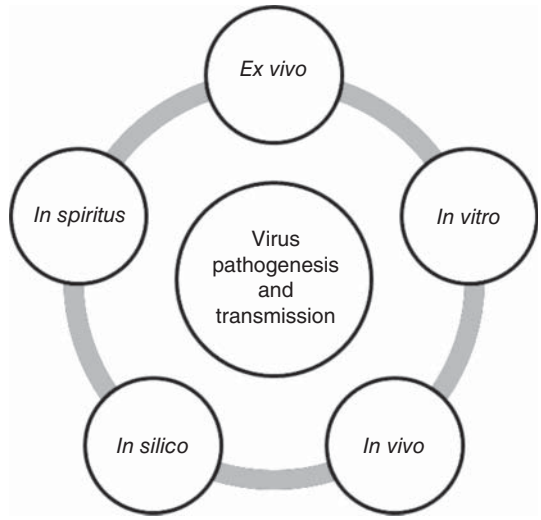

Figure 24.1 Laboratory modeling of influenza virus infection in mammals. Examples of different research platforms that can contribute towards a greater understanding of influenza virus pathogenicity, transmissibility, and tropism. *In spiritus* = in breath.

Factors relating to choice of an appropriate model

Mammalian experimental models can contribute invaluable information to the study and understanding of influenza viruses. However, the choice of species to be used can have a major impact on the meaningfulness of the data obtained. There is no one model that is ideally suited for all research applications. Rather, each mammalian species has specific advantages and disadvantages, with different species being suitable for different research aims, so it is important to decide which species are most appropriate for modeling desired properties (Figure 24.2). Several areas that need to be considered when choosing a model are described below.

Facilities

Primary factors that determine which animal model is most appropriate for investigation are the size, cost, and ease of handling of the species in question. Small rodents such as mice offer numerous advantages, including a relatively low cost and easy husbandry. Ferrets require larger caging and stringent air filtration to maintain seronegativity to circulating influenza viruses prior to use. Guinea pigs have emerged as an alternative to ferrets in this context, due to their relatively smaller size [82]. Larger higher-order mammalian species such as non-human primates are typically cost prohibitive, and few laboratories possess appropriate facilities for accommodating these species. The selection of virus(es) to be studied may also influence this decision, as research on many viruses, including highly pathogenic avian influenza viruses, must be performed in facilities that meet appropriate biosafety requirements [28].

Experimental readout

The type of data to be obtained from the experiment will strongly influence the choice of species used for investigation. For example, not all species support replication of human influenza viruses without prior adaptation, present with similar clinical signs of infection to humans, cause comparable pathology in the respiratory tract to that observed in humans, or transmit virus efficiently to naive contact animals [69]. Furthermore, the availability of species-specific reagents varies widely between models, limiting the ability to study certain properties or features in select species. For example, ferrets are typically considered the best model for the coincident study of influenza virus pathogenesis and transmission [9], but a paucity of reagents available for the study of immunological properties

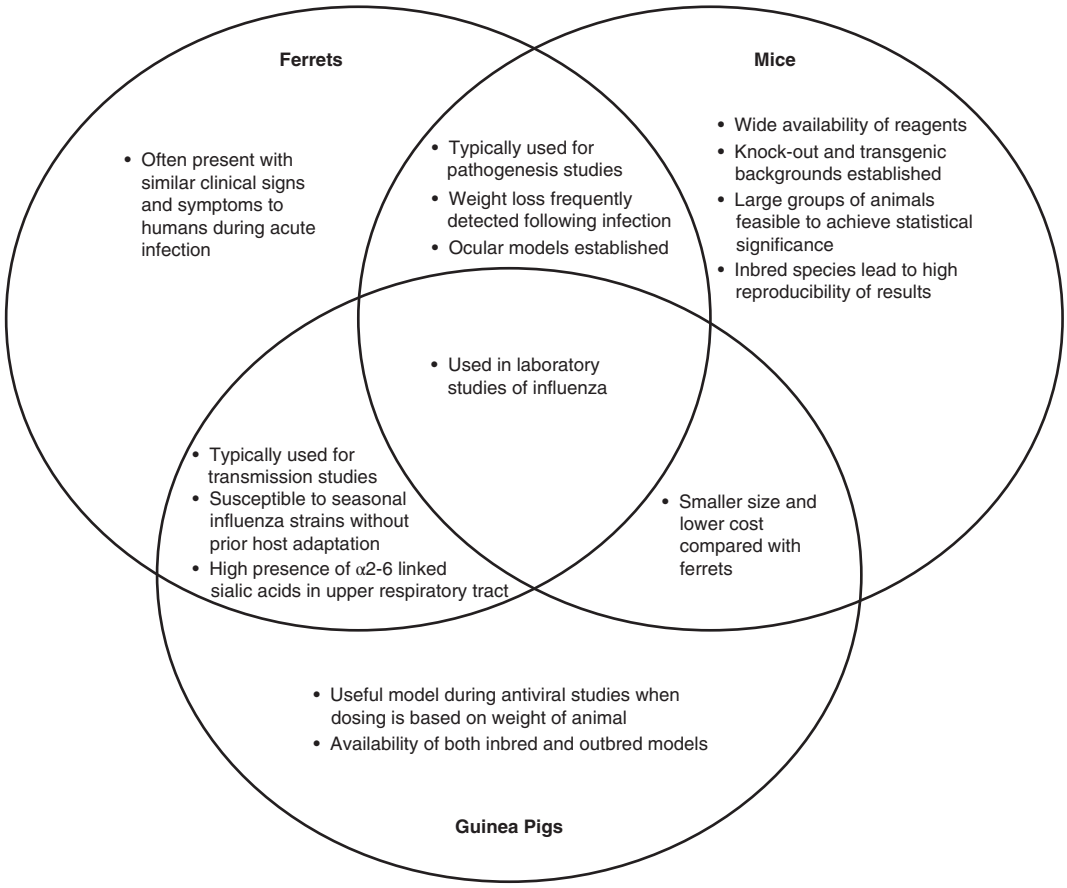

Figure 24.2 Advantages and disadvantages of mouse, ferret, and guinea pig models for use in influenza virus research.

currently limits the utility of this species for certain assays. Advanced consideration of the critical parameters of the proposed experiments can help govern the choice of model used in each case.

Receptor-binding specificity and virus attachment

Influenza viruses bind to glycoconjugates containing terminal sialic acids in either a Neu5Acα(2,3)-Gal or Neu5Acα(2,6)-Gal conformation [123]. The epithelia of the human upper respiratory tract possess an abundance of α2,6-linked sialic acids; the lower respiratory tract epithelium bears sialosides in both α2,6 and α2,3 conformations (Figure 24.3) [70]. Generally, human influenza viruses preferentially bind α2,6-linked sialic acids, facilitating productive virus replication in the human upper respiratory tract, whereas avian influenza viruses preferentially bind α2,3-linked sialic acids, resulting in efficient virus replication in the lung. Receptor-binding preferences are therefore frequently associated with the tropism of influenza viruses in humans. Accordingly, the distribution of sialic acid moieties in different mammalian models can influence the virulence observed following experimental inoculation. For example, the attachment pattern of human and avian influenza viruses to ferret respiratory tract tissues closely matches that in humans, whereas the attachment pattern to murine respiratory tract tissues does not [119, 151]. Thus the receptor-binding specificity of the virus(es) to be studied, as well as the distribution of α2,3- and α2,6-linked sialic acids in the epithelium of the respiratory tract of the mammalian model chosen, can influence the resulting host range, virulence, and transmissibility [34].

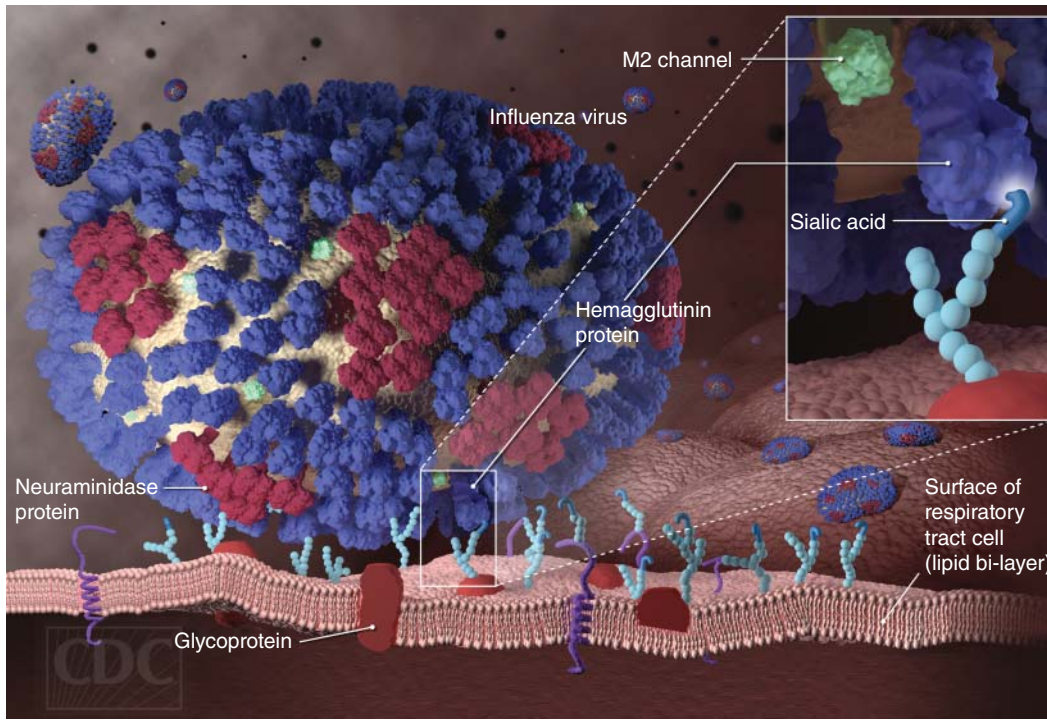

Figure 24.3 Binding of influenza virus to host epithelial cell. The influenza hemagglutinin (HA) binds to sialic acids present on the epithelia of host cells in the respiratory tract. Source: Dan Higgins/PHIL CDC. *See Plate section for color representation of this figure*.

Sample size and statistical power

Scientists who conduct animal research have an ethical obligation to use the minimum number of animals necessary to achieve valid and reproducible results. As mice are inbred, widely available, and relatively inexpensive, this model is typically employed when statistical rigor is necessary. However, meaningful data can be obtained from all mammalian species, despite the need to balance numerous constraints on sample size (including size, cost, space, and ethical considerations, as discussed in this section). When statistical power is not feasible, or sample sizes are very limited, researchers must exercise caution when interpreting their findings [11, 101]. Meta-analyses, which derive statistical power from analyzing data generated from numerous separate smaller studies, provide an additional option for more stringently interpreting the results obtained from experiments that were of necessity limited to smaller sample sizes [127, 164].

Ethical considerations

Animal research has a critically important role in many aspects of influenza virus study, and researchers must be vigilant with regard to issues of animal welfare and responsible research. Therefore adherence to the "3 R's" of animal research – reduction, replacement, and refinement – should be emphasized and taken into consideration when designing *in-vivo* experiments [114]. The concurrent use of *in-vitro* and *ex-vivo* assays greatly enhances and enriches *in-vivo* experimentation [11, 78].

Mammalian models of traditional intranasal and intratracheal inoculation

There are numerous mammalian species which, although they are not natural hosts of influenza virus, can support influenza virus infection and

Table 24.1 Differential virulence of closely related influenza viruses in different species.

Virus	Subtype	Patient outcome[a]	Mouse virulence[a]	Ferret virulence[a]	627 PB2[b]	Total amino acid changes
A/HK/483/97	H5N1	Fatal	High	High	K	50
A/HK/486/97	H5N1	Recovered	Low	High	E	
A/NL/219/03	H7N7	Fatal	High	High	K	15
A/NL/230/03	H7N7	Recovered	Low	Low	E	
A/Thai/16/04	H5N1	Fatal	High	High	K	13
A/SP/83/04	H5N1	Fatal	Low	Low	E	
A/VN/1203/04	H5N1	Fatal	High	High	K	8
A/VN/1204/04[c]	H5N1	Fatal	High	High	E	

[a] Patient information and mammalian pathotyping results described in the literature [10, 85, 91, 163].
[b] Amino acid at position 627 of PB2. K, lysine; E, glutamic acid.
[c] Virus isolate obtained from the same individual.

replication. Inoculation by the intranasal or intratracheal route using a liquid inoculum of diluted virus typically causes a productive infection in each species. However, as will be discussed below, the virulence and disease presentation can vary widely between species (Table 24.1). Although there is a need to balance the gender ratio of mammalian models used in research, as gender differences can influence numerous biological parameters [30], studies of influenza virus pathogenesis and transmission are typically restricted to one gender within an experiment, in order to minimize any potential variation.

Mice

Mice (*Mus musculus*) are utilized ubiquitously in laboratory research due to, among many other features, their low cost, ease of handling and housing, well-characterized genome, the wide array of reagents available for this species, and the availability of transgenic backgrounds. However, mice are not a natural host of influenza virus, and contemporary human influenza A viruses (H1 and H3 subtypes) often require host adaptation to achieve high levels of virus replication. Furthermore, mice do not present with many of the clinical signs and symptoms that are observed during human infection, and transmission studies are not typically conducted in this species, although select studies have reported transmission from infected to naive mice [81, 141]. Despite these limitations, mice represent a useful model for influenza pathogenesis studies, as symptom onset, lung pathology, and induction of host responses are temporally related to virus replication. In addition, the inbred background of most murine laboratory strains allows the study of subtle alterations to the virus, the phenotype of which may be more difficult to observe in outbred species.

Influenza virus pathogenesis

The most clinically obvious sign of disease in mice inoculated with non-adapted human or LPAI viruses is mild to moderate weight loss, which generally resolves by 14 days post inoculation (DPI). Transient lymphopenia, lethargy, and a drop in body temperature may also occur during the acute phase of infection, temporally associated with peak viral titers. Virus replication is typically restricted to the respiratory tract, notably the lung and nose. Numerous proinflammatory cytokines and chemokines can be detected in the infected mouse lung or bronchoalveolar lavage fluid.

Severe disease resulting in lethality in mice can occur following inoculation with mouse-adapted human viruses, the reconstructed 1918 pandemic virus, or wild-type avian viruses, generally but not exclusively limited to HPAI [54, 91, 93, 146]. Mice infected with these viruses typically lose more than 20% of their initial body weight before succumbing to infection when inoculated with high doses. Virus replicates to high titer in the lung, and systemic spread of virus to the brain and other organs may occur, especially during infection with H5N1 HPAI

viruses [91]. As in severe human cases, dysregulated immune responses are commonly detected, with pronounced lymphopenia and leukopenia in peripheral blood and hypercytokinemia in the lung [143, 147]. The pathology of viral pneumonia in the lungs of mice infected with virulent viruses is generally similar to that observed in humans, with histological lesions detected throughout the respiratory tract [69]. This histopathology is consistent with the attachment pattern of H5N1 HPAI virus in the murine lung [151].

Infection of transgenic mice

Unlike other mammalian models, the mouse model offers a unique opportunity to investigate the role of specific immune mediators in influenza virus pathogenesis via the use of transgenic animals. Mice that lack individual cytokine receptors and/or signaling pathways have enabled a more precise study of the contribution of individual host responses to viral pathogenesis [14, 136]. Furthermore, the importance of factors that contribute to the establishment of a robust antiviral state, such as Mx proteins, have been examined by studying inbred mice that were engineered to carry a functional *Mx1* allele [115, 149]. In addition to genetic manipulation, mice have been used to study the effects of pregnancy, malnutrition, obesity, diabetes, and other comorbidities on influenza virus pathogenesis [25, 37, 138, 156].

Ferrets

Although not as ubiquitously employed as mice, ferrets (*Mustela putorius furo*) represent an established and widely used model for influenza virus pathogenicity and transmissibility. Unlike mice, influenza A viruses readily infect naive ferrets without prior host adaptation. However, their larger size, higher cost, and incompletely characterized genome limit the utility of ferrets for some laboratories or for particular research applications, especially those that require large sample sizes. Furthermore, the high susceptibility of ferrets to influenza virus infection mandates that investigators always wear respiratory protection when in the same room as the animals, to ensure that the ferrets are not exposed to any respiratory pathogens originating from exposure to humans. Although ferrets are outbred, leading to potential animal-to-animal variation within an experiment as compared with inbred mouse strains, the benefit of pathotyping a virus in a species in which the signs and symptoms of disease so closely mirror those in humans often outweighs this variability. Thus ferrets represent a mammalian model that is well suited for the concurrent study of virus pathogenesis and transmission [9]. The utility of the ferret model for evaluating vaccine effectiveness also validates the use of this species in initial characterizations of influenza viruses.

Influenza virus pathogenesis

Ferrets infected with human influenza viruses typically exhibit a mild and transient illness, presenting with numerous clinical signs and symptoms of infection that also occur during human infection, including sneezing, rhinorrhea, and fever. Mild to moderate weight loss is common. Replication of seasonal influenza viruses and other low-virulence strains is typically restricted to the upper respiratory tract of ferrets. However, virus replication in the lung has been detected for select human viruses, notably pandemic strains [90, 148].

Avian and swine influenza viruses of low virulence generally resemble human influenza viruses with regard to their pathogenicity in ferrets, with moderate weight loss and fever detected during the acute phase of infection, although strain-specific differences leading to enhanced pathogenicity do occur [10, 90, 91, 107, 153]. Viral replication throughout the respiratory tract is common, with generally localized and mild inflammation detected in the lungs. In contrast, some HPAI viruses of the H5 and H7 subtypes are capable of causing systemic and fatal disease in ferrets, characterized by severe weight loss, sustained fever, pronounced lethargy, dyspnea, diarrhea, and potential neurological involvement, although this property can be strain-specific (Figure 24.4) [14]. Ferrets inoculated with highly virulent viruses typically die during the acute phase of infection, often due to reaching humane endpoint thresholds of morbidity, lethargy, and/or neurological symptoms. Systemic spread of virus to the brain and other extrapulmonary organs is often detected [91]. Severe disruption of lymphohematopoietic parameters is common. Inflammation of pulmonary tissues can be severe, with bronchopneumonia and acute bronchiolitis detected early after infection, especially following

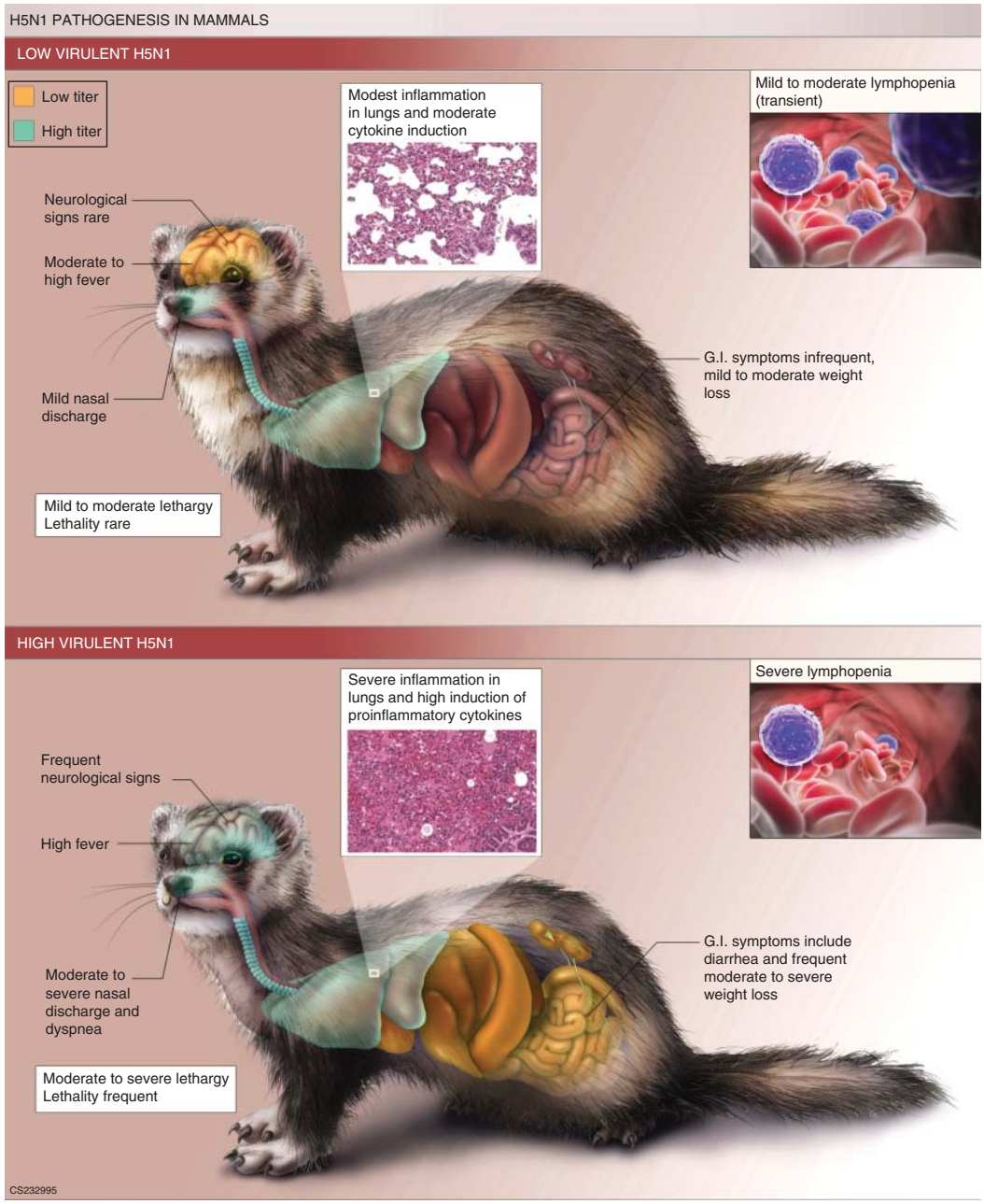

Figure 24.4 Use of the ferret model to study H5N1 virus pathogenesis. Numerous virus and host features studied in the laboratory in ferrets following infection with avian influenza viruses such as H5N1 are shown. Many LPAI viruses exhibit similar features to the low virulent H5N1 viruses depicted in the top panel. Illustration by Alissa Eckert. From Belser, J. A. and T. M. Tumpey. 2013. H5N1 pathogenesis studies in mammalian models. *Virus Research* 178:168–185, with permission of Elsevier. *See Plate section for color representation of this figure*.

infection with highly pathogenic H5N1 viruses or the reconstructed 1918 virus [69, 163].

Influenza virus transmission

The transmissibility of influenza viruses between ferrets has been documented since 1941 [2]. Due to the high susceptibility of ferrets to infection with influenza viruses, the presentation of clinical signs and symptoms of illness that correspond to those observed in humans, and the general agreement of data from experimental transmission modeling with those obtained from the human population, ferrets have become a critically important model for transmission studies. They can be used to model both transmission that occurs in the presence of direct contact (i.e. co-housing an infected ferret with a naive ferret), and transmission that occurs via droplet or aerosol routes (i.e. separating infected and naive ferrets by means of a perforated barrier, excluding direct or indirect contact with infected food, water, or bedding, but allowing air exchange between the animals) [12]. Although mammalian modeling cannot re-create all of the social and physical behaviors observed between humans that may contribute to the incidence of virus transmission, and the use of serologically naive ferrets in the majority of these studies does not fully represent a human population that possesses varying degrees of pre-existing immunity to circulating strains, the ferret transmission model has nonetheless enabled the close study of viral and host determinants that contribute to influenza virus transmissibility [12, 64].

Seasonal influenza A viruses are transmitted efficiently between ferrets both via respiratory droplets and through direct contact [52, 89]. Pandemic influenza viruses, including H1N1 viruses from the 1918 and 2009 outbreaks and H2N2 viruses from the 1957 outbreak, also demonstrate transmissibility in ferrets by these routes, as do select viruses of swine origin [56, 90, 97, 106, 107, 148]. In contrast, HPAI viruses such as H5N1 and H7N7 are not readily transmitted between ferrets, or are only transmitted in the presence of direct contact, consistent with the observed lack of sustained human-to-human transmission [5, 89, 158]. Numerous LPAI viruses associated with human infection, including viruses within the H7N2 and H9N2 subtypes, have demonstrated a capacity for transmission between ferrets in direct contact [5, 153]. H7N9 viruses have also shown some capacity for transmission by respiratory droplets [8, 111, 154, 162]. Although no non-human species can precisely model in a laboratory setting the transmissibility of influenza viruses in humans, these studies collectively show that ferrets generally recapitulate the transmissible phenotype of both avian and human influenza viruses, providing critically important information for public health.

Guinea pigs

Anecdotal accounts of the susceptibility of guinea pigs (*Cavia porcellus*) to influenza virus infection date back to the 1918 pandemic, but only in recent years has the utility of this species for pathogenesis and transmission studies been fully examined in a research setting [81]. The relatively low cost and small size of guinea pigs make them an attractive species for laboratory study. Furthermore, they are a permissive host to many influenza A viruses, and the receptor distribution of sialic acid receptors in the guinea pig respiratory tract is similar to that in humans [133]. Although guinea pigs do not present with many detectable clinical signs of infection, limiting their utility for traditional pathogenesis experiments, they are frequently used to assess influenza virus transmissibility.

Influenza virus pathogenesis and host responses

Unlike mice and ferrets, infected guinea pigs do not show overt signs of disease following infection with influenza virus, and do not exhibit fever, sustained weight loss, or lethargy during the acute phase of infection. Consequently, viruses that are lethal in mice and ferrets (including the reconstructed 1918 virus and some H5N1 HPAI viruses) do not maintain a virulent phenotype in this species [150]. Despite this, both human and avian influenza viruses are capable of infecting guinea pigs without prior host adaptation, with peak viral titers in upper respiratory tract samples being detected at 1–4 DPI [81, 150]. Replication of influenza viruses, including the H5N1 subtype, in guinea pigs is restricted to the respiratory tract [43, 150]; systemic spread to the brain has not been detected. Pulmonary lesions and bronchointerstitial pneumonia have been reported in H5N1 virus-infected guinea pigs, but

the overall severity of histopathology is reduced compared with the mouse or ferret models [72, 150]. The presence of a functional Mx protein in guinea pigs may contribute to the resistance of this species to severe disease following influenza virus infection. The lung physiology of guinea pigs is similar to that of humans. However, as with the ferret model, a paucity of commercially available reagents for this species limits the use of this model for some applications [141]. Induction of innate immune responses has been studied in this model [80], as has the role of exogenously administered type 1 interferon [130, 150].

Influenza virus transmission

As with the ferret model, unadapted seasonal and pandemic influenza viruses replicate to high titers in the respiratory tract of guinea pigs and are capable of transmission to naive animals both by direct contact and via the airborne route [81, 96]. Transmissibility of influenza B viruses has also been demonstrated in guinea pigs [109]. Swine influenza viruses and an H9N2 LPAI virus were not readily transmissible in this model, although an H7N9 virus demonstrated transmissibility between guinea pigs in a direct contact model, similar to results in the ferret [42, 133]. Interestingly, although H5N1 viruses typically do not exhibit transmissibility between ferrets, several H5N1 HPAI viruses have been shown to be transmitted to naive guinea pigs via direct contact [43, 160].

Cotton rats

Cotton rats (*Sigmodon hispidus*) are susceptible to infection with a range of human pathogens, and have been employed to model numerous respiratory viruses [21, 45]. Although not used as ubiquitously as mice, due in part to the limited reagents available for this species, cotton rats do offer certain advantages over mice, and therefore represent the model of choice for some laboratories. They are permissive to many influenza A viruses which cannot replicate in mice without prior adaptation, facilitating the study of wild-type viruses in this model. This may be due in part to a distribution of sialic acids throughout the respiratory tract of the cotton rat which more closely mimics the distribution found in humans, with greater expression of α2,6-linked sialic acids in the trachea and lung, than that in mice [16]. Furthermore, unlike many strains of mice, this species carries a functional set of *Mx* genes [21]. However, the lack of a sneeze reflex limits the utility of cotton rats for transmissibility studies [38].

Influenza virus pathogenesis

Cotton rats that have been inoculated with human or LPAI viruses exhibit mild transient weight loss, a transient drop in body temperature, and mild lethargy, with histopathology evident in lung tissue [16, 71, 104]. Virus typically replicates to high titers in both nose and lung, with the peak viral load being observed 1–3 DPI. Histopathology of the lungs can be moderate to severe, with peribronchiolitis and interstitial pneumonia detected by 7 DPI. [16, 71]. HPAI viruses maintain a lethal phenotype in cotton rats when inoculated at high doses, with greater weight loss and hypothermia observed compared with LPAI virus infection [16].

Immunity to influenza virus

The expression of biologically active antiviral *Mx* in inbred cotton rats permits the evaluation of early innate responses, as viral infection post-inoculation is typically controlled prior to the onset of the adaptive immune response [38]. The cotton rat model has been used to study antibody responses and heterosubtypic immunity elicited by inactivated influenza vaccines, live-attenuated vaccines, or prior infection, yielding results similar to those observed in human studies [117, 131, 159]. Although not as frequently studied in cotton rats, assessment of influenza virus antiviral efficacy is also possible using this model [124].

Pigs

Pigs (*Sus scrofa*) have long been regarded as a "mixing vessel" for influenza viruses, as they are able to support replication of both avian and human influenza viruses [87]. This capacity is probably conferred by the expression of both α2,3- and α2,6-linked sialic acids present in the respiratory tract of pigs, with a distribution similar to that observed in humans [145]. Further highlighting the role of swine in the study of influenza viruses, numerous *in-vitro* models of swine epithelial cells and swine respiratory tissue explants exist, which complement *in-vivo* results [33, 59]. However, the

specialized housing and husbandry requirements for conducting research in this model limit the use of this species to selected laboratories.

Influenza virus pathogenesis

Influenza virus infection in swine typically presents as respiratory disease, and can contribute to secondary bacterial pneumonia in this species, although subclinical infections often occur [33, 152]. The 2009 H1N1 viruses are capable of causing moderate disease in pigs, presenting with numerous clinical symptoms of infection, including sneezing, coughing, nasal discharge, dyspnea, and lethargy [73, 86]. Virus replication in the absence of clinical symptoms has been documented in miniature pigs [56]. In contrast to other mammalian models, such as the mouse and ferret, infection of pigs with H5N1 HPAI viruses typically results in a mild or asymptomatic infection, with virus replication generally restricted to the respiratory tract [77]. A similar presentation has been observed in pigs, but not in miniature pigs, infected with an H7N9 virus [154, 162]. Characterization of the immune responses elicited following infection with avian, swine, or human influenza viruses has further improved the utility of this model for pathogenesis studies [33].

Influenza virus transmission

The findings of studies of the transmissibility of influenza viruses between pigs, and from pigs to other susceptible mammalian species, have obvious implications for the spread of viruses that could pose a threat to human health, especially in view of the fact that the last four pandemic viruses in humans originated partly or entirely from non-human reservoirs [35]. In support of this, numerous studies have documented the cross-species transmission of influenza viruses from pigs to humans, often due to occupational exposure to swine [44, 161]. Studies that re-created pig-to-pig virus transmission events have found that swine H3N2 isolates and a human H3N2 variant strain isolated from a human were both transmissible between pigs when the animals were placed in direct contact [65]. Furthermore, transmission of H7N9 virus from infected to naive contact pigs (either by virus isolation or by seroconversion) has been demonstrated [79, 162]. These and others studies provide experimental evidence of the ability of pigs to support virus spread throughout a swine population, leading to greater potential for human exposure.

Non-human primates

Non-human primates, including cynomolgus macaques (*Macaca fascicularis*) and squirrel monkeys (*Saimiri sciureus*), have been used to evaluate the virulence of both avian and human viruses [99, 112]. Viral transmission from infected to naive non-human primates in the presence of direct contact has also been reported [94]. However, due to cost, size, and ethical constraints, this model is not frequently used for traditional pathogenesis or transmission studies.

Influenza virus pathogenesis

Although only infrequently used for studies evaluating the virulence of influenza viruses with pandemic potential, pathotyping studies conducted in non-human primates have provided important information. Cynomolgus macaques infected with influenza viruses display numerous clinical signs and symptoms that are also observed in humans, such as fever, coughing, anorexia, and lethargy [56, 112]. The 2009 H1N1 and H7N9 LPAI viruses evaluated in this model were found to replicate throughout the respiratory tract, unlike seasonal viruses, which are generally restricted to upper respiratory tract tissues [56, 154]. In contrast, severe disease leading to acute respiratory distress syndrome and death in this species has been reported following infection with either the reconstructed 1918 virus or H5N1 HPAI viruses [66, 112]. Despite the severe and fatal disease observed following infection with these virulent viruses, extrapulmonary spread of virus to the brain is not typically observed in non-human primates, and not all H5N1 viruses exhibit a lethal phenotype [14].

Host responses to influenza virus

The close physiological similarity between humans and non-human primates makes this model a desirable one for the study of systems biology and host responses following influenza virus infection. These studies have proved valuable for understanding the differences in host responses elicited

by viruses that cause mild and severe disease in mammals [98]. These models have also been used to identify the role of molecular determinants associated with detrimental host responses and alteration of lymphohematopoietic parameters among viruses associated with severe human disease, notably the reconstructed 1918 virus and H5N1 HPAI viruses [4, 29]. The use of models that so closely recapitulate human disease has enabled a greater understanding of influenza virus-induced pneumonia, providing valuable information that can contribute to improved treatment of virus infection in humans [120].

Other mammalian models

Although other non-human mammalian species are not frequently employed as laboratory models, it is still important to study the susceptibility of these species to influenza virus infection in order to gain an understanding of the potential risk of handling infected animals, especially for individuals who may be at risk of exposure due to occupational tasks. Species barriers typically restrict the permissiveness of many of these species to influenza virus infection, although the establishment of stable lineages of influenza viruses in selected instances (e.g. H3N8 canine influenza or H7N7 equine influenza) has demonstrated the potential for cross-species involvement [32, 142]. Notably, H5N1 viruses have been shown to productively infect a wide range of mammalian species, including dogs, cats, Syrian hamsters, and rabbits, maintaining a capacity to cause lethal disease depending on the virus strain and species evaluated [14, 69]. Although influenza virus infection of these species in a laboratory setting has been studied only infrequently, these studies nevertheless make an important contribution to our understanding of the animal–human interface.

Variation of inoculation route and dose

The above-mentioned studies are largely representative of animals inoculated by the intranasal route, which is a reliable and consistent method of infecting animals with a typically high dose of virus. However, many mammalian models can be adapted to study alternative exposure routes that pose a threat to human health. Furthermore, modulation of inoculation conditions can provide greater insight into the virulence of influenza viruses and allow for targeted infection of anatomical sites.

Modulation of liquid instillation

Dilution of virus in a liquid inoculum that is subsequently administered to the respiratory tract is the traditional route of inoculation for small mammalian models of influenza. However, variation in the dose, volume, and location of this inoculation can have a major impact on the severity of infection post inoculation. Many viruses that exhibit a lethal phenotype in mammals when administered at a high dose do not cause lethal disease when administered at lower doses [91]. Intranasal instillation is the most common route of inoculation in mice, but the volume administered varies widely between laboratories, ranging from less than 25 μL up to 100 μL deposited in the nares of the animal. Comparative studies have demonstrated that differences of as little as 10 μL in the volume applied are sufficient to modulate virus morbidity and mortality following infection [92]. Similarly, reducing the volume of inoculum from 1 mL to 500 μL or 100 μL during intranasal inoculation of ferrets limits the respiratory tract tissues that are initially exposed to virus [7, 17, 47]. Bypassing the nares of the animal altogether and inoculating ferrets by the intratracheal route can also achieve site-specific pathology [17, 18]. Inoculation at multiple anatomical locations (typically including both respiratory tract and conjunctival sites) is often used in the non-human primate model to achieve consistent infection with influenza virus [112]. As the choice of an appropriate inoculation method and volume can strongly influence the disease presentation and pathology observed following influenza virus infection, close attention should be paid to these parameters, especially when comparing studies between laboratories that utilize different inoculation protocols.

Aerosol inhalation

Although instillation of a liquid inoculum is an established practice in the field, there is a concurrent need to study mammalian models that recapitulate inoculation routes which occur in

nature. As inhalation of virus-containing aerosols represents one of several modes of influenza virus transmission in humans [139, 140], aerosol inhalation inoculation methods have been established in multiple mammalian species. Not only do aerosol inhalation models more closely resemble natural exposure than do liquid instillation inoculation methods, but also they mitigate the possibility of the animal diverting inoculum away from the respiratory tract as a result of swallowing [46]. Numerous studies have been conducted to compare aerosol delivery of influenza viruses with the intranasal route in order to better understand differences in infectivity, disease onset and progression, virus shedding kinetics and duration, and elicitation of immune responses.

Experimental inoculation of mice with influenza virus by the aerosol inhalation route was established over 50 years ago [116]. Since this time, more sophisticated instrumentation to generate, sample, and analyze aerosols has improved our ability to investigate the role of infectious dose and particle size in governing virus deposition in the mammalian respiratory tract [46, 67, 88]. Inoculation of mice, ferrets, guinea pigs, and squirrel monkeys by the aerosol inhalation route has been documented [23, 46, 96, 126]. However, the majority of this research was conducted using historic laboratory strains of influenza virus, or viruses that have undergone mammalian adaptation. There is a need to extend these studies to include contemporary influenza viruses and strains with pandemic potential. In this context, recent studies have found that ferrets inoculated with H5N1 virus by the aerosol inhalation route maintain the same high infectivity and lethality as intranasally inoculated ferrets, but that aerosol-inoculated ferrets present with more severe disease when exposed to low doses of virus [47, 74].

Ocular inoculation

Although not routinely studied in the context of influenza virus infection, the eye represents both a potential site of virus replication and a portal of entry to establish a productive respiratory infection [13]. This is largely due to the distribution of permissive sialic acids on both the human corneal and conjunctival epithelial cells, as well as the nasolacrimal duct, which bridges the ocular and nasal tissues [70]. The predominance of α2,3-linked sialic acids in ocular tissue may partially govern the ocular tropism displayed among select avian influenza viruses in humans, but the ability of both human and avian influenza viruses to bind to human ocular tissue indicates that other properties contribute to the tropism of some viruses for this tissue [7, 103].

To date, ocular inoculation models for influenza virus have been established in mice and ferrets, the majority of which utilize a liquid suspension instilled onto the surface of the eye with or without prior corneal scarification [13]. Mice generally recapitulate the apparent ocular tropism observed among select virus subtypes in humans [15, 132, 137], while both human and avian influenza viruses can mount a productive and transmissible respiratory infection in ferrets following ocular inoculation with a liquid or aerosol inoculum [1, 6, 7]. Mice and ferrets that have been inoculated by the ocular route typically lack the macroscopic ocular signs that are observed in humans, such as conjunctivitis, but exhibit a course of respiratory disease that is generally observed following traditional intranasal inoculation.

Alternative inoculation routes

Respiratory exposure and ocular exposure represent the two most frequent routes of influenza virus infection in humans, but several other inoculation routes are employed in the laboratory to investigate virus infectivity and tropism. Intragastric inoculation of mice, ferrets, guinea pigs, and hamsters has been used to model the ability of H5N1 viruses to cause disease following digestive exposure [72, 76, 121]. Highlighting the ability of HPAI viruses to cause severe disease and death following multiple inoculation routes in mammalian models, H5N1 influenza viruses have been shown to maintain a lethal phenotype following intravenous or intracranial inoculation [14, 24]. Understanding the relative risk of different potential routes of human exposure allows both a more in-depth assessment of the virulence of influenza viruses that pose a threat to human health, and guidance on mitigating that risk in humans [135].

The importance of established models for public health

Well-characterized mammalian models serve numerous invaluable roles with regard to the prevention, treatment, and study of influenza viruses in humans (Box 24.1). The addition of whole-system *in-vivo* data provides a critical bridge between *in-vitro* and *ex-vivo* experimentation, and allows the study of complex virus–host interactions that would not be possible by other means. Several examples of the role of established mammalian models in public health are described below.

Box 24.1 Importance of mammalian models for the study of complex virus–host interactions.

Examples of research applications that are greatly enhanced by or unachievable without the use of mammalian models

- Analysis of virus deposition throughout respiratory tract following exposure
- Modulation of lymphohematopoietic parameters following infection
- Induction of proinflammatory cytokine and chemokine mediators
- Capacity of virus for extrapulmonary or systemic spread
- Tropism of virus in respiratory and non-respiratory tissues and cell types
- Elicitation of clinical signs and symptoms following virus infection
- Virus transmissibility (direct or airborne) to naive contacts
- Induction of adaptive and long-lived memory immune responses
- Analysis of immune correlates of protection following infection or vaccination
- Efficacy of novel antiviral and vaccine approaches, including adjuvants
- Study of aerosolized virus released during mammalian respiratory activities
- Virus infectivity following use of alternative (non-respiratory) inoculation routes
- Generation of *de-novo* mutations during acute virus infection

Evaluation of vaccines and vaccine candidates

Testing of candidate vaccine viruses which are considered for vaccine production relies on numerous assays to demonstrate attenuation compared with the parental virus [155]. Among these tests is the need to demonstrate virus attenuation in ferrets or other suitable mammalian models, such as mice. Compared with the parental strain, the candidate vaccine virus must possess substantially reduced viral titers in respiratory tract tissues, a lack of systemic spread beyond the respiratory tract, and diminished clinical signs of disease. Comprehensive pathotyping of wild-type viruses from which candidate vaccine viruses are derived is therefore essential in order to ensure thorough assessment of the safety profile of these vaccine candidates prior to their release to vaccine manufacturers [19]. Use of this paradigm has resulted in the evaluation of many candidate vaccine viruses generated by both classical reassortment and reverse genetics in mammalian models following recent pandemics and outbreaks [27, 113].

Mammalian models have also been critically important for the assessment of preclinical novel vaccine approaches. Alternative vaccine approaches that do not require propagation in embryonating eggs, do not require the use of live virus, and/or include conserved influenza virus antigens for increased cross-protection represent an area of intense research that invariably includes *in-vivo* preclinical evaluation [128]. The efficacy of novel adjuvants to boost the immunogenicity and/or cross-reactivity of existing and experimental influenza vaccines has also been evaluated in these models [3, 36]. Alternative delivery methods have been similarly compared with conventional intramuscular injection or nasal spray in mammalian species for effectiveness and heightened immunogenicity [68, 125]. Although ferrets are typically used in these evaluative studies, the development of additional ferret-specific reagents to better quantify host immune responses is needed for the more detailed assessment of immune correlates of protection in this work [110].

Mammalian models continue to play a role after vaccines have been licensed for human use. Human serological studies evaluating cross-protection of previous seasonal vaccination against novel heterologous strains have been aided by parallel research in mammalian models [26, 53]. Several studies involving the ferret and guinea pig models have assessed the ability of vaccination to block transmission of homologous or heterologous influenza viruses to naive contacts, providing an experimental complement to human studies that have evaluated the effectiveness of vaccination of healthcare workers in order to reduce the transmission of virus to patients [84, 100, 108]. Although less frequently employed, due to size, cost, and ethical considerations, vaccination of non-human primates with candidate vaccine viruses has yielded valuable information about humoral and cellular immune responses following vaccination [39]. The comparison of protective responses between young and aged mice has provided an *in-vivo* model for studying the efficacy of vaccination in different populations and for investigating novel ways to improve vaccines for greater immunogenicity in immunocompromised populations [60].

Efficacy of antiviral treatments

Small mammalian models are typically used for preclinical assessment of influenza virus inhibitors and other antiviral approaches once efficacy has been demonstrated *in vitro* [122]. As such, establishing in advance well-characterized *in-vivo* models against contemporary influenza viruses, especially those that possess pandemic potential, maximizes the ability to determine the efficacy of the antiviral agent under investigation. The choice of species and challenge virus(es) can strongly influence the assay parameters available to quantify an antiviral effect, and care must be taken to design studies to balance a robust viral challenge in the animal without overwhelming the ability of the antiviral agent to function as it is designed to do.

Mice are frequently used to study the efficacy of novel antiviral treatments against influenza virus, as they permit the measurement of many parameters associated with antiviral activity. These include reduction and/or protection against morbidity, mortality, viral replication in respiratory tract tissues, systemic spread of virus, lung pathology, and induction of detrimental host responses, among others [124]. Many of these observations may also be evaluated in ferrets, although the larger size of this species can make dosing based on the weight of the animal cost-prohibitive. Guinea pigs and cotton rats have both been utilized for antiviral testing, but the lack of viral pathogenesis typically observed in these species generally limits the parameters examined to viral titers. These studies have nonetheless examined the ability of antiviral treatment to reduce the severity of influenza virus infection as well as to assess the overall viral fitness of antiviral-resistant viruses [20, 22, 118].

Recent studies have examined the ability of antiviral treatments not only to limit virulence in the infected host, but also to inhibit virus transmissibility [82]. Ferrets have been used to model household transmission of a 2009 H1N1 virus by treating either infected or naive contact ferrets with oseltamivir and measuring transmission efficiency between animals [102]. Similar studies have been used to examine alternative antiviral approaches, as interferon treatment disrupted the transmissibility of a 2009 H1N1 pandemic virus between guinea pigs when either the donor or contact animals were treated [130]. These studies provide a much needed complement to retrospective epidemiology studies evaluating antiviral efficacy in human populations, thereby improving our understanding of virus transmissibility with and without pharmaceutical interventions.

Surveillance and risk assessment

Active surveillance of influenza virus across the globe provides a snapshot of which viruses are currently circulating in wild birds, gallinaceous poultry, and other species. Elucidation of molecular determinates that confer virus pathogenicity and transmissibility has improved our ability to examine sequence data from surveillance isolates in order to identify markers of mammalian virulence or adaptation that may increase the threat of human infection. Furthermore, virus transmissibility in laboratory animals is a component of the Influenza Risk Assessment Tool (IRAT), an evaluation tool designed to assess the potential pandemic risk of influenza A viruses [31]. Determination of antiviral susceptibility, antigenic relatedness to vaccine candidates, and disease severity can also

be aided by experimental mammalian inoculation with these viruses. Risk assessments have been conducted for previously pandemic viruses that still pose a potential threat to human health, such as H2N2, wild-type avian influenza viruses that have caused sporadic infection in humans to date, and reassortant human–avian influenza viruses [57, 58, 61].

As currently available antiviral agents represent the first line of defense against novel influenza viruses, the evaluation of viruses that have been shown by *in-vitro* assays to be resistant to these antiviral products has an important role in pandemic preparedness efforts. Numerous mammalian models have been utilized to determine whether oseltamivir-resistant viruses exhibit diminished virulence compared with sensitive strains [49, 157]. The transmissibility of oseltamivir-resistant viruses has also been evaluated in the ferret and guinea pig models, and it has often been found that resistant strains are capable of maintaining a transmissible phenotype [118, 157]. An understanding of the risk posed by viruses that are resistant to currently available antiviral products, and the assessment of mutations that may alter virus fitness *in vivo*, both provide vital information for public health officials.

Role of environmental conditions

Although the seasonality of influenza viruses varies across the globe, it is likely that both environmental conditions and human behavior influence the periodicity of annual epidemics [95]. Temperature and humidity have been identified as two parameters that contribute to influenza virus seasonality in general, and transmission in particular [83]. Several studies utilizing guinea pigs and ferrets have been conducted to model the effect of environmental conditions on virus transmission to naive contacts. These studies have provided experimental data indicating that cold and dry conditions permit a higher frequency of H1N1 and H3N2 virus transmission by respiratory droplets compared with conditions of high relative humidity and high temperature [80, 129]. In addition to studying the incidence and efficiency of virus transmission between animals, housing animals in well-controlled environmental chambers permits the study of aerosol and virus shedding profiles from infected mammals under a range of humidity and temperature conditions [48]. These *in-vivo* studies permit the close examination of environmental parameters that require tightly controlled settings, and could not be easily conducted in any other context.

Determination of pandemic potential

Avian influenza viruses of multiple subtypes, notably H5 and H7, have caused documented sporadic human disease and death for decades. However, these virus subtypes have not caused a pandemic to date, probably due to their poor transmissibility between humans. Understanding how a virus acquires a transmissible phenotype, and whether this phenotype can be acquired without a resulting loss of virulence in mammals, represents an urgent public health need. Using the ferret model, two independent studies found that H5N1 viruses are capable of acquiring a transmissible phenotype, highlighting the public health threat posed by this virus subtype should these changes occur in nature [51, 55]. Similar results have been obtained with an H7-subtype virus [134], revealing the pandemic potential of multiple subtypes of influenza virus. These *in-vivo* "gain-of-function" studies provide essential information that not only identifies genetic changes in the virus which confer a transmissible phenotype, but also guides surveillance efforts, vaccine strain selection and stockpile decisions, and antiviral efficacy testing [62, 75].

Despite the wealth of information that can be obtained from these studies, there are numerous biosafety, biosecurity, and ethical issues which must be taken into consideration in order to guide the responsible generation and manipulation of viruses that pose a potential heightened risk of human infection [78]. *In-vivo* experimentation provides the most comprehensive and reliable assessment of virus transmissibility, but conducting concurrent and supporting work on numerous research platforms represents an important step in this process (Figure 24.1). There has been a move towards increased transparency among researchers, public health experts, regulatory boards, funding sources, and the lay public when planning, conducting, and disseminating the results of these studies [40, 50]. As human infection with avian influenza viruses persists, and novel viruses emerge that

pose a threat to human health, continued close scrutiny and assessment of the potential public health benefits and risks of this line of research will be necessary.

Conclusion

Influenza viruses pose a continuous threat to human health, necessitating constant monitoring and investigation in order to achieve optimal preparedness for inevitable outbreaks and pandemics. This chapter has described how the use of mammalian models represents a vital element of these efforts. Given the important role played by *in-vivo* data in the prevention and control of influenza virus infection, there is a need for continuing refinement of the existing models in order to optimally study and mimic human exposure and disease. Advances in aerobiology that have led to increased use of mammalian inoculation via inhalation rather than liquid instillation are just one example of such innovation [46]. Bioluminescent imaging with replication-competent influenza reporter viruses, enabling the analysis of real-time infection dynamics *in vivo*, represents another novel approach that will yield a greater understanding of virus–host interactions in the future [105, 144]. During the pursuit of these important advances there are several areas pertaining to the use of animal models in influenza virus research which must not be overlooked. More detailed reporting of experimental and statistical methods in published studies using mammalian models is needed in order to improve understanding, interpretation, and reproducibility of the data contained therein [63]. Similarly, it is important for individual studies to incorporate adequate sample sizes of animals used, analysis of multiple viruses to avoid unintentional strain-specific results, and varied experimental readouts [11, 78]. Careful attention to these and other considerations will greatly aid our study and interpretation of the zoonotic potential of animal influenza viruses in the years ahead.

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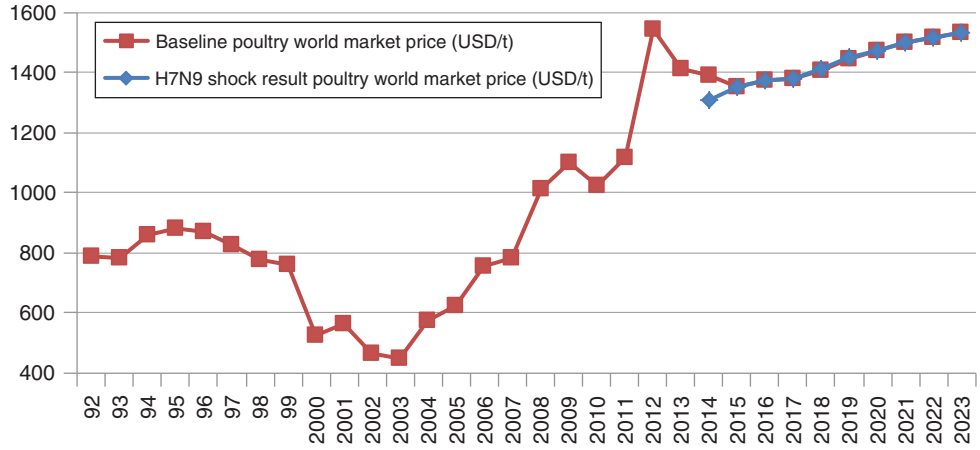

Figure 3.3 World poultry market prices and simulated impacts from 50% demand decline and export ban in Asia. Source: OECD-FAO. 2013. *OECD-FAO Agricultural Outlook 2013*. OECD Publishing. Available at 101718/agr_outlook-2013-en. Holger Matthey, Market and Trade Division. Used with permission from the FAO.

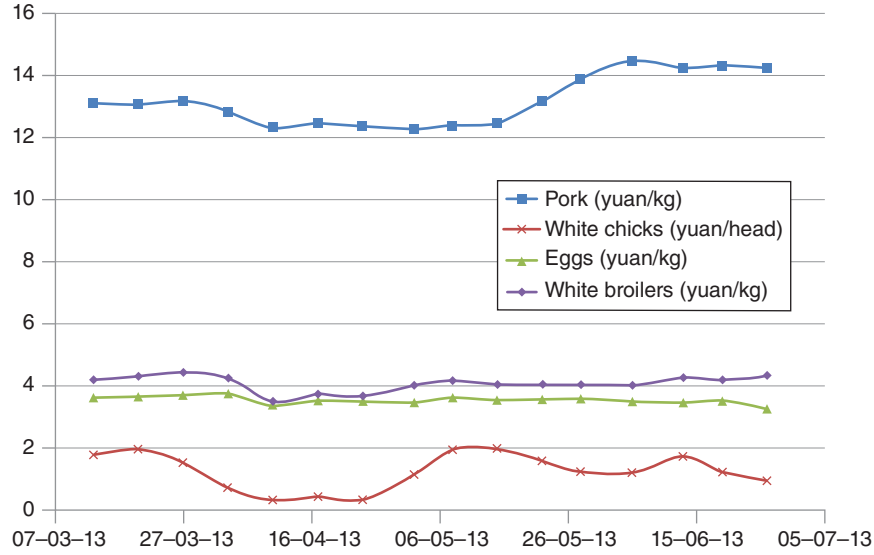

Figure 3.4 National pork and poultry prices in 2013 in China [12].

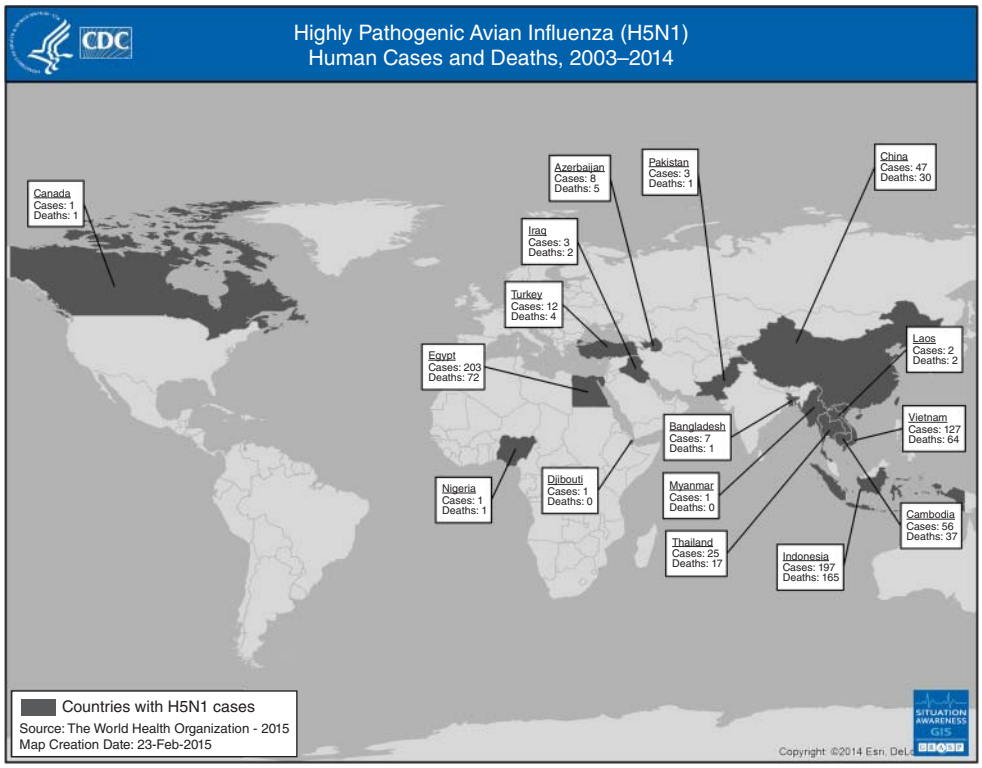

Figure 5.1 Geographic distribution of human H5N1 HPAI cases reported to the World Health Organization between November 2003 and December 2014. Source: World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC).

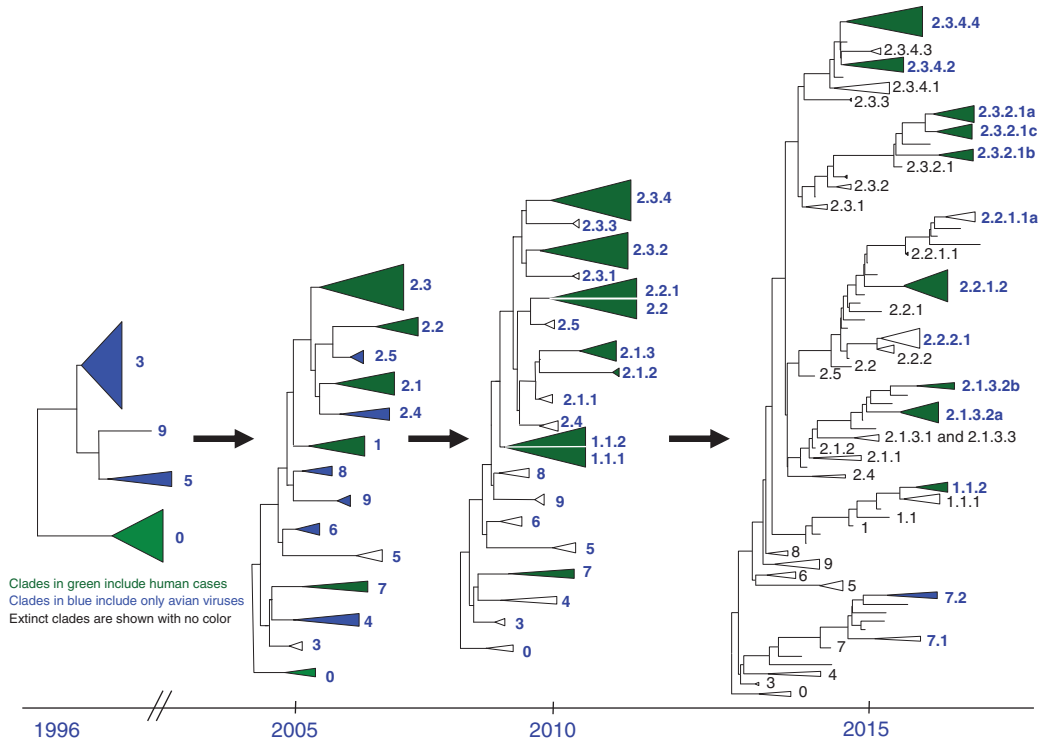

Figure 9.1 Phylogenetic relationships of the HA gene of Gs/GD-lineage H5N1 HPAIVs over time. The WHO/OFFLU H5 Evolution Working Group has kept under continuous review the nomenclature for Gs/GD-lineage H5 HPAIVs as they have evolved since their first emergence and detection in 1996. Discrete monophyletic groups appear within a specific clade, and when those groups meet the nucleotide divergence criteria (as well as having bootstrap values greater than 60, and within-clade average pairwise distances of less than 1.5%) they are split into second-order clades (but still considered part of the first-order clade). As a second-order clade continues to evolve it may reach a similar level of genetic diversity, at which point it may be split into third-order clades, and so on. The same clade designation criteria apply to first-, second-, and any higher-order clade designations. Extinct clades that are believed to be no longer circulating are shown without color, clades that have only been reported in avian species are shown in blue, and clades that include viruses which have been detected both in humans and in avian species are shown in green. Courtesy of Todd Davis, CDC Atlanta.

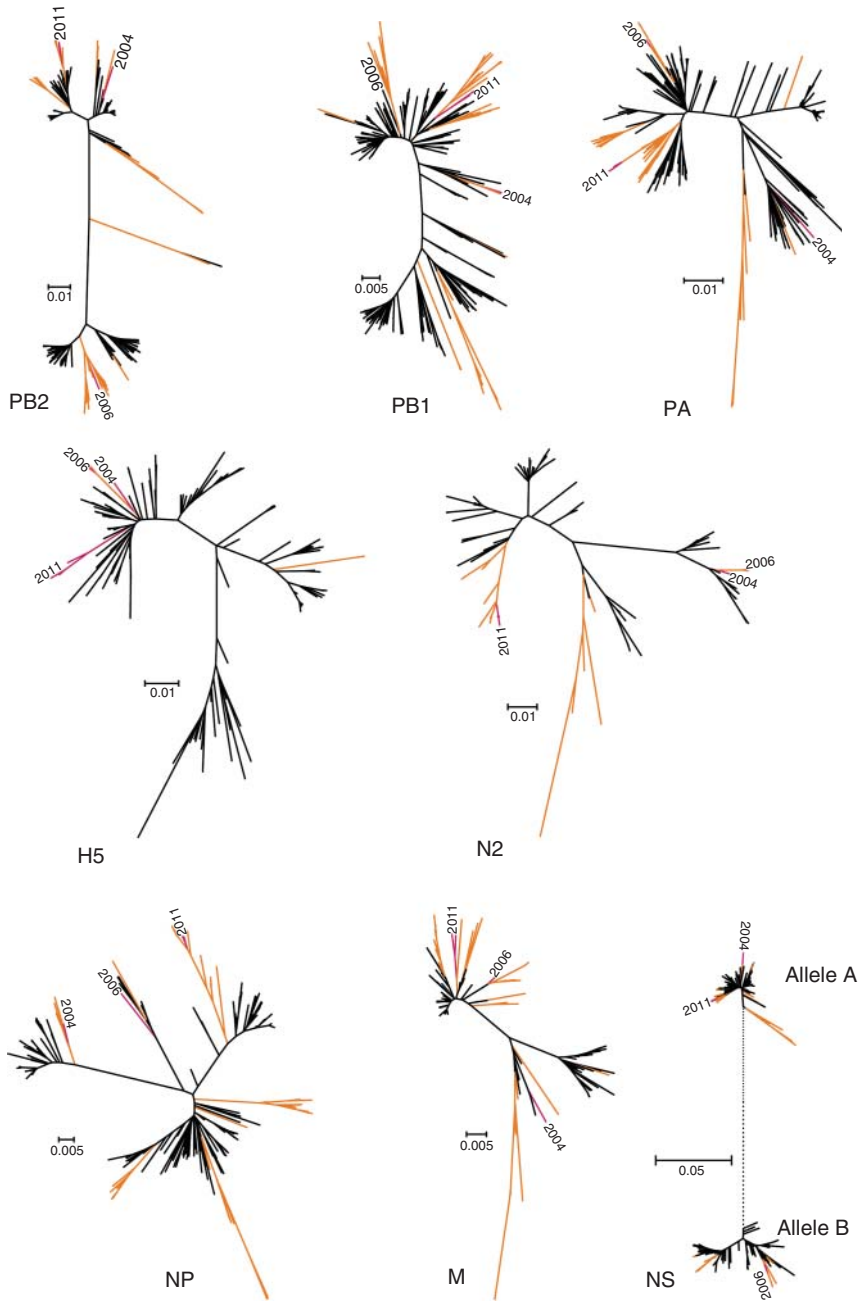

Figure 10.1 Maximum likelihood trees of full gene sequences derived from South African HPAI H5N2 isolates (shown in red), other southern African wild duck and ostrich isolates (shown in orange), and Eurasian strains (shown in black).

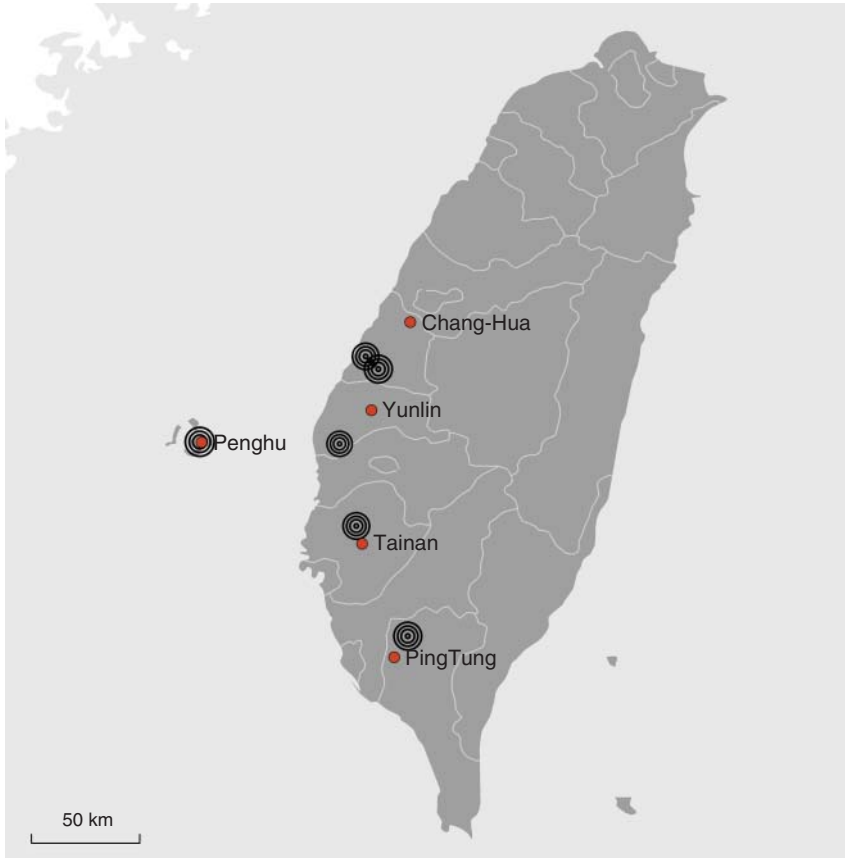

Figure 10.2 Distribution of H5N2 LPAI-affected premises in Chinese Taipei during 2012. Courtesy of the World Organisation for Animal Health.

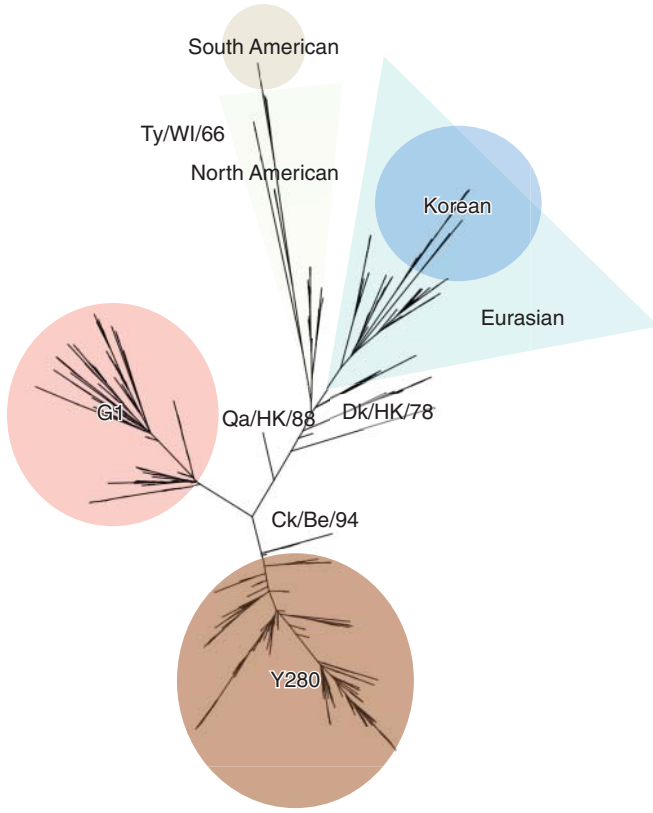

Figure 11.1 H9 HA phylogenetic tree showing major phylogenetic groups. Phylogenetic analyses were performed using online tools available at the Influenza Research Database (www.fludb.org). The unrooted tree was generated using the Archaeopteryx software tool as described elsewhere [337]. Labeling and colors were added using PowerPoint software (Microsoft, Inc.).

Figure 11.2 H9 HA phylogenetic tree showing major phylogenetic relationships of H9N2 viruses isolated from various animal species. Phylogenetic analyses and editing were performed as described for Figure 11.1. H9 HA position 226 in the receptor-binding site (site) with leucine is shown in red, with glutamine is shown in black, and with mixed virus populations carrying leucine and/or glutamine is shown in green. Note that a single virus isolate from a human case with methionine 226 is shown in blue. Light red box corresponds to G1-lineage viruses, and light brown box corresponds to Y280-lineage viruses.

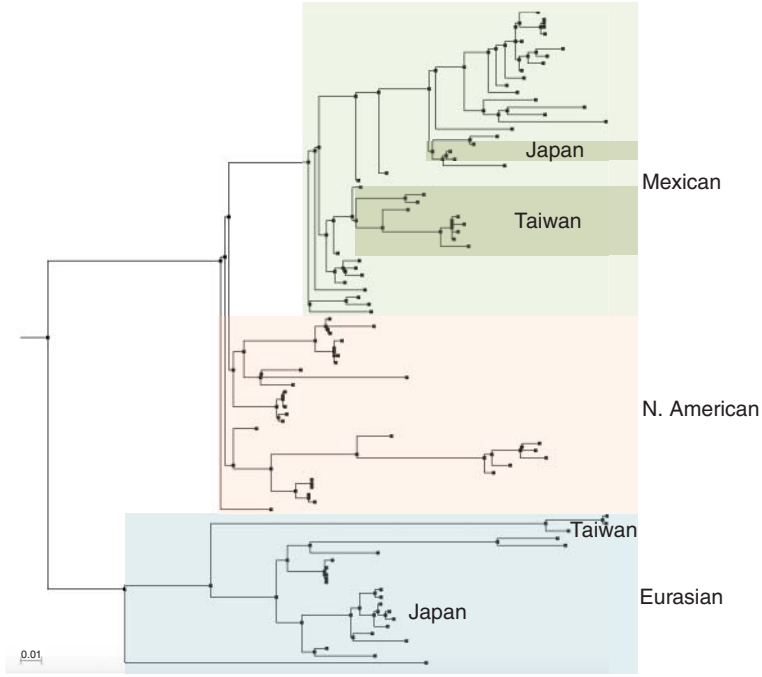

Figure 11.3 H5 HA phylogenetic tree showing major phylogenetic relationships of H5N2 viruses of North American (light orange) and Eurasian (light blue) lineages. Phylogenetic analyses and editing were performed as described for Figure 11.1. The Mexican viruses (shown in light green) form an independent evolutionary path stemming from an ancestor in the North American lineage. Mexican H5N2-vaccine-derived viruses were isolated from independent outbreaks of LPAI in Japan and Taiwan. In Taiwan, Mexican-derived H5N2 surface gene segments have reassorted with Taiwanese LPAIVs, and their endemic nature remains uncertain.

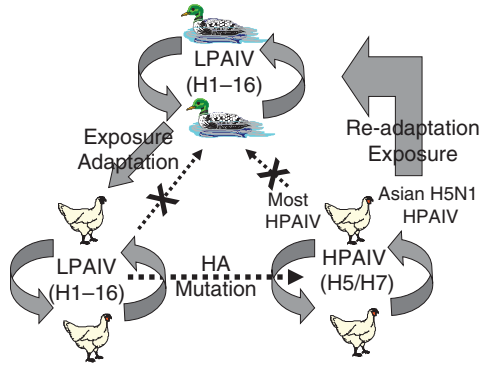

Figure 12.4 Epidemiology of LPAIVs and HPAIVs between free-living aquatic birds and poultry. Source: D. Swayne, U.S. Department of Agriculture/Agricultural Research Service.

Figure 13.1 Two-week-old Pekin ducks showing severe neurological signs at 3 days after IN inoculation with A/egret/HK/757.2/02 H5N1 HPAIV. Reprinted with permission from *Avian Diseases*. Copyright held by the American Association of Avian Pathologists, Athens, Georgia, USA. Source: USDA – M. Pantin-Jackwood [103].

Figure 13.2 Two-week-old Pekin ducks showing severe neurological signs at 3 days after IN inoculation with A/egret/HK/757.2/02 H5N1 HPAIV. Reprinted with permission from *Avian Diseases*. Copyright held by the American Association of Avian Pathologists, Athens, Georgia, USA. Source: USDA – M. Pantin-Jackwood [103].

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Figure 13.3 Bile-stained loose droppings from a 2-week-old Pekin ducks at 3 days after IN inoculation with A/egret/HK/757.2/02. Reprinted with permission from *Avian Diseases*. Copyright held by the American Association of Avian Pathologists, Athens, Georgia, USA. Source: USDA – M. Pantin-Jackwood [103].

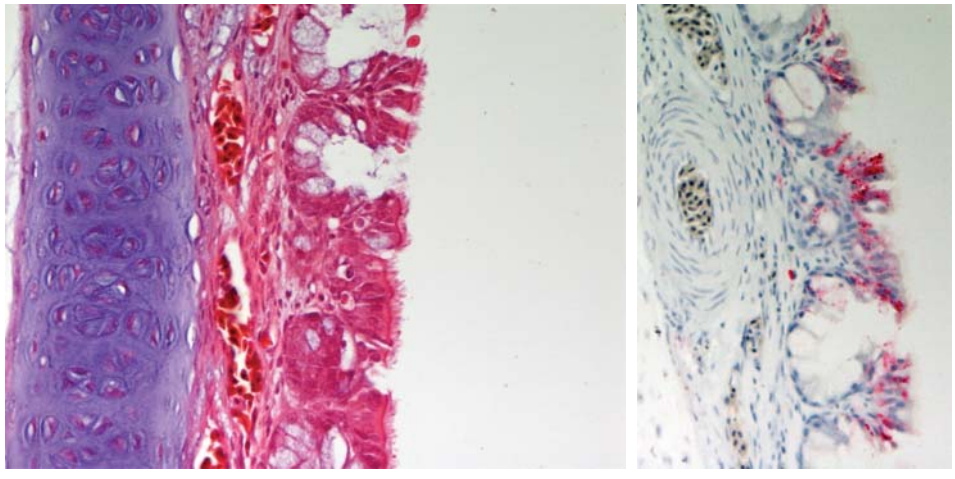

Figure 13.4 Moderate necrotizing rhinitis, with submucosal congestion and edema, and glandular hyperplasia of the nasal epithelium of a 2-week-old duck that died 3 days after IN inoculation with A/crow/Thailand/04 H5N1 HPAIVAIV. HE. Inset. Demonstration of viral antigen in the epithelial cells (shown in red). Reprinted with permission from *Avian Diseases*. Copyright held by the American Association of Avian Pathologists, Athens, Georgia, USA. Source: USDA – M. Pantin-Jackwood [103].

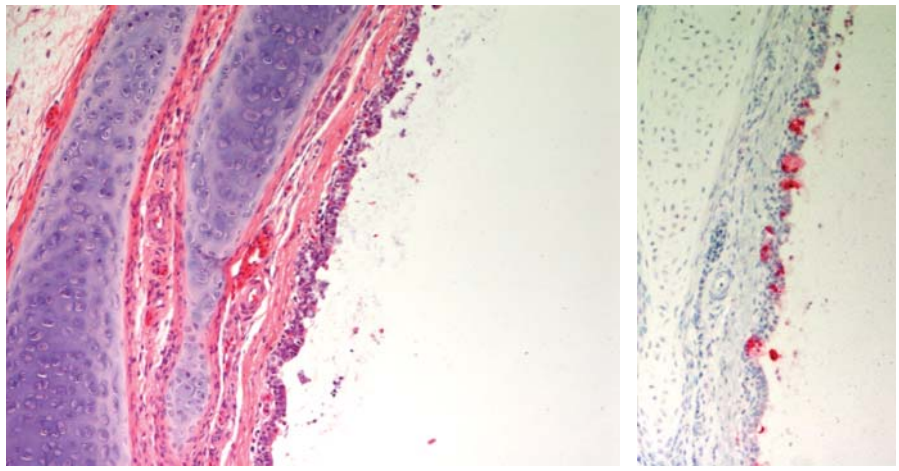

Figure 13.5 Degeneration and necrosis of the tracheal epithelium with mucocellular exudate containing sloughed epithelial cells of the trachea of a 2-week-old duck IN inoculated with A/crow/Thailand/04 and found dead at 4 days after inoculation. HE. Inset. AI viral antigen staining (shown in red) present in the epithelial cells. Reprinted with permission from *Avian Diseases*. Copyright held by the American Association of Avian Pathologists, Athens, Georgia, USA. Source: USDA – M. Pantin-Jackwood [103].

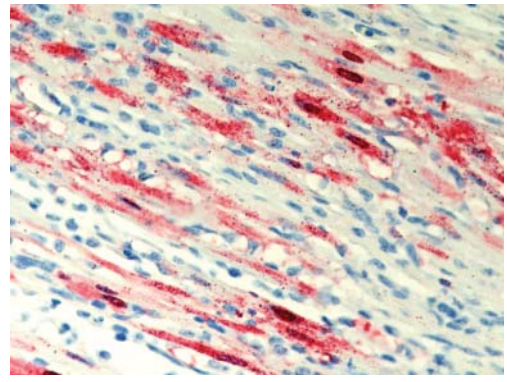

Figure 13.6 Extensive intranuclear and intracytoplasmic AI viral antigen (shown in red) in degenerated and necrotic myocytes of the heart of a 2-week-old duck IN inoculated with A/Thailand PB/6231/04 H5N1 HPAIV and found dead at 5 days after inoculation. Reprinted with permission from *Avian Diseases*. Copyright held by the American Association of Avian Pathologists, Athens, Georgia, USA. Source: USDA – M. Pantin-Jackwood [103].

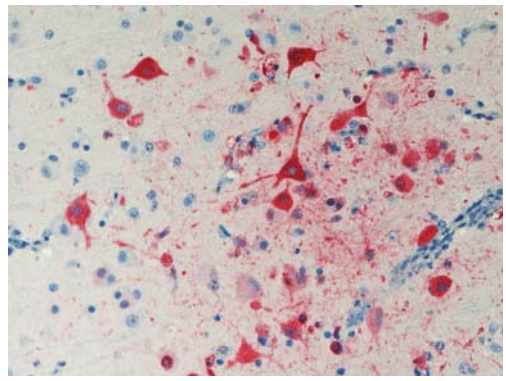

Figure 13.7 Strongly positive AI viral staining (shown in red) present in neurons of the cerebrum of a 2-week-old duck IN inoculated with A/Vietnam/1203/04 H5N1 HPAIV and found dead at 4 days after inoculation. Reprinted with permission from *Avian Diseases*. Copyright held by the American Association of Avian Pathologists, Athens, Georgia, USA. Source: USDA – M. Pantin-Jackwood [103].

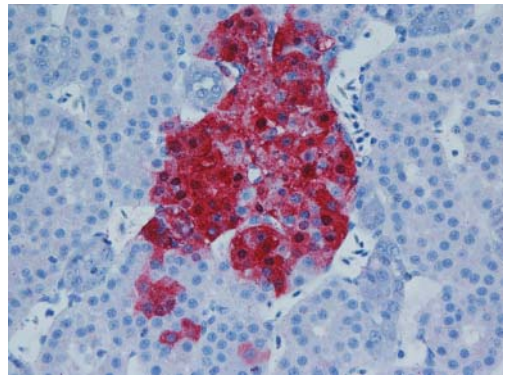

Figure 13.8 AI viral staining (shown in red) of the corticotrophic cells of the adrenal gland of a 2-week-old duck IN inoculated with A/Vietnam/218/05, 2 days after inoculation. Reprinted with permission from *Avian Diseases*. Copyright held by the American Association of Avian Pathologists, Athens, Georgia, USA. Source: USDA – M. Pantin-Jackwood [103].

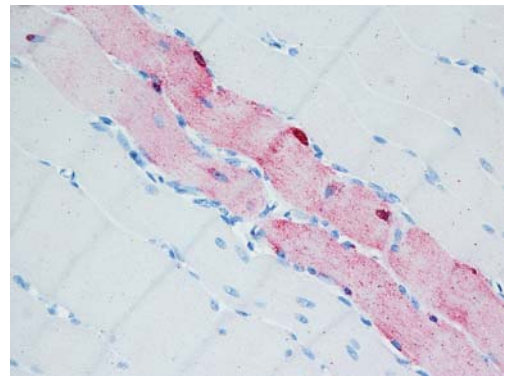

Figure 13.9 AI viral staining (shown in red) of the myocytes of skeletal muscle of a 2-week-old duck IN inoculated with A/crow/Thailand/04 and euthanized at 4 days after inoculation. Reprinted with permission from *Avian Diseases*. Copyright held by the American Association of Avian Pathologists, Athens, Georgia, USA. Source: USDA – M. Pantin-Jackwood [103].

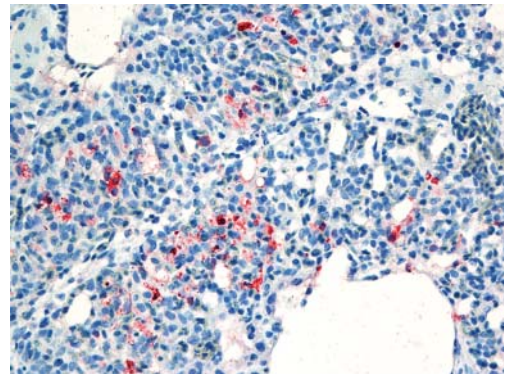

Figure 13.10 AI viral staining (shown in red) of phagocytic cells and alveolar epithelium of the lung of a 2-week-old duck IN inoculated with A/chicken/Egypt/08124S-NLQP/2008.

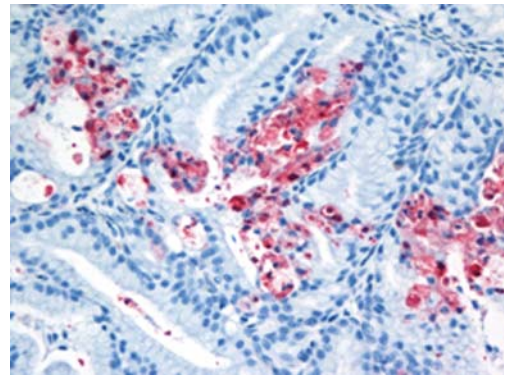

Figure 13.11 Vacuolar degeneration and AI viral staining (shown in red) of the Harderian gland epithelia of a 2-week-old duck IN infected with A/chicken/Egypt/08124S-NLQP/2008.

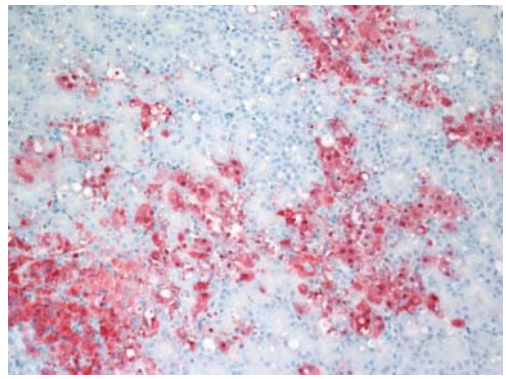

Figure 13.12 Severe multifocal cellular swelling and necrosis of the pancreatic acinar epithelium with viral staining (shown in red) of a 2-week-old duck IN infected with A/chicken/Egypt/08124S-NLQP/2008.

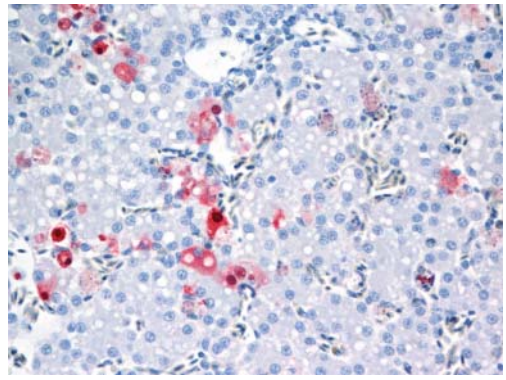

Figure 13.13 Viral staining (shown in red) in hepatocytes and Kupffer cells in the liver of a 2-week-old duck IN infected with A/chicken/Egypt/08124S-NLQP/2008.

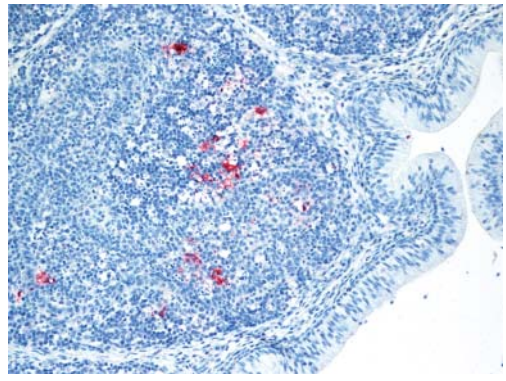

Figure 13.14 AI viral antigen (shown in red) in resident and infiltrating phagocytes in a bursa follicle of a 2-week-old duck IN infected with A/chicken/Egypt/08124S-NLQP/2008.

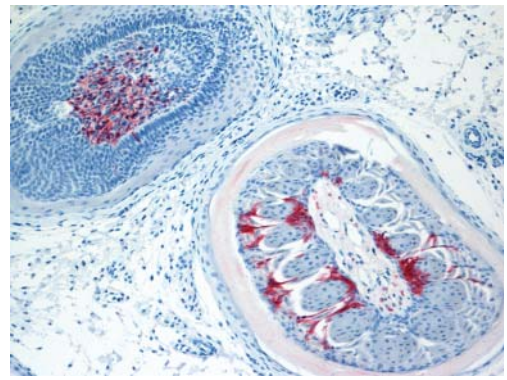

Figure 13.15 AI viral antigen (shown in red) in the epithelium and pulp of feathers of a 2-week-old duck IN infected with A/duck/Vietnam/218/2005.

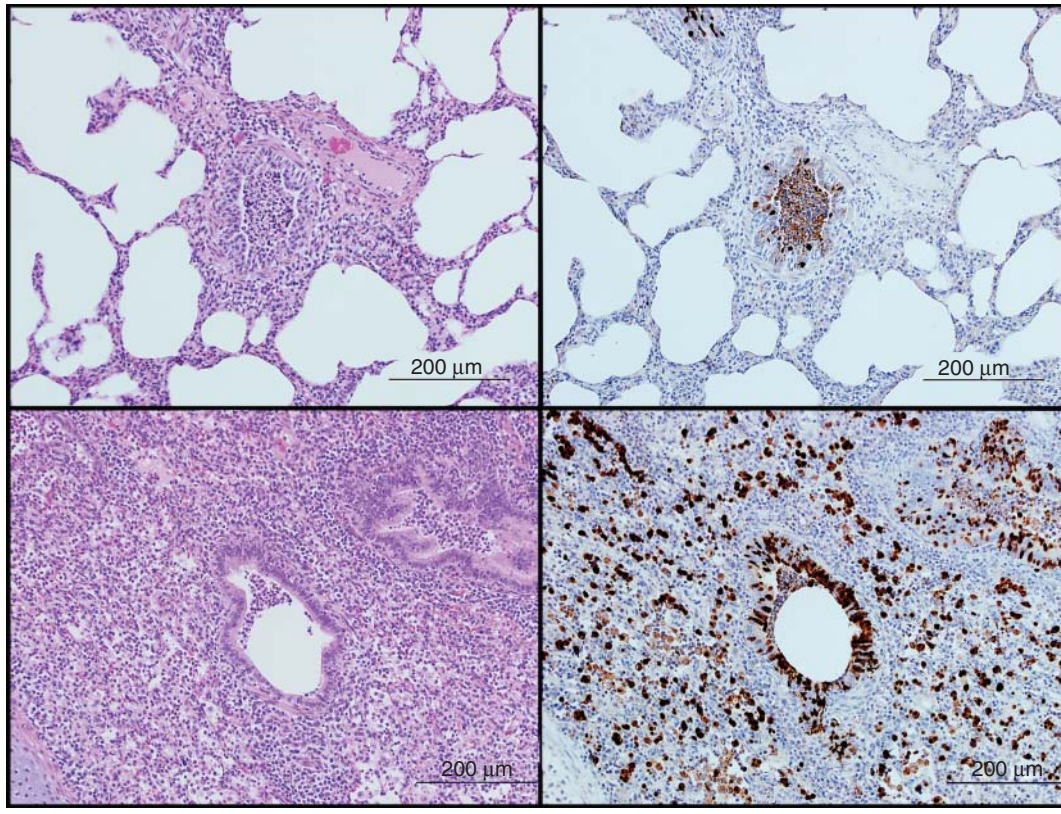

Figure 16.2 Experimental inoculation of a 4-week-old pig with A/swine/Texas/4199-2/1998 H3N2 virus 48 hours PI. Courtesy of Susan Detmer. **Figure 16.2a.** Severe, necropurulent bronchiolitis with mild interstitial pneumonia; hematoxylin and eosin (H&E), 200×. **Figure 16.2b.** Moderate bronchiolar epithelial cell and intraluminal immunoreactivity to anti-Influenza A nucleoprotein; immunohistochemistry (IHC) with diaminobenzidine (DAB), 200×. **Figure 16.2c.** Necropurulent bronchiolitis with severe alveolar pneumonia (lobular consolidation); H&E, 200×. **Figure 16.2d.** Strong bronchiolar and alveolar immunoreactivity to anti-Influenza A nucleoprotein; IHC with DAB, 200×.

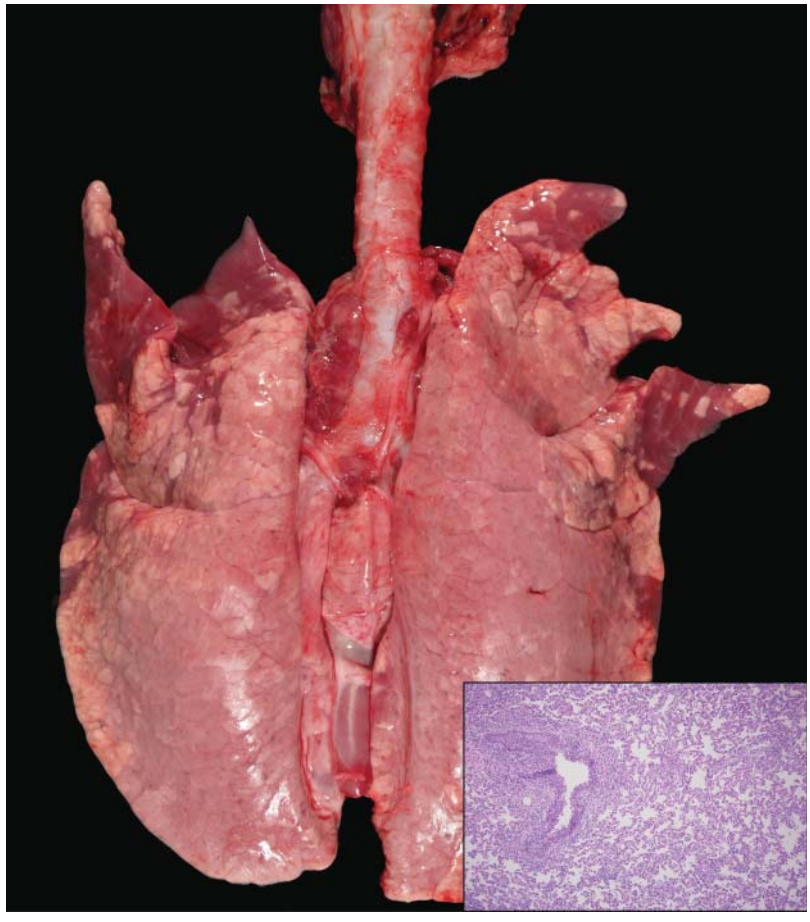

Figure 16.3 Macroscopic lesions in the lung of a 4-week-old pig experimentally inoculated with A/swine/Illinois/02450/2008 H1N1 virus 5 days PI. The depressed, dark red, multifocal to coalescing lobular lesions are in the cranioventral portions of the lungs, and reflect the microscopic lesions (shown in inset) of atelectasis. Courtesy of Susan Detmer.

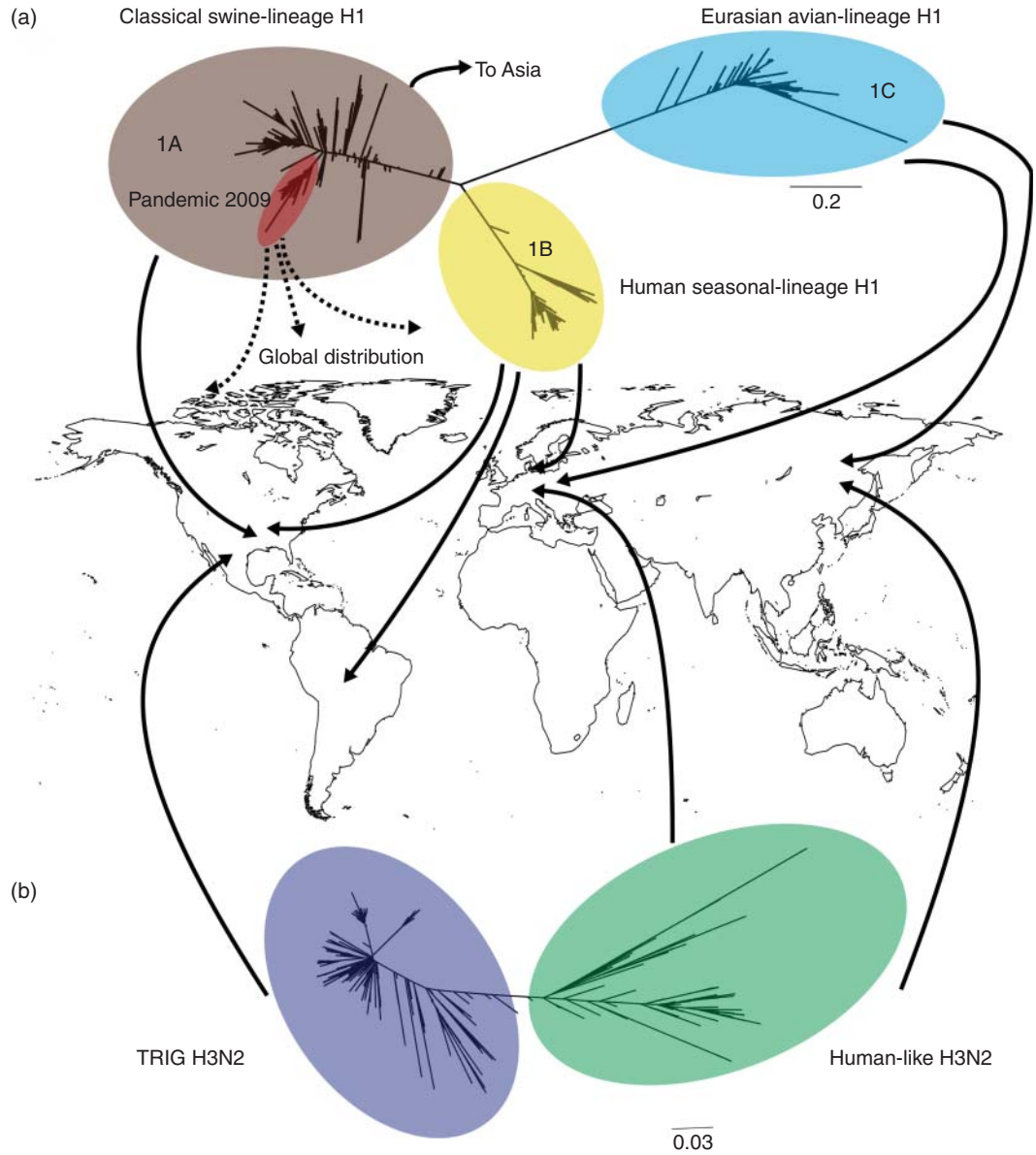

Figure 18.2 The major H1 and H3 genetic lineages and their geographic distribution in swine. (A) The phylogenetic relationships of H1 sequences. (B) H3 sequences are depicted in respective trees and color coded by lineage. In Europe, the HA genes are derived from Eurasian avian-like H1N1 (shown in blue), a human-like H3N2 (shown in green), or a human-like H1N2 (shown in yellow). Classical H1 (shown in brown), human-like H1 (shown in yellow), and human-like H3-TRIG (shown in purple) co-circulate. In Asia, the predominant HA lineages reflect the dynamics observed in North America and Europe, with co-circulating viruses classified as a classical swine lineage, human-like H3, or Eurasian avian-like H1. The H1N1pdm09 arose from the classical swine-lineage H1 (shown in red), and underwent global dissemination through human-to-swine transmission. Used with permission from Vincent, A. L., K. M. Lager, and T. K. Anderson. 2014. A brief introduction to influenza A virus in swine. *Methods in Molecular Biology* 1161:243–258.

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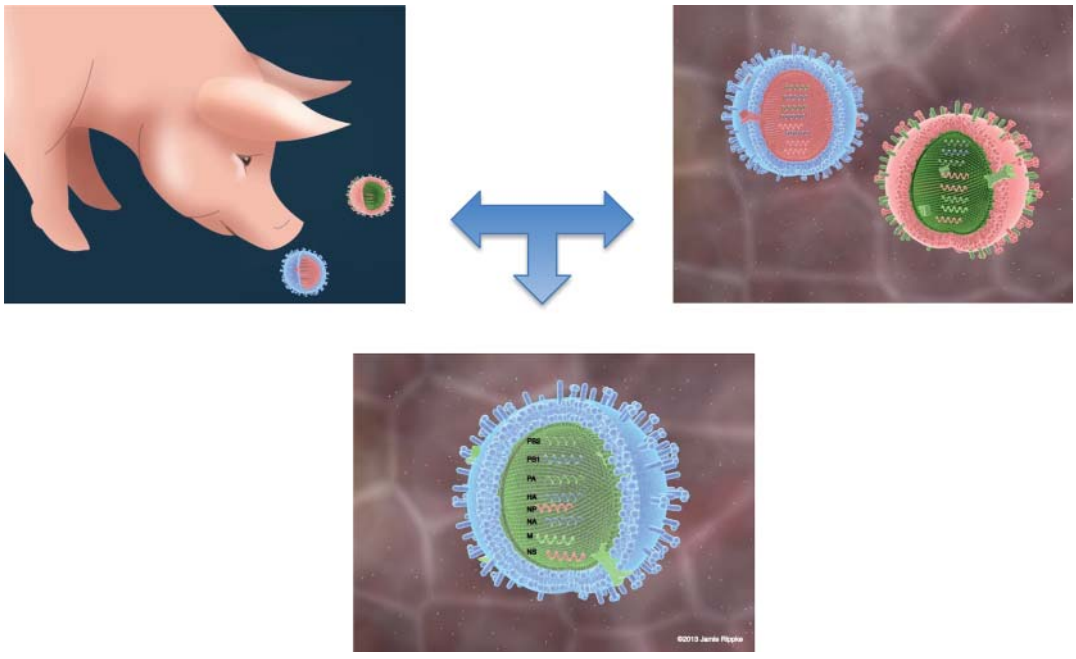

Figure 18.3 Putative generation of H3N2 variant in pigs. Pigs infected with two strains of IAV – H3N2-TRIG (shown in blue) and H1N1pdm09 (shown in pink) – allowed for reassortment of each parent virus's specific whole-genome constellation to generate novel progeny virions. One virus resulting from the potential reassortment patterns contained seven gene segments derived from the H3N2-TRIG and a single gene segment derived from H1N1pdm09 (the M gene shown in light green). An H3N2 with the genome constellation depicted here has been found in almost 350 human cases in the USA since 2010, termed H3N2 variant (H3N2v). Reassortants between endemic swine viruses and the H1N1pdm09 have been detected frequently in pig populations around the world since 2009. Source: Amy Vincent and Jamie Rippke.

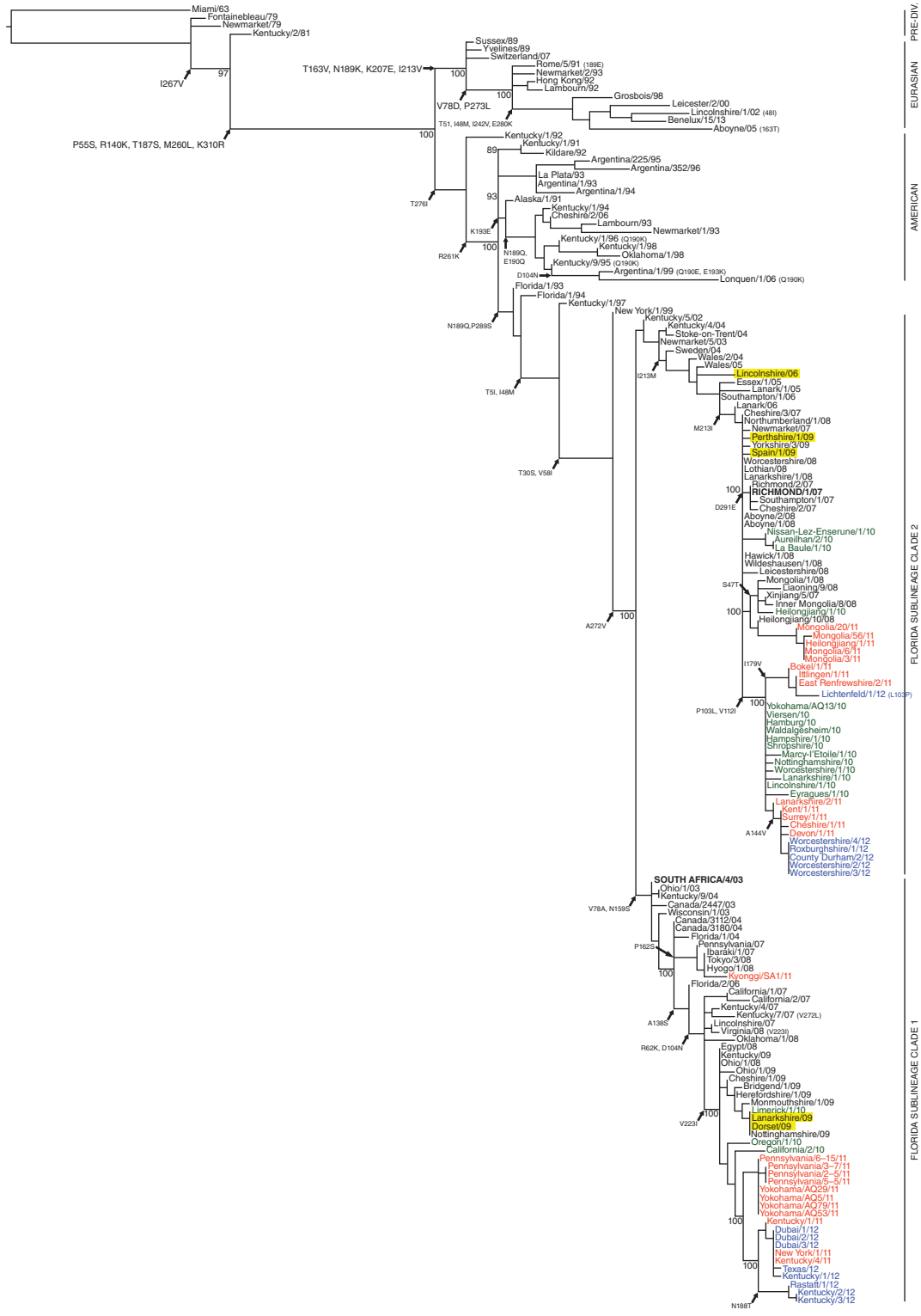

Figure 20.1 Phylogenetic tree of EIV H3N8 HA1 nucleotide sequences. This maximum-likelihood tree was generated using PhyML version 3. Bootstrap values obtained after 100 replicates are shown at major nodes. Amino acid substitutions are shown in parentheses or indicated at branch points. Phylogenetic groups (Pre-divergence, American lineage, Eurasian lineage, and Florida sub-lineage clades 1 and 2) are shown on the right. Sequences are color coded by date of isolation for the years 2010 (green), 2011 (red), and 2012 (blue), with the older isolates shown in black. The present OIE-recommended representative vaccine strains A/eq/Richmond/1/07 and A/eq/South Africa/4/03 are shown in bold. Reassortant strains that were identified containing HA from one Florida clade and NA from the other are highlighted in yellow. Reprinted from Woodward A. L. *et al.*, Development of a surveillance scheme for equine influenza in the United Kingdom and characterisation of viruses isolated in Europe, Dubai and the USA from 2010–2012. *Veterinary Microbiology* 169:113–127, 2014, with permission from Elsevier. We thank Dr. Adam Rash for providing the figure.

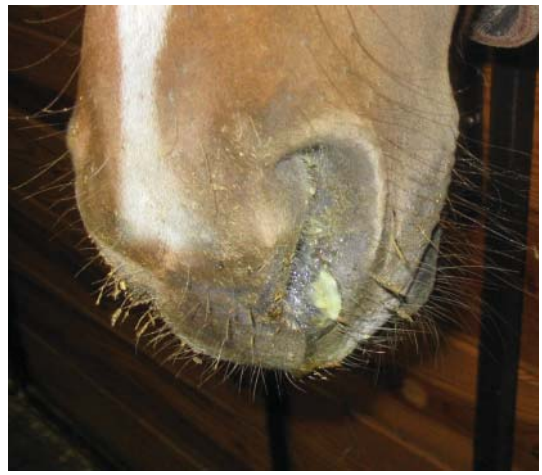

Figure 20.2 Equine influenza virus-infected horse exhibiting typical mucopurulent nasal discharge. Experimental infection with A/equine/Ohio/2003 (H3N8) virus, 5 days post infection. The horse was not febrile at this point, but had a cough and was still shedding detectable virus. Some horses in this study developed secondary spikes of pyrexia, and mucopurulent nasal discharge persisted as late as 9 days post infection. Photo courtesy of Thomas Chambers.

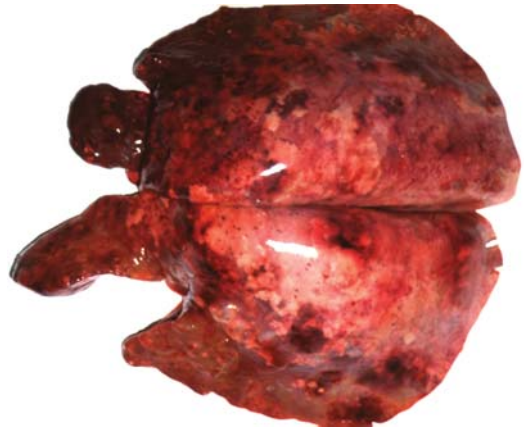

Figure 20.3 Acute severe bronchointerstitial pneumonia with edema and hemorrhage in a horse with equine influenza A virus infection and secondary bacterial infection. Photo from Noah's Arkive, University of Georgia.

Figure 23.1 Natural infection of tigers with H5N1 highly pathogenic avian influenza virus in Sri Racha, Thailand, in 2004. Affected animals had high fever, respiratory distress, and (in some cases) nervous signs, and died with serosanguinous nasal discharge. Photograph courtesy of Dr. Roongroje Thanawongnuwech, Chulalongkorn University, Thailand.

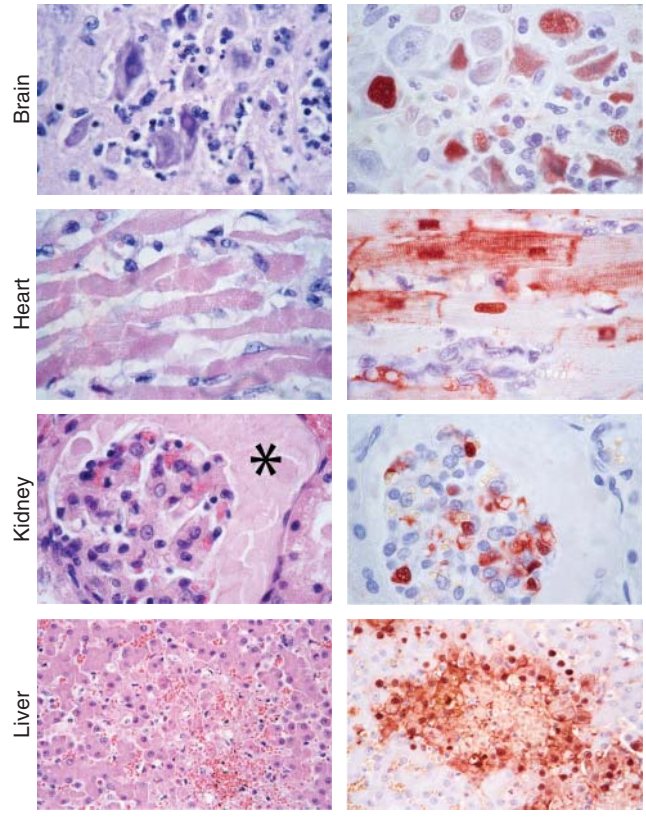

Figure 23.2 Systemic histological lesions in domestic cats after experimental HPAIV H5N1 infection. The left-hand column shows necrotizing inflammatory foci present in multiple tissues stained with hematoxylin and eosin. The right-hand column shows influenza virus antigen (red-brown staining) present in serial sections of the same tissues, stained for nucleoprotein by immunohistochemistry. Reprinted from *The American Journal of Pathology*, January 2006, Vol. 168, No. 1, pp. 176–183, Rimmelzwaan G. F., van Riel D., Baars M., Bestebroer T. M., van Amerongen G., Fouchier R.A., Osterhaus, A. D., Kuiken, T. Influenza A virus (H5N1) infection in cats causes systemic disease with potential novel routes of virus spread within and between hosts, with permission from Elsevier.

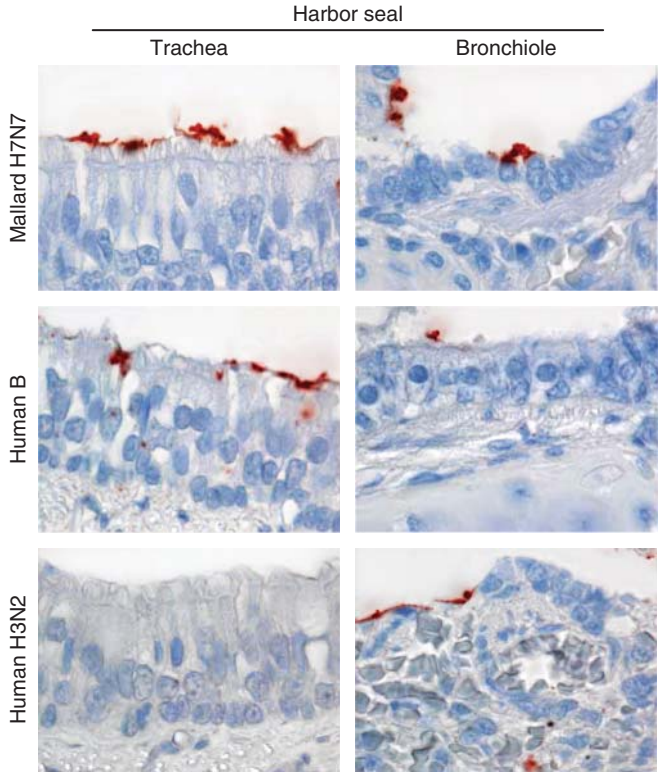

Figure 23.3 Low pathogenic avian influenza A virus (H7N7), human seasonal influenza A virus (H3N2), and human influenza B virus show different degrees of attachment to the trachea and bronchiole of a harbor seal (*Phoca vitulina*). Red staining indicates virus attachment to the epithelial cell surface. Reprinted from Ramis A. J., van Riel D., van de Bildt M. W. G., Osterhaus A., Kuiken T. Influenza A and B virus attachment to respiratory tract in marine mammals. *Emerging Infectious Diseases* [serial on the Internet]. 2012 May [date cited]. Available from 10.3201/eid1805.111828. With permission of EID.

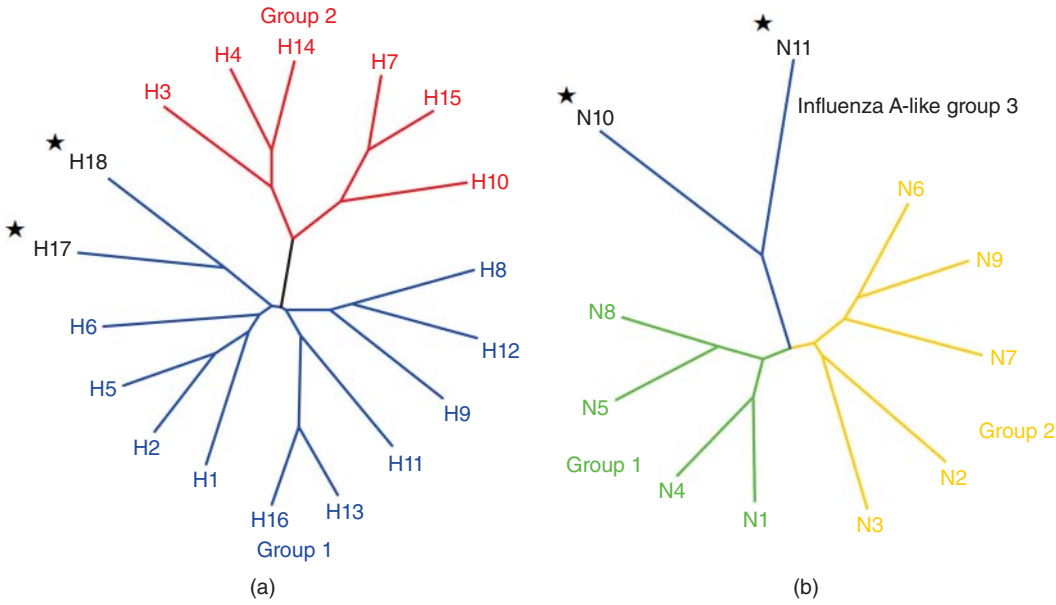

Figure 23.4 Phylogenetic trees displaying the hemagglutinin (HA) and neuraminidase (NA) genes of bat-derived H17N10 and H18N11 influenza viruses (denoted by asterisks) compared with the relative distance of HAs and NAs of all previously known influenza A virus subtypes. Reprinted from *Trends in Microbiology*, April 2014, Vol. 22, No. 4, pp. 183–191, Wu Y., Wu Y., Tefsen B., Shi Y., Gao G. F. Bat-derived influenza-like viruses H17N10 and H18N11, with permission from Elsevier.

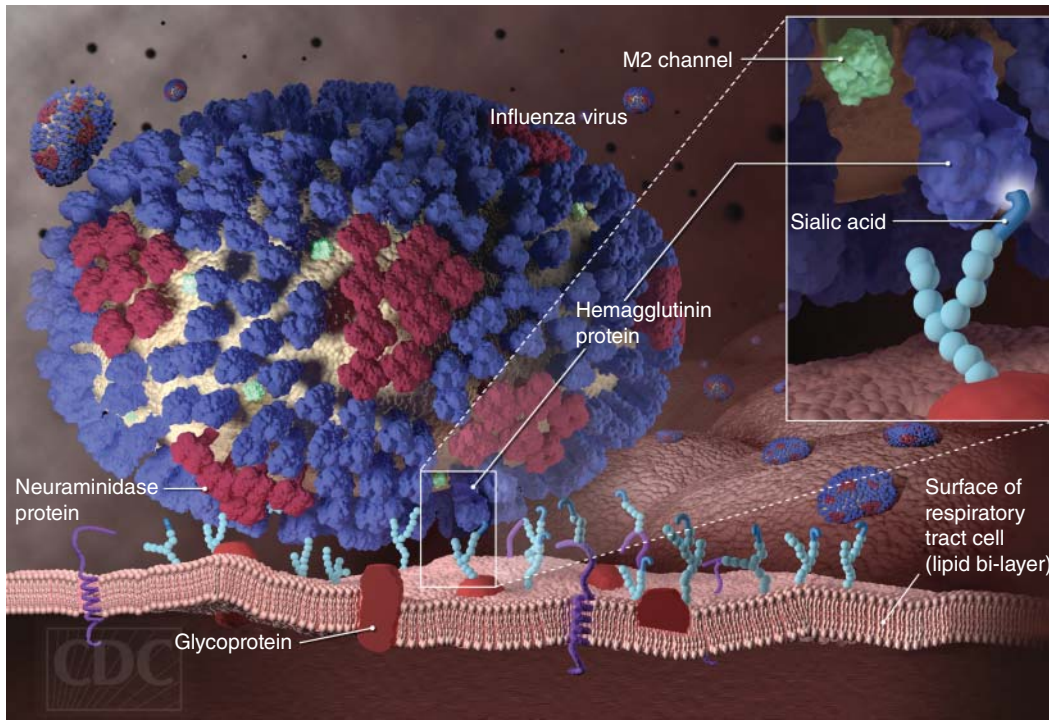

Figure 24.3 Binding of influenza virus to host epithelial cell. The influenza hemagglutinin (HA) binds to sialic acids present on the epithelia of host cells in the respiratory tract. Source: Dan Higgins/PHIL CDC.

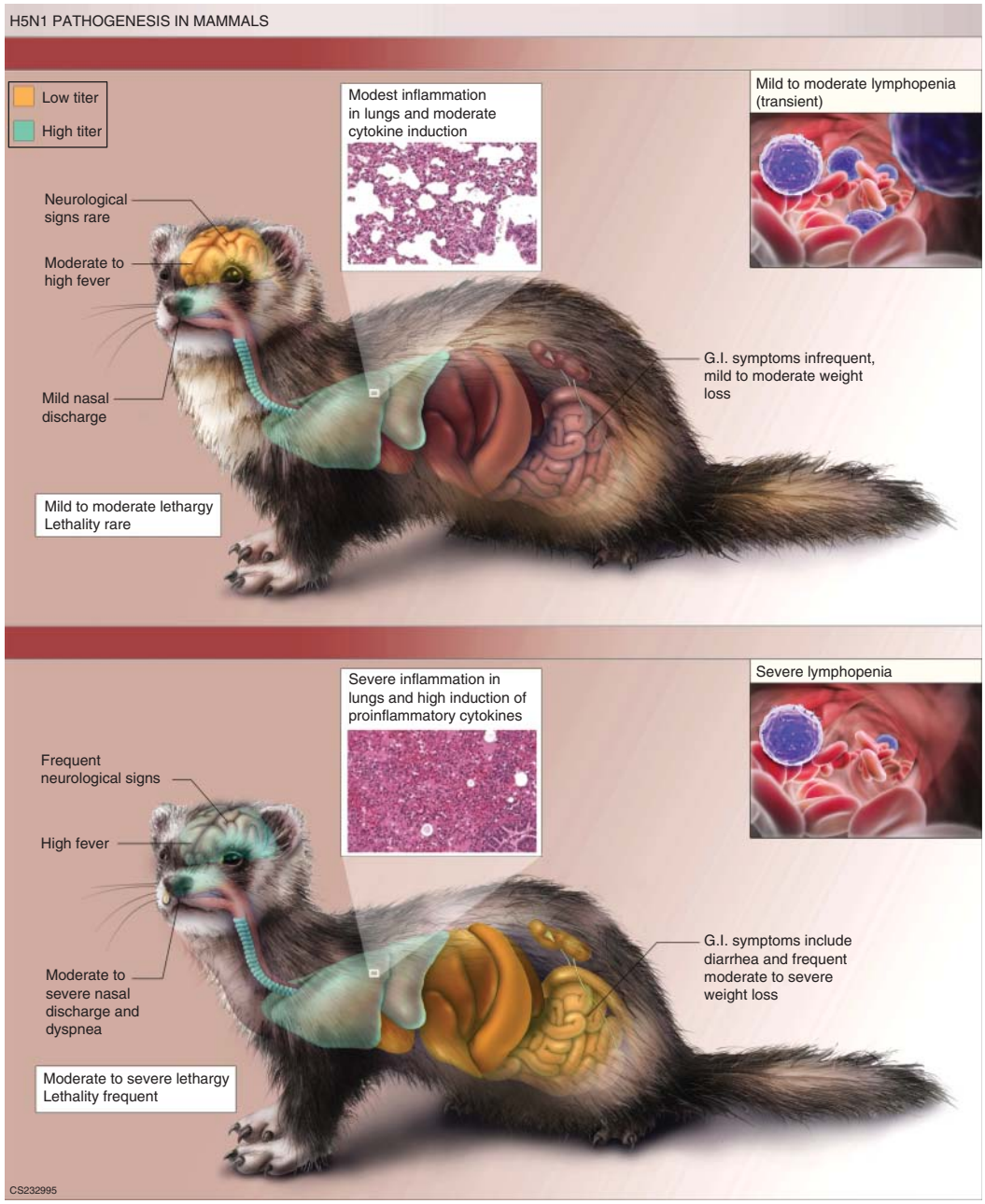

Figure 24.4 Use of the ferret model to study H5N1 virus pathogenesis. Numerous virus and host features studied in the laboratory in ferrets following infection with avian influenza viruses such as H5N1 are shown. Many LPAI viruses exhibit similar features to the low virulent H5N1 viruses depicted in the top panel. Illustration by Alissa Eckert. From Belser, J. A. and T. M. Tumpey. 2013. H5N1 pathogenesis studies in mammalian models. *Virus Research* 178:168–185, with permission of Elsevier.