

TRENDS IN
EMERGING VIRAL
INFECTIONS
OF SWINE

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ANTONIO MORILLA

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 **Iowa State Press**
A Blackwell Publishing Company

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Iowa State Press
2121 State Avenue, Ames, Iowa 50014

Orders: 1-800-862-6657
Office: 1-515-292-0140
Fax: 1-515-292-3348
Web site: www.iowastatepress.com

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 Printed on acid-free paper in the United States of America

Trends in emerging viral infections of swine/edited by Antonio Morilla, Kyoung-Jin Yoon, and Jeffrey J. Zimmerman.—1st ed.

p.;cm.

Includes bibliographical references and index.

ISBN 0-8138-0383-7 (alk. paper)

1. Swine—Virus diseases.

[DNLM: 1. Swine Diseases. 2. Virus Diseases—veterinary. 3. Communicable Diseases, Emerging—veterinary. SF 977.V57 T794 2002] I. Morilla, Antonio. II. Yoon, Kyoung-Jin.

III. Zimmerman, Jeffrey J.

SF977.V57 T74 2002

636.4'089'6925—dc21

2002002912

The last digit is the print number: 9 8 7 6 5 4 3 2 1

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Foreword

Trends in Emerging Viral Infections of Swine is the first comprehensive description of economically significant evolving virus-associated conditions of pigs. As with disease in other animal species and humans, new viral agents are constantly being discovered and previously described agents are reappearing with devastating consequences. The editors have gathered contributors with worldwide experience and perspectives from a vast array of subdisciplines. When viewed in its entirety, this book spans an interface ranging from both veterinary and medical clinicians to epidemiological investigators and scientists studying molecular aspects of viruses and their pathogenicity.

The book will be of particular interest to a broad spectrum of readers because it was written both before and after September 11, 2001. As a result, this publication provides the most up-to-date information regarding possible agroterrorism viral agents. In addition, disease caused by foot-and-mouth disease virus and classical swine fever virus continue to threaten global economies.

Perhaps, more importantly, several chapters delegate attention to a multitude of emerging viruses, including Nipah, West Nile, Menangle, and Circo. These virulent viral pathogens will undoubtedly wreak havoc on both human health and swine production for many years.

This volume is a significant contribution to the scientific community of veterinarians, academicians, and government regulators. Its organization allows it to be examined either in its entirety or as a chapter-specific reference. Instructors may find the publication useful as a basis for a graduate course and/or as a reference text for courses at various levels in microbiology, pathology, and epidemiology.

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22 October 2001

Preface

The title *Trends in Emerging Viral Infections of Swine* indicates our focus both on international *trends* (recent significant developments) in swine health and on *emerging viral infections of swine*, since we seem to be at a point in history when previously unrecognized viral agents are being discovered and old ones are reasserting their presence or expanding their geographical range. It was not our intent to provide a comprehensive epistle on virology, since several excellent tomes are already available. Regardless of whether the viral disease process is analyzed at the micro level or the macro level, our ultimate focus is pigs and pig health.

Pork producers constantly strive for improvements in genetics, husbandry, and animal health with the goal of providing safe, wholesome, and useful products to consumers and society. To achieve this end, local, regional, and international trade in animals, semen, embryos, biologics, feedstuffs, and a variety of commodities is required. The significant level of economic activity that stems from this activity means that swine production has become an important enterprise in many countries. Though not to the exclusion of other interests and issues, an ever-growing concern of pork producers and health authorities has been the protection of animals from diseases and the threat that infectious agents pose to economic, social, and political sectors, as well as to human health. As is eloquently recounted in several chapters of this book, failure in this regard can have tragic consequences.

Traditionally, the most destructive viral infections of swine are the Office International des Epizooties (OIE) List A diseases: foot-and-mouth disease, African swine fever, classical swine fever, and swine vesicular disease. Capable of spreading quickly and inflicting massive economic damage, the List A diseases have been targets of eradication and control programs in all countries with significant swine production. Perhaps once considered conquered, or at least in retreat, in most parts of the swine-producing world, the reemergence of classical swine fever and foot-and-mouth disease in areas of Europe and Asia and the resurgence of classical swine fever in parts of the Americas has shaken this perception.

In addition, new viral diseases or infections have appeared or been identified throughout the world in recent years: blue eye disease, Menangle virus, Nipah virus, porcine circovirus type 2, porcine reproductive and respiratory syndrome, and porcine respiratory coronavirus, among others. After emerging, some have remained local, whereas others have spread widely. These events seem to parallel similar developments in human and animal health, i.e., the appearance of immunodeficiency viruses and highly virulent influenza A viruses in humans, parvovirus in canines, ranaviruses in amphibians (Daszak et al. 1999), and more. Overall, and particularly after September 11, 2001, these events add to our sense of disquiet and vulnerability.

However, it is important to acknowledge that the appearance and/or recognition of new infectious agents is not unique to our era. Classical swine fever, the bane of swine producers and a preeminent member of List A, was unknown until it appeared in Indiana, USA, some time prior to 1833 (Bierer 1955). Likewise, influenza in swine was unreported until the fall of 1918, when similarities between clinical signs in swine in Iowa, USA, and “Spanish” influenza in humans led to the term “hog flu” (McBryde 1927). If the discussion is expanded to include other species and bacterial agents, the history of the movement of infectious agents becomes massive: leptospirosis expanding out of Southeast Asia in the 17th century (Gsell 1984), syphilis appearing in Europe in the 16th century, and the great cholera (*Vibrio cholerae*) pandemics that have swept the globe for the last 170 years.

Thus, we are looking at the latest in a series of developments. Understanding how and why these agents appear is a complex subject beyond the scope of this preface, but one that deserves our attention. In brief, factors responsible for current events fit in the classical subheadings of agent (virus), host (pigs), and environment. New viruses appear for one of three reasons: evolution of an existing virus into one with new virulence characteristics; movement of a virus from one animal species into another; or movement of a virus previously restricted to an isolated or inaccessible group into a wider population. Relative to pigs and their environment, a number of

important changes have occurred. In particular, consolidation of production into fewer, larger herds of genetically homogeneous animals, while required for economic viability, may have increased the vulnerability of herds to infectious agents. In addition, extensive movement of live animals and semen, necessary for genetic improvement, has exposed herds more to the introduction of infectious agents. Further, the movement, both legal and illegal, of swine and swine-derived products at national and international levels has given viruses the opportunity to spread worldwide. And, of course, there is the possibility of the intentional introduction of an exotic disease into a country (i.e., bioterrorism).

With the reality of a global economy coming to pass, it has become evident that emerging diseases have been elevated from a national to an international issue, since control at the international borders will ultimately fail. Consequently, for improved preparedness, it is crucial that we better understand the microbiological and epidemiological characteristics of these agents and share our experiences in their prevention and control. Therefore, we offer *Trends in Emerging Viral Infections of Swine* to producers, veterinary professionals, educators, animal health authorities, legislative bodies, and all those responsible for animal health.

The Editors
January 2002

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1 INTRODUCTION

1.1

Transmission of Viruses Through Pigs and Products of Pig Origin

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SUMMARY

The transmission of viruses to other pigs or to humans through contact with live-infected pigs and/or contaminated products of porcine origin is an area of increasing awareness. Trade and transport of semen, embryos, pork products, and/or tissues for xenotransplantation bring both potential benefits and risks. Issues to consider and measures to reduce risk are discussed.

INTRODUCTION

If animals were born, lived, and died all on the same farm, and if their products traveled no farther than to the direct community associated with that farm, transmission of viruses and other pathogens would be limited to the indirect route, or on the wind. With emphasis on efficiency and least-cost production, and the advent of a global market, agricultural practice has become increasingly complex. It is no longer unusual for animals to travel several times during their lives, their journeys often taking them many miles from their herd of origin and sometimes even across national boundaries; frozen semen and embryos may travel even farther. Consumers now have access to every type of food at any time of the year, supplied by massive and thriving international trade. The advent of transgenic pigs produced as human organ donors opens the doors to further widespread distribution of animal products, albeit in a very specialized and controlled environment.

Any movement of a live animal or a product derived from a live animal presents a potential risk of transmission of any virus resident in the animal to be moved, or the donor animal. In each case, the potential risk must be weighed against the benefits derived from the movement. In some situations, zero risk is the only acceptable option and, therefore, movement of the animal or its products unacceptable. Traditional methods of minimizing risk of disease spread were based on such *zero risk* principles, with international trade agreements preventing movement of animals into disease-free areas (Kelly and Wooldridge 1999). Another factor in the equation is the level of need. If one's life depends on provision of a pig's heart for transplantation, the level of acceptable

risk may alter. The same might be true of a starving population in need of food. When attempting to balance the advantages against the disadvantages, consideration must be given to the size and nature of the population at risk, the types of pathogen involved, and the predicted outcome of transmission. It is also relevant to review the outcome of not taking the risk. Rather than attempting to produce a comprehensive list of viruses that may be transmitted through the movement of pigs or pig products, we have approached the subject in terms of the relative risks, and systems for minimizing those risks, while recognizing the potential benefits of such movements. The product and the purpose of the movement can be summarized as follows:

1. Live pigs: Necessary in order to supply breeding stock gilts and boars to pork producers. Required as animals progress through a multisite production system. Delivery of commercial pigs to slaughter.
2. Semen (fresh, frozen): Gene movement with increased biosecurity and welfare.
3. Embryos: Genome movement with increased biosecurity and welfare.
4. Meat and meat products: Provision of food (human and otherwise).
5. Xenotransplants: Benefit to human health.

MOVEMENT OF LIVE PIGS

One of the most effective methods by which a virus may be spread is through the movement of infected animals into a naive population (Amass and Clarke 1999). This could be the movement of a single animal from one herd to another, or the importation of hundreds of animals into another country; the impact varies accordingly. International trade in pig genetics has resulted in the need for stringent biosecurity procedures for this very reason (Torrison 1998), recognizing that essentially all pig viruses (known and unknown) may be transmitted in this way. The viruses of importance are those endemic in the source population (along with those that might infect the pigs in transit) that are not present in the recipient population.

Taking porcine reproductive and respiratory syndrome virus (PRRSV) as an example, introduction of infected pigs into a seronegative population would present a high risk of introducing the virus into the naive population, which would produce a major (negative) impact on herd health and productivity. Seronegative pigs being introduced into a seropositive herd would be presented with a major infective challenge resulting in their infection. Managing incoming naive pigs through their integration into a seropositive herd can control the impact of disease (White 1999), although such discussions are not within the remit of this chapter.

The concern here is the problem of transmitting virus to a previously unexposed population. The aforementioned examples demonstrate the importance of having precise information about the health status of both source and recipient herds whenever live animals are moved, with every effort made to match health status (Potter 1999).

In practice, viruses of concern to a naive herd include Aujeszky's disease virus (ADV), classical swine fever (hog cholera) virus (CSFV), porcine circovirus type 2 (PCV2), porcine parvovirus (PPV), PRRSV, swine influenza virus (SIV), porcine epidemic diarrhea virus (PEDV), and transmissible gastroenteritis virus (TGEV). Other viral agents of relevance in specific countries are African swine fever virus (ASFV), foot-and-mouth disease virus (FMDV), and swine vesicular disease virus (SVDV).

The primary purpose of moving pigs between herds is to deliver high genetic merit breeding stock. As maintaining the health status of the recipient herd is of paramount importance (Potter 1999), a biosecurity protocol must be in place to avoid introduction of infection. The protocol, which cannot eliminate the risk, but in most cases bring it to an acceptable level, must include

1. Matching the health of the source herd and the recipient herd.
2. Regular health monitoring of the source herd, including at the point of dispatch.
3. Routine health checks and specific serology of the source herd.
4. Health monitoring of the recipient herd.
5. Protection of the pigs to be moved from the point of selection to delivery into the recipient main herd (including isolation and transportation).
6. A period of isolation of the incoming pigs from the resident population while further health testing is carried out.

Note that veterinary checks, monitoring, and serology can only represent a "snapshot" view of herd health. In many cases, animals shed virus before clinical signs of disease appear, highlighting the importance of effective isolation for incoming pigs. Minimizing the number of

intakes reduces the risk of transmission. In some cases, a single repopulation intake is preferred, although this carries the disadvantage of genetic lag behind the continually improved source population. Limiting the intakes to a single source herd is also important. The risk may be reduced and certain incompatibilities overcome by attempting to "clean up" the potentially infected population before movement. The supply of specific pathogen free (SPF) stock is one approach to this (Amass and Clarke 1999), where animals are derived from herds of a specific controlled health status. An alternative is segregated early weaning (SEW) where transmission of a pathogen at a specific stage in a piglet's life from its dam may be avoided by weaning the piglet before this can occur; the key here is to understand the *at risk* age for a particular pathogen (Muirhead and Alexander 1997).

Piglets can be snatched at birth, or delivered by hysterectomy, never being exposed to their dams externally. While such practices are expensive, they are an option where gene movement with minimal disease risk is essential. A total depopulation-repopulation eliminates contact between incoming and outgoing pigs and consequent risk of virus transmission. In practice, this is of more value when the incoming population is cleaner than the resident population, and must be accompanied by a thorough cleansing and disinfection program before the new pigs are delivered.

MOVEMENT OF SEMEN

Movement of both fresh and frozen semen for artificial insemination (AI) offers certain health benefits over the movement of live animals, although gene movement is restricted to the sire's contribution. Semen is a potential vehicle for all viruses that may produce systemic infection in the donor boar, and for this reason every effort must be made to protect the health of AI boars (Philpott 1993). Virus may be shed directly into the ejaculate from the testes or accessory glands, or may contaminate the ejaculate during or after collection. Virus can be difficult to detect in semen as proteolytic enzymes in seminal plasma can interfere with their detection in the laboratory, and an infected boar may only shed virus intermittently (Hare 1985).

Viruses found in boar semen include adenovirus, ADV, ASFV, CSFV, porcine cytomegalovirus, enterovirus, FMDV, Japanese encephalitis virus (JEV), PPV, PRRSV, *Reovirus*, SIV, SVDV, and transmissible genital papilloma virus (Almond et al. 1998).

It is reasonable to expect a semen dose to be pathogen free. Transmission of disease through AI is dose dependent. During processing, the ejaculate is diluted by a factor of 1:4 to 1:12. Dilution reduces the concentration of virus in the final inseminate but increases the potential for spread of virus, as up to 30 insemination doses may be prepared from one ejaculate, and in

theory at least, each dose may be dispatched to a different farm. Fresh semen may be dispatched from a stud to hundreds of different farms every day of the week (Connor et al. 1994). It is also possible that a recipient herd may be receiving semen from multiple AI studs. When a live animal is moved, it is delivered to a single location, so while the risk of virus spread through semen is lower than through movement of a live animal, there is greater potential for spread to several populations.

Approximately 99% of boar semen is used in fresh form; that is, prepared and usually dispatched on the day of collection (Glossop 1998). Semen extenders in current use preserve the life of the sperm cells for 3 to 7 days, allowing no time for semen quarantine prior to dispatch. Most AI biosecurity protocols require that boars are clinically healthy on each day that semen is collected (EC Council Directive 90/429/EEC), but this wise precaution can take no account of virus shed prior to emergence of clinical signs. Freezing semen allows long-term storage in liquid nitrogen, introducing the option of a quarantine period before dispatch, with postcollection serology and health monitoring of the donor boars. As sperm cells are damaged during freezing and thawing, the sperm concentration per dose is doubled to compensate. This reduces the number of doses produced, but effectively concentrates any virus present. Freezing and storing boar semen in liquid nitrogen preserves many viruses. While not an issue for known viruses, which can be tested for in the donor boar before or after semen collection, this raises risks associated with transmission of as-yet-unknown viruses.

Although the risk of disease spread through AI is low, it cannot be eliminated completely. Stud biosecurity is of great importance and must address all methods by which a disease agent may be distributed through the semen. Stud health should be under the permanent supervision of a veterinarian (Connor et al. 1994). The stud should be sited in a biosecure location surrounded by security fencing, with visitors and wildlife deterred (Glossop 1995). Staff must adhere to regulations regarding freedom from contact with other pigs and follow a “shower in” policy. Deliveries must be made in clean trucks, and the biosecurity of incoming feed and other materials carefully controlled (Spronk and Glossop 1997). Boars should be selected from a minimum of different populations (ideally, one) of satisfactory health status subject to continual monitoring. Where boars come from different sources, each intake should be restricted to a single source, and the number of intakes minimized (Glossop 1998). Provision of an isolation facility places a barrier between the source population and the stud. It should be a totally separate unit sited at least one kilometer away from the main stud, operated on an “all in, all out” basis and thoroughly cleansed and disinfected between batches. Only boars of the same health status should be held at the same time in isolation

(except when a sentinel program is in operation). Boars should spend at least 30 days in isolation, during which time serological tests can be conducted.

Boars should be checked for clinical signs of disease daily and semen never collected from a sick boar. If infectious disease is suspected, the stud should be closed immediately. The health status of the stud should be monitored regularly by serology (monthly or quarterly) for diseases including PRRS, AD, CSF, brucellosis, and leptospirosis. The stud should operate under strict hygiene precautions, and the facilities should be constructed of materials that may easily be cleansed and disinfected to minimize the risk of semen contamination. Cross-contamination between two ejaculates in the laboratory should be avoided. Many studs no longer pool ejaculates for this reason. Routine microbiological examination of extended semen (Colenbrander et al. 1993) and polymerase chain reaction (PCR) testing of semen for viruses, such as PRRSV, may also be applied.

EMBRYOS

Recent technological advances in swine embryo transfer and especially the successful cryopreservation and non-surgical transfer of swine embryos (Beebe et al. 2000; Cameron 2001; Cameron et al. 2000; Dobrinsky 2000) have raised concerns about the possibility of transmission of virus when using embryo transfer. The range of infections that might be transmitted by embryo transfer is vast, but the risks are very small if precautions are taken, and far less than those from the movement of post-natal animals, semen, and most animal products (Wrathall 1995). Studies show that infection or seroconversion of recipients following transfer of infected embryos has occurred only when the embryos have been exposed to much higher levels of the pathogen *in vitro* than they would under the most extreme condition *in vivo* (Singh 1987). International Embryo Transfer Society (IETS) surveys indicate that 300,000 to 400,000 bovine embryos and a smaller but not insignificant number of ovine, caprine, and porcine embryos are transferred annually within and between countries (Wrathall and Sutmoller 1998). There have been no substantiated reports of disease transmission to an uninfected recipient by commercial transfer of an *in vivo*-derived embryo in any of these species. Despite this outstanding safety record, these authors advise that “there still are good reasons to be cautious,” and that besides the remote risk of transmitting diseases from infected donors, there is also the possibility of transmission via contaminated equipment or media used to collect, wash, freeze, and thaw embryos.

Viral diseases are transmitted to embryos either by carriage in or on the surface of the gametes as a result of the virus infecting the preovulated ovum or when attached to the spermatozoa so that infection occurs at

fertilization (vertical transmission), or when the developing embryo is infected in the reproductive tract (Shelton and Morris 1985). The probability of this happening depends on the disease pathogenesis, the characteristics of the pathogen, and the immune status of the donor (Sutmoller 1996). Vertical transmission of virus to swine embryos has not been reported, and the possibility of it occurring is considered remote. With the possible exception of PPV, infection of the developing pig embryo before hatching, between days 6 and 7, is most unlikely because the zona pellucida is an effective barrier against virus. However, viruses may adhere to the surface of the zona pellucida or even penetrate beneath the surface. Thus, the status of the zona pellucida is critical in determining the health status of the embryo. After hatching from the zona pellucida, the embryo could also become infected. In pigs, both enveloped and nonenveloped viruses readily stick to the zona pellucida and cannot easily be removed by washing or trypsin treatment (Wrathall 1995).

Porcine viruses researched apropos the risk of transmission through embryo transfer have been categorized by the IETS Import/Export Research subcommittee according to the likely risk of transmitting disease as follows:

Category 1

Disease agent for which there is sufficient evidence to show that risk of transmission is negligible provided that the embryos are properly handled between collection and transfer.

James et al. (1983) transferred 804, day-3 to day-4 zona pellucida-intact porcine embryos collected from 38 donors seropositive for ADV to 34 recipients from an ADV-seronegative herd. All recipients remained seronegative, and all of the 208 piglets born were also found to be seronegative. ADV was not isolated from the flushings or uterine/oviductal cells recovered with the embryos. Other studies have produced similar results (Veselinovic et al. 1991). Singh and Thomas [unpublished data cited in Stringfellow and Seidel 1998 (appendix 4)] reported that when zona pellucida-intact, ADV-exposed embryos were treated with 0.25% trypsin, at pH 7.6 to 7.8 for 60 to 150 seconds, none of the embryos were found to carry ADV.

Category 2

As for category 1, but for which additional transfers are required to verify existing data.

When 171 zona pellucida-intact embryos were exposed to CSFV for 2 to 18 hours and then washed 10 times in phosphate-buffered saline, virus was isolated from 165 of them, although the amount of virus was small. However, when 24 were cultured to allow replication to occur, all of the embryos were rendered noninfectious (Dulac and Singh 1998). The same authors found

that when embryos were exposed to less than 10^6 fluorescent forming units/ml of CSFV and then treated with trypsin, they were rendered noninfectious.

Category 3

Disease agents for which preliminary evidence indicates that the risk of transmission is negligible provided that the embryos are properly handled between collection and transfer [Stringfellow and Seidel 1998 (chapter 6, page 82)], but for which additional in vitro and in vivo experimental data are required to substantiate the preliminary findings.

In the only reported study on transmission of FMDV using porcine embryos, Singh et al. (1986) found that when 194 zona pellucida-intact embryos were exposed to 10^6 plaque-forming units/ml and washed, there was no effect on development, but 5% of the embryos carried the virus. When Singh and Thomas (1987a) exposed zona pellucida-intact porcine embryos to SVDV and then washed them, infectious virus was still isolated from all of the embryos. When treated with pronase or trypsin, the number of embryos carrying the virus was reduced and the amount of virus lessened on each embryo. None of the treatments, however, were capable of disinfecting every embryo. Nevertheless, when Singh et al. (1987) transferred porcine embryos from donors infected with SVDV to two noninfected recipients, all of the recipients and the piglets subsequently born remained seronegative for SVDV. When porcine zona pellucida-intact embryos were exposed to 10^6 hemadsorption doses of ASFV for 18 hours, washed, and then cultured, Singh et al. (1984) found that 38 of 40 embryos retained infectious virus. However, when they were treated with trypsin or pronase, the number of embryos carrying virus was reduced from 95% to 30%. There were fewer viruses on each embryo, and most if not all were bound to the zona pellucida.

Category 4

Diseases on which preliminary work has been conducted or is in progress.

Trypsin (0.25%) has been found to be effective in inactivating or removing vesicular stomatitis virus (VSV) from porcine embryos in vitro. Most, if not all, of the virus is considered to bind to the zona pellucida (Singh and Thomas 1987b). Wrathall and Mengeling (1979) transferred 76-day-old zona pellucida-intact embryos exposed to PPV for 21 hours in culture to four seronegative recipients after washing twice. All of the recipients seroconverted within 8 days. Fifty percent of the embryos died, and PPV-specific fluorescent material was found in trophoblast cells in 6 of 11 blastocysts. Early porcine embryos (four to eight cells) were infected with virulent and avirulent strains of PPV by both microinjection and incubation (Bane et al. 1990). Infection did not significantly inhibit in vitro development when compared with

uninfected controls, but the identification of virus associated with the embryos indicated the presence of infectious virus within viable embryos. Using PCR techniques, Gradil et al. (1994) detected PPV in association with 4-day-old porcine embryos incubated in vitro in the presence of a strain of PPV, despite attempts to rid the embryos of virus by either washing or treatment with pronase or trypsin.

The effect of PRRSV on the development of porcine embryos in vitro was studied by Prieto et al. (1996). Thirty-two-hour-old embryos were microinjected with PRRSV and cultured for 72 hours or cultured in the presence of PRRSV. The result was that microinjection or incubation of the embryos with PRRSV did not significantly inhibit development in vitro when compared with the controls. Also, the virus was not detected by either PCR or fluorescent antibody techniques in association with any of the embryos. They concluded that 4- to 16-cell-stage porcine embryos are not susceptible to infection in vitro.

In summary, of those porcine viruses that have been studied, many (ADV, ASFV, CSFV, FMDV, PPV, SVDV, and VSV) become firmly adherent to the zona pellucida and cannot be removed simply by washing. Trypsin treatment is effective in inactivating or removing ADV, CSFV, and VSV, but not as effective in removing ASFV or SVDV. Effective washing or trypsin treatment has not been demonstrated with PPV or PRRSV. There are still a considerable number of important porcine viruses that have not been studied in relation to transmission via the embryo, e.g., encephalomyocarditis virus, JEV, PCV2, porcine respiratory coronavirus, SIV, and Paramyxoviridae, in particular, the Nipah and Menangle viruses causing reproductive failure in swine. From research reported to date, it is reasonable to conclude that by exposing porcine embryos to high concentrations of viruses in vitro they are likely to adhere to the intact zona pellucida and may not be entirely removed or inactivated by washing or treatment with trypsin or pronase. Except possibly for PPV, however, viruses are unable to penetrate the zona pellucida in vitro and infect the embryo and/or inhibit development. Therefore, the risk of zona pellucida-intact porcine embryos becoming infected by viruses in vivo is remote. Although remote, the possibility of small numbers of virions becoming attached to the zona pellucida in vivo is real, however, sanitary handling of in vivo-derived embryos as recommended in the IETS Manual is considered effective in preventing transmission of viruses from donors to recipients (Stringfellow 1998).

When embryos are collected surgically under sterile conditions, the risk of contamination of flushings and embryos at the time of collection is further reduced. Highly sensitive and rapid PCR techniques can also be used to detect specific viruses on embryos and in flushing and washing fluids. Therefore, PCR may be used in addition to sanitary handling for specific viruses that

may be of particular concern. In pigs, PCR was used to confirm that washings and enzyme treatment (using trypsin or pronase) had failed to remove PPV from embryos exposed to the virus in vitro. Further, it was used to demonstrate that embryos collected from PPV-viremic donors were not infected (Wrathall and Suttmoller 1998). The removal of the entire zona pellucida of porcine embryos after washing, freezing, thawing, and immediately before transfer (Dobrinsky et al. 2001; L. F. S. Beebe personal communication) may provide additional safety in the risk management of embryo transfer in swine.

Suttmoller (1996) outlines three lines of defense against the introduction of diseases by embryos. The disease situation in the country and on the farm of origin, along with the health of the donor, must be satisfactory. Correct sanitary handling and processing of the embryos must be applied. The health status of the herd of origin must be monitored during the time the embryos are in postcollection storage, and postcollection tests carried out on flushing fluids and any unfertilized embryos should be considered.

MEAT AND MEAT PRODUCTS

Any form of food production must be cost effective, efficient, and part of a carefully controlled chain from the raw material to the end user. In the case of meat, the source is the farm, and the end user is the consumer or caterer, with control and responsibility for the quality and safety of the end product changing hands many times throughout the process. Movement of meat may be simply the purchase of fresh pork direct from the farm, or it may be the importation of whole container loads of bacon into a country. With importation, government authorities exercise their right to avoid introduction of foreign viruses to the population as a whole, recognizing that risks to both public health and animal biosecurity posed by the introduction of meat products contaminated with virus are potentially serious. As with moving live animals, the important considerations relate to the health of the source population on the production unit, and the risk of spreading any particular virus to the recipient population, in this case the consumer. Although the consumer usually is human, there are occasions when a meat product may be fed back to animals (e.g., swill feeding). This outdated practice can present a serious risk and is considered the source of the 2001 outbreak of FMD in Britain (personal observation). Pigs are particularly important in this respect as they are omnivorous and may be fed scraps of human food.

In order for a meat product to act as a vehicle for virus transmission, the virus needs either to be present in the animal at the time of slaughter or to contaminate the product within the abattoir or meat-processing works. It must survive any subsequent processing and storage, and be capable of infecting susceptible animals or humans

when presented as food (AQUIS Report 2000). As most viruses found in meat gain access through the blood or lymphatic supply, thorough exsanguination can reduce virus load in the product. Survival of virus during processing and storage depends on its physical characteristics, the nature of the processing, the period of storage, and the conditions of pH and temperature. Muscle pH drops postmortem due to accumulation of lactic acid, although final pH will vary between breeds, and with certain other factors, e.g., time of last meal and antemortem handling (Monin 1981), and may be increased if meat is frozen too soon after slaughter (MacDiarmid 1991). The pH of blood clots, bone marrow, lymph nodes, and viscera does not fall to the same level as in the muscle, and, therefore, survival of virus in these tissues may differ (Blaha 1989).

Methods of meat preservation aim to reduce the activity of the microorganisms that cause the deterioration of the product. These methods may destroy the pathogen, or reduce the numbers present in meat, but in some cases such measures may also preserve the pathogen (AQUIS Report 2000). Refrigeration is the most common method of meat preservation. Low temperatures retard microbial growth and enzymatic and chemical reactions. Freezing occurs when the temperature of the meat falls below -2°C (28°F), but although this may inactivate viruses, it will not reduce the number of pathogens. Thermal processing is in widespread use for killing microbial agents in meat. Products may be subjected to a moderate level of heat (e.g., curing) to extend shelf life, and this may destroy any pathogens present, or reduce their number. More severe ($>100^{\circ}\text{C}$) heat treatment (e.g., canning) results in a product that may be stored without refrigeration, almost all pathogens having been destroyed. Studies into the effect of temperature on virus survival have detailed exacting conditions under which certain viruses may be inactivated by heating (Blackwell 1984; Blackwell et al. 1988; Blackwell and Rickansrud 1989; McKercher et al. 1978) or low-temperature long-time (LTLT) thermal treatment (Masana et al. 1995).

Dehydration is used to preserve meat products and may be combined with a fermentation stage that reduces pH (e.g., salami). A hot-air process or a freeze-drying process may also be used. Dehydration may have little effect on animal pathogens present in meat, and freeze-drying (lyophilization) may actually preserve viruses. Ionizing radiation may be used to kill microorganisms in meat without appreciably raising its temperature. Curing is a traditional process for the preservation of meat, using a range of chemicals including sodium chloride, sodium nitrite, and nitrate. Sugar and organic acids inhibit gram-negative bacteria but have little effect on other microorganisms. Organic acids are occasionally used for their bacteriostatic and fungistatic properties. They act to lower the pH of a product and, in doing so, may in-

activate some important livestock pathogens (e.g., FMDV). Mebus et al. (1993) highlighted the importance of specific curing regimens for virus inactivation, stating that FMDV can survive for 170 days in hams salted and dried using the “Prosciutto di Parma” process, a method in which the curing process takes 365 days, for 190 days in bacon, 183 days in ham fat, and 89 days in ham bone marrow. Lasta et al. (1992) showed that combined treatment by irradiation, heat, and pH modification was effective in inactivating FMDV in meat products.

The Office International des Epizooties (OIE) identifies two categories or “lists” of animal or zoonotic diseases of concern with regards to meat movement between countries:

List A. Transmissible diseases which have the potential for very serious and rapid spread, irrespective of national borders, which are of serious socioeconomic or public health consequence and which are of major importance in the international trade of animals and animal products.

List B. Transmissible diseases which are considered to be of socioeconomic and/or public health importance within countries and which are significant in the international trade of animals and animal products.

In establishing barriers to reduce the risk of virus transmission through meat, customers need assurances regarding the health status of each production unit and, for imports, the health status of the country of origin. Consideration must be given to how the meat should be processed and handled from slaughter to delivery. This may include a requirement for de-boning, removal of all tissues of the central nervous system, and possibly even heat treatment to a specified temperature for a specified length of time.

Many viruses are sensitive to changes of pH, e.g., FMDV is sensitive to both acid and alkaline conditions (most stable at pH 7.4 to 7.6), and all strains are rapidly inactivated below pH 4 and above pH 11 (Geering et al. 1995; Wittmann 1989); below freezing, the virus is stable almost indefinitely. Suspensions of virus will retain infectivity for 8 to 10 weeks at ambient temperatures of about 22°C (72°F), and for up to 10 days at 37°C (99°F). Above this temperature, inactivation is more rapid. Acid and alkaline formulations are the most effective for disinfection of meat and meat products (Geering et al. 1995).

Despite the role of meat products in the transmission of virus, striated muscle tissue is not considered to be a site for virus multiplication (Geering et al. 1995). However, where animals are viremic at the time of slaughter, muscle tissue may be contaminated by virus particles within the muscle vasculature. FMDV is readily

inactivated in skeletal muscle as the pH decreases below 5.8 within 48 hours of the onset of rigor mortis (Blackwell 1984). However, where animals are stressed prior to slaughter, or meat frozen without allowing for maturation, the pH of muscle tissue may not fall sufficiently to inactivate the virus (Monin 1981). Meat products may contain tissues other than muscle, either as ingredients or because these tissues (e.g., bone marrow and lymphoid tissue) may be difficult to separate from the meat itself. Viable FMDV has been isolated from both lymph nodes and bone marrow and may be protected within these tissues from the low pH in surrounding muscle (Blackwell and Rickansrud 1989).

Each country has its own established policies and protocols for the importation of meat and meat products. This must always be regarded as a national issue, rather than a matter for individual choice, and must always be subject to strict regulations.

XENOTRANSPLANTATION

The pig is regarded as the most likely future source of cells, tissues, and organs for human transplantation. This rapidly developing area of science is a major focus of attention in terms of the risks of disease spread to humans, particularly since the recipients of xenotransplants will be immunocompromised. There is additional concern that the risk may not be restricted to the recipient but may also spread to the wider community (Onions et al. 2000). The process of organ transplantation bypasses many of the body's defense mechanisms against infection. A major area of consideration is the endogenous porcine retrovirus present in the pig cell genome that would inevitably be transferred with any transplant.

The approach being developed to minimize the risk of pathogen dissemination through xenotransplantation is based on establishing a list of organisms from which donor animals must be free—and it is indeed a lengthy list. It is recognized, however, that there is greater potential for controlling the precise health status of a population of donor pigs than for controlling the health status of potential human donors. The use of gnotobiotic pigs is not regarded as practicable for this purpose due to the size of animals involved and the impact this would have on their welfare. The value of cohorts within the donor animal group for health testing is important, and this would be rendered impossible by such a requirement. The way forward, therefore, appears to be the establishment of populations of SPF pigs managed as breeding herds for the production of xenotransplant tissues.

Although many viruses are species specific, cross-species transmissions have been recorded that could have a devastating effect on a xenotransplant-recipient human. Onions et al. (2000) regard this as particularly significant because many newly emerging viral diseases of relevance to humans appear to have begun by cross-

species transmission, e.g., Nipah virus, pandemic influenza, and the human immunodeficiency virus. It would be essential to any transplant program that the donor herd of pigs be screened meticulously for viruses as part of a strict biosecurity protocol.

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1.2

The Role of International Agencies in the Control of Contagious Animal Diseases

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SUMMARY

A brief overview is given of the international agencies responsible for global animal health: Office International des Epizooties (OIE), Food and Agricultural Organization of the United Nations (FAO), International Atomic Energy Agency (IAEA), World Health Organization (WHO), Pan-American Health Organization (PAHO), Organismo Internacional Regional de Sanidad Agropecuaria (OIRSA) [International Regional Organization for Animal and Plant Health], Inter-American Institute for Cooperation on Agriculture (IICA), and Organization of African Unity/Inter-African Bureau for Animal Resources (OAU/IBAR).

INTRODUCTION

Contagious diseases have been a threat to livestock since ancient times. These agents have traversed among livestock without respect to international borders. A classic example is the spread of rinderpest in Europe causing tremendous losses of cattle several times in the 1800s (Hanson and Hanson 1983). Outbreaks of foot-and-mouth disease (FMD) and classical swine fever (hog cholera) continue to occur occasionally in Europe and other parts of the world. The highly contagious nature of some of these agents seems to defy typical border control measures. The rapid exchange of breeding stock among sophisticated production operations, while promoting desirable genetic traits, may also lead to the exchange of infectious agents. Porcine reproductive and respiratory syndrome was first reported in North America in 1989 (Keffaber 1989); within 5 years, pig operations from most countries of the world had contracted the virus.

The economic impact of outbreaks of these infections can be great and, in some cases, preventable. International organizations provide the framework for cooperation among countries in the prevention of transboundary transmission and control of outbreaks of these infections. The core funding of these organizations is through membership dues; they seek additional funding from donor groups or organizations for targeted

actions. They provide forums for discussion, technical assistance, training, standards for trade/control/eradication, and laboratory activities. Occasionally, they directly supply resources. There are also organizations with primarily economic focus, such as the World Bank, that assist in many different areas of agricultural development and crisis.

Nongovernmental organizations (NGOs) also assist in efforts to prevent and control the spread of infections from country to country and deserve mention. NGOs are funded privately as charitable or business organizations and increasingly act as agencies for implementing donor-funded projects. In recent years, several veterinary NGOs have been formed, such as *Vétérinaires sans Frontières*, *Vetaid*, and *Vetwork*. Since they are not attached to a specific government or government program, they play a unique role. They have been able to accomplish control procedures in the absence of a cooperative or effective government and have provided innovations for the delivery of animal health services. For example, an NGO may train local villagers to vaccinate cattle for rinderpest in countries torn by civil strife, where governmental organizations would not choose to work.

This chapter presents some of the major international governmental organizations involved in livestock disease prevention. This listing is not meant to be complete or in order of importance, and will become outdated as the names and missions of the organizations change. It is intended to provide examples of how these organizations address the control of serious contagious animal diseases.

OFFICE INTERNATIONAL DES EPIZOOTIES (OIE)

Founded in 1924, the OIE maintains its headquarters in Paris, France. As one of the most influential international organizations in animal health, its membership in December 1999 included 155 countries. Its main missions are the dissemination of animal disease information and harmonization of trade regulations in animals and animal products. The OIE compiles formal reports and

informs member countries on the occurrence and course of animal diseases throughout the world. It also coordinates and facilitates scientific studies and publications on surveillance and control measures. The OIE collaborates with the other organizations listed and maintains working relationships with universities, research institutions, laboratories, and other academic groups that act as collaborating centers or reference laboratories. Most of the activities of the organization are based at its headquarters and these collaborating/reference centers.

The OIE has traditionally categorized contagious diseases as List A (those with rapid spread and serious socioeconomic consequences, such as FMD, rinderpest, classical swine fever, and African swine fever) and List B (those with socioeconomic importance, such as *Aujeszky's*, rabies, and brucellosis). A complete outline of List A and List B diseases is available on the OIE web site. The OIE, through the International Animal Health Code, provides standards for disease reporting, surveillance, and risk analysis, as well as guidelines for trade in animals and animal products for most List A and List B diseases. The *Manual of Standards for Diagnostic Tests and Vaccines*, an OIE publication, is an important element in the harmonization of diagnostic procedures and the production of biologics.

The OIE has a regional presence in Africa, Asia and the Pacific, the Americas, Eastern Europe, and the Middle East with coordinators/offices in Mali, Japan, Argentina, Bulgaria, and Lebanon. A Regional Coordination Unit of the South-East Asia Foot-and-Mouth Disease Campaign (RCU-SEAFMD) has the goal of the eradication of FMD in Southeast Asia.

FOOD AND AGRICULTURAL ORGANIZATION OF THE UNITED NATIONS (FAO)

Founded in 1945, the FAO maintains its headquarters in Rome, Italy. It is the largest autonomous agency in the United Nations system, with 180 member countries at the time of this writing. The mission of the Animal Health Service of the Animal Health and Production Division is to provide basic veterinary services and control infectious and parasitic diseases. The FAO focuses on assisting developing countries. FAO personnel are located in several regional offices, in addition to their headquarters, in order to forward their missions in the field.

The FAO EMPRES program (from Emergency Prevention System) focuses on serious epidemic livestock diseases; that is, diseases with the potential to cause catastrophic production losses, constrain international trade in livestock and livestock products, and even threaten the security of food. One of these, rinderpest, is currently the subject of a worldwide eradication campaign led and coordinated by EMPRES.

Like OIE, FAO collaborates with the other organizations, academic centers, and laboratories. FAO also

maintains information systems for disease occurrence and tracking and, in addition, provides infrastructure, education, and other resources with the intent of enabling countries to track and report their own disease information. They do this by sponsoring international workshops, initiating cooperative field programs within countries, providing computer software, conducting ad hoc investigations of disease outbreaks, distributing periodic bulletins and other publications, etc.

INTERNATIONAL ATOMIC ENERGY AGENCY (IAEA)

Also in the United Nations family, the IAEA is an independent, intergovernmental, science- and technology-based organization headquartered in Vienna, Austria. Regarding animal diseases, they were originally involved with isotope-based diagnostic tests. Presently, they assist other UN agencies in providing member countries with training, diagnostic test kits, laboratory reagents, technical assistance, and other laboratory-related resources. They are closely linked with the FAO.

WORLD HEALTH ORGANIZATION—PAN-AMERICAN HEALTH ORGANIZATION (WHO-PAHO)

Principally concerned with human health, WHO is part of the United Nations and is headquartered in Geneva, Switzerland. Its mission is to attain the highest possible level of health for all people. The WHO assists governments with direction, technical cooperation, teaching, training, direct aid, etc. In the area of livestock health, they are focused on zoonotic diseases, including FMD in South America. In South America, the Pan-American Center for Foot-and-Mouth Disease (PANAFTOSA) assists countries with laboratory services and epidemiological expertise in the campaign to rid the continent of FMD virus. Food-borne diseases, Rift Valley fever, rabies, brucellosis, bovine tuberculosis, and the transmissible spongiform encephalopathies are traditional diseases of focus. The branch of the WHO in the Americas is PAHO, which is headquartered in Washington, DC.

ORGANISMO INTERNACIONAL REGIONAL DE SANIDAD AGROPECUARIA (OIRSA) [INTERNATIONAL REGIONAL ORGANIZATION FOR ANIMAL AND PLANT HEALTH]

OIRSA was created in 1953 after the success of the International Committee for the Combat of Locusts in the late 1940s and is headquartered in San Salvador, El Salvador. It is a regional organization with eight member countries: Mexico, Guatemala, Belize, Honduras, El Salvador, Nicaragua, Costa Rica, and Panama. OIR-

SA is directed by the Comité Internacional Regional de Sanidad Agropecuaria (CIRSA). CIRSA is composed of the Ministers of Agriculture from each country, meets twice a year, and is responsible for the decision-making process and the organization's policies. An executive director carries out the mandate of CIRSA within four technical areas: animal health, plant health, international quarantine and, more recently, food safety. In addition, OIRSA has representatives within each country.

OIRSA is a unique organization in that it largely generates its own income and is financially sustainable. Several years ago, the governments of the member countries conceded to OIRSA the application of all treatments of agricultural products in ports, airports, and borders and allowed the organization to charge for these services. The income generated is redistributed and invested in animal and plant health projects in each country, and a portion is allocated to fund the regional headquarters in El Salvador. Member countries also contribute an annual fee.

OIRSA's mission is to promote the modernization and strengthening of animal and plant health infrastructures in order that member countries may achieve compliance with international agreements, in particular the Agreement on the Application of Sanitary and Phytosanitary Measures of the World Trade Organization. The organization develops and coordinates activities for the prevention, control, and eradication of animal and plant diseases in order to support international trade in animal and plant products from the region.

INTER-AMERICAN INSTITUTE FOR COOPERATION ON AGRICULTURE (IICA) [INSTITUTO INTERAMERICANA DE COOPERACIÓN PARA LA AGRICULTURA]

Founded in 1945, IICA is the agriculture agency of the Organization of American States (OAS). IICA's membership includes every country in the Americas, with the exception of Cuba. The agricultural health program has a network of animal and plant health specialists that cover North, Central, and South America, as well as the Caribbean. Headquartered in Costa Rica, IICA's mission is to provide cooperative services for agriculture and to strengthen and facilitate inter-American dialogue on agricultural issues. IICA's first goal is to support its member states in bringing about sustainable development in agriculture and rural areas; the second is to situate this development within the context of hemispheric integration.

Relative to agricultural health, IICA's mission is to assist member countries in developing and maintaining optimal agricultural health and food safety so that their animal and plant products can meet the most stringent

food safety requirements and compete successfully in international markets. IICA seeks to achieve this goal by cooperating with national agricultural health systems in modernizing their infrastructure, organization, and operation.

The institute's cooperation services are grouped into strategic areas: policies and trade; science, technology, and natural resources; agricultural health; rural development; training and education; and information and communications.

IICA's most important responsibilities within the areas of agricultural health and food safety include the following:

1. IICA assists countries in updating or modernizing their agricultural health and food safety systems and, with the participation of both the public and private sectors, develops strong technical, regulatory, and institutional components at the regional and country levels.
2. IICA works with member countries in the implementation of the World Trade Organization (WTO) Agreement on the Application of Sanitary and Phytosanitary Measures (SPS) Agreement. IICA also cooperates and participates with the WTO/SPS Committee and reference organizations (International Plant Protection Congress, OIE, and Codex Alimentarius) in the implementation of SPS standards at the country and regional levels.
3. IICA promotes technical competencies and leadership development in the area of food safety and the development of domestic food safety policies and systems within the food chain.
4. IICA is required to be alert to emerging animal health and food safety issues, as well as immediate disease and pest emergencies. Part of this responsibility includes review and support of actions approved by the Agricultural Health and Food Safety Emergencies and Emerging Issues Fund (FAO).
5. IICA collects and provides information on agricultural health and food safety, including its importance to animal and public health, tourism, commerce, and the environment. In part, this involves enhancing and promoting the use of the hemispheric network, Agri-Health.

SECRETARIAT OF THE PACIFIC COMMUNITY (SPC)

This is a cooperative effort in the area of animal health issues between the FAO and 22 Pacific island countries and territories.

MIDDLE EAST ORGANIZATIONS

In the Middle East, the Regional Oversight Committee (ROC), established by the animal health component of the Middle Eastern Regional Cooperation (MERC) program, coordinates FMD and brucellosis surveillance and control. The members are the Directors of Veterinary Services of Egypt, Israel, Jordan, and the Palestinian Authority. The Regional Animal Disease Surveillance and Control Network (RADISCON), a FAO project, seeks to facilitate the development of national and regional disease reporting systems in 29 countries of the Middle East, Arabian Peninsula, Maghreb, Sahel, and the Horn of Africa (www.fao.org/ag/AGA/AGAH/ID/Radiscon).

ORGANIZATION OF AFRICAN UNITY/ INTER-AFRICAN BUREAU FOR ANIMAL RESOURCES (OAU/IBAR)

A regional organization, headquartered in Nairobi, Kenya, OAU/IBAR is involved with animal production and with disease surveillance and control within Africa. OAU member states include all countries on the continent of Africa and several surrounding islands. OAU/IBAR facilitates information exchange and holds general biennial meetings with the Ministers of Agriculture and Directors of Veterinary Services. IBAR currently coordinates integrated disease control activities with funding from the European Union and other sources. These activities include rinderpest eradication and an integrated tsetse control program, Farming in Tsetse Controlled Areas (FITCA), in East Africa.

In 1986, OAU/IBAR began the coordination of the Pan African Rinderpest Campaign (PARC), a compilation of national and regional projects involving 34 countries designed to eradicate rinderpest from Africa. The sustainability of control and eradication was assured through the restructuring and strengthening of veterinary services. The PARC program has been largely successful, and rinderpest was contained within two foci in East Africa by 1996. The PARC program sponsored the introduction of the thermostable Vero cell-adapted rinderpest vaccine and the development of the community-based vaccination programs that have facilitated rinderpest eradication in many remote or insecure areas.

The OAU/IBAR is currently implementing the Pan African Campaign for the Control of Epizootics (PACE), a program designed to expand upon the successes of PARC. The PACE program seeks to promote the national epidemiological capacity of member states, consolidate the progress made against rinderpest, and develop achievable strategies for the control of other OIE List A diseases. A component of PACE, the Community-Based Animal Health and Participatory Epidemiology Project (CAPE) specifically targets the development of appropri-

ate animal health and epidemiological services adapted to extensive African production systems.

LIST OF OFFICIAL WEB SITES

OIE: www.oie.int
 RCU-SEAFMD: www.seafmd-rcu.oie.int/
 FAO: www.fao.org
 IAEA: www.iaea.org
 WHO: www.who.int
 PAHO: www.paho.org
 OIRSA: www.oirsa.org.sv
 IICA: www.iicanet.org
 SPC: www.spc.int
 RADISCON: www.fao.org/ag/AGA/AGAH/ID/Radiscon

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1.3

The Social Impact of Disease Control and Eradication Programs: Case Studies

Cristóbal Zepeda

Disease control and eradication is both a science and an art, the art lies in an ability to orchestrate the application of knowledge in a manner acceptable to many specialized interest groups. (Schnurrenberger et al. 1987)

SUMMARY

Control and eradication programs are generally perceived to be beneficial to producers. However, if eradication plans are not carefully planned, the social impact of programs can be enormous, especially for the poorest farmers. Two contrasting case studies involving disease eradication in small-scale subsistence farms are presented. The factors involved in the perception of animal disease risk and previous experience with official programs play an important role in the success or failure of an eradication program.

INTRODUCTION

The success of disease control and eradication programs is usually measured by the decrease in incidence of a specific disease in the first case, and by the elimination of the causal agent in the second (Schnurrenberger et al. 1987). In general terms, it is assumed that control and eradication of disease leads to increased benefits for producers and society as a whole. A crucial question to answer before implementation of a program, especially if the program requires the application of drastic measures such as the destruction of infected animals (stamping out) is “Who is responsible for the costs and who will receive the benefits of the program?” This question is especially relevant in developing countries where intensive production systems coexist with subsistence farming. In many countries, intensive production systems account for approximately 80% of production, but involve only a minority of producers. On the other hand, subsistence farming, although marginal in terms of production, constitutes a very important source of income and nutrition for a vast majority of producers.

Disease eradication programs have not always benefited the producers for whom the program was intended. The success or failure of a disease eradication program is

hence dependent on the perception of the importance of a disease. Several factors influence the relative importance of diseases for different producer groups. This chapter illustrates, through examples, the relative success and failure of several disease control and eradication programs and intends to draw lessons and conclusions from them.

THE PERCEPTION OF THE RISK OF ANIMAL DISEASES

The relative importance of animal diseases in different production systems depends largely on how risk is perceived by the producers. Several factors influence the way people and societies perceive risk. Slovic (1987) summarized some of these factors and grouped them into two broad categories.

The first category, labeled the *dread factor*, includes the degree of control (perceived or real) over the situation, the potential for catastrophic or fatal outcomes, the spread of risk (e.g., will the hazard affect few or all?), the degree to which exposure may be avoided, and the ease for control. The second category includes factors such as the level of knowledge about the risk and the time required to observe the impact. A risk can be plotted against these two categories of factors. Risks scoring high on both categories receive the most attention and are perceived to be more important.

A similar process can be followed to assess the perceived importance of the risk of a disease. Small-scale subsistence producers will assign a greater importance to diseases that cause death, severely limit production, or affect human health; less importance will be placed on mild diseases that have only a potential effect on the long term. Large-scale intensive producers will similarly place considerable importance on diseases that cause death, severely limit production, or affect human health, but also on diseases that affect production in an inapparent, subclinical fashion, and which may be observable only through close monitoring of production parameters.

Uncertainty factors, such as the presence or absence of immediate effects, the effectiveness of control measures, the level of knowledge about the disease and, most importantly, the level of trust in the official intervention

programs, will also play an important role in the perception of the risk of disease for both types of producers.

Several techniques have been developed to assess the relative importance of different diseases in small-scale production systems in developing countries (Catley 2000; Chambers et al. 1989). Participatory appraisal methods have been used to incorporate small-scale producers in the identification and ranking of animal diseases as well as in the design and implementation of control and eradication programs (Catley 2000).

CASE STUDIES

African Swine Fever in Haiti

The Dominican Republic and Haiti share the island of Hispaniola, the second largest island in the Caribbean. In 1978, an outbreak of African swine fever (ASF) was detected in the Dominican Republic. The source of the outbreak was possibly food waste from an airport that was fed to swine. The Haitian government ordered the preventive slaughter of all swine in a 15-km area along the border with the Dominican Republic to prevent the introduction of the disease. Unfortunately, this effort proved to be insufficient; in late 1978, ASF was detected in Haiti and spread rapidly throughout the country (IICA 1981).

To eradicate ASF, Haiti signed an agreement with the Inter-American Institute for Cooperation on Agriculture (IICA). The United States, Canada, and Mexico, fearing the introduction of the disease into the mainland, financed the eradication program. To date, no vaccines against ASF virus have been developed. The only option for eradication was to slaughter the entire swine population in both countries. The objectives of the official project were to slaughter all swine and restock with "improved" breeds that would allow the development of a productive swine industry (IICA 1981). This had a devastating social and economic impact on Haiti's rural population (Zepeda 1989).

To understand the impact of this decision fully, it is important to note that Haiti's 1978 swine population, estimated at 1.2 to 1.9 million, was owned by 86% of the rural population. The type of swine raised in Haiti were *creole* swine. These rugged animals had a very high resistance to the harsh environment. The traditional subsistence farm in Haiti had one or two swine, generally tied to a stake and fed grass and agricultural by-products to maintain the animals at a constant weight until the owner decided to fatten them. Fattening was done with seasonal fruit surpluses (mangoes or avocados), tubers, agricultural by-products, and kitchen waste. From a weaning weight of around 4 kg (8.8 pounds) the animals reached 30 kg (66 pounds) in a period of 16 months. Once at this weight, the animals could be kept for another year or fattened to 60 kg (132 pounds) in 3 to 4 months (GRD 1981). The efficiency of this system was poor if compared with

modern swine production parameters, but was perfectly adapted to the rural production system.

Swine represented the most important source of cash income. To spread the risk of losing animals to disease or malnutrition, Haitian peasants had developed a very interesting system consisting of lending one of their animals and at the same time receiving an animal from another peasant. In this way, if disease struck the swine of one holding, the farmer would not lose his entire savings. This sort of agreement implied a payment in species, usually a piglet. Swine were slaughtered in times of need or situations requiring a substantial amount of cash, such as weddings or funerals. The loss of this vital part of the subsistence system severely damaged the livelihood of the rural population in one of the world's poorest countries.

Through the program, ASF fever was effectively eradicated from the island of Hispaniola in 1984 (OIE 2000), allowing the initiation of the repopulation phase of the program. Whereas the estimated value of the swine population in 1978 was between 70 to 90 million dollars (Zepeda 1989), the repopulation phase of the project had a budget of only 8.5 million dollars (IICA 1981). Swine were imported from the United States and Canada. The project expected farmers to construct swine-raising facilities requiring a substantial investment that was beyond the means of the majority. Those animals that were not kept in this type of facility generally died from disease or malnutrition. The essence of the problem was that the imported animals were bred to perform under a more controlled livestock production infrastructure that included specialized facilities, vaccines, medication, veterinary attention, and good-quality feed. In Haiti, those conditions could not be met, and the animals could never reach their productive potential.

As a result of this situation, two alternative independent projects were designed to reintroduce the creole pig into Haiti. The first project had the objective of creating a new variety of swine that was hardy and productive. French Gascon and Chinese breeds, known to be resistant and prolific, were bred to creole swine from the island of Guadeloupe. To comply with Haitian animal health regulations, the first offspring of these animals were obtained by hysterectomy from sows bred in France and then shipped to Haiti (Delate 1985). Although the animals seemed to be adapted to the Haitian conditions, the success of the project was limited by the enormous cost of each piglet, inadequate distribution, and the lack of training (Zepeda 1989).

The second project attempted to select a breeding herd of creole swine in Jamaica that would produce piglets to be shipped to Haiti. Jamaica was chosen as a source country due to its privileged sanitary situation, being free of the major swine diseases including ASF, classical swine fever (CSF), and Aujeszky's disease (pseudorabies). The type of swine predominant on the

island was the product of Berkshire, Large Black, and Chinese breed crosses that resulted in a hardy medium-sized animal ideally suited for Haiti. A herd of breeding stock was gathered in holding pens and tested. Unfortunately, the first ever occurrence of Aujeszky's disease in Jamaica was detected in this herd. The project ended when Jamaican authorities depopulated the herd.

Although ASF was eradicated from Hispaniola, the social and economic implications of the eradication program are still being felt. Almost 20 years after the ASF outbreak, the swine population in Haiti in 1996 ranged between 500,000 to 600,000 animals (Jeannot 2000), a figure much lower than the 1.2 to 1.9 million that existed prior to 1978. In 1996, CSF was identified in pigs in Haiti. The strategy adopted this time was not to stamp out the disease, but to vaccinate, an option not available for ASF. A report by Jeannot (2000) stressed that the main constraint to the success of the vaccination program was the mistrust generated by the ASF experience.

The Eradication of Classical Swine Fever in Southern Nicaragua

Classical swine fever is endemic in Nicaragua (Zepeda 2000). In 1994, a pilot project to eradicate CSF in the department of Rivas in southern Nicaragua was initiated. The European Union and the Organismo Internacional Regional de Sanidad Agropecuaria (OIRSA) funded this project. The objective was to establish a methodology of disease control that could later be applied to the rest of the Central American countries where CSF is endemic.

Nicaragua's swine population in 1994 was estimated at 500,000 head. Between 92% and 96% of these animals were kept in small-scale subsistence holdings (Rooijakkers 1999; Zepeda 2000). The department of Rivas was chosen as a pilot site because it had ideal conditions to control CSF. Costa Rica, a CSF-free country, lies on the south, with the Pacific Ocean on the west, and Lake Nicaragua on the east. There is only one major road and few secondary roads linking Rivas with the neighboring departments of Granada and Carazo to the north.

A serological study, conducted at the beginning of the project and in the absence of vaccination, indicated a seroprevalence of 22%. The project's strategy was to vaccinate a large proportion of the animals in successive years. In addition to vaccination, an important component of the project was producer education. The emphasis was on swine rearing, basic sanitary measures, and the importance of disease reporting.

Vaccination was carried out systematically in the area for 2 years, the number of CSF cases dropped, and the disease eventually ceased to occur. At this point, vaccination was stopped. After a year without cases, without vaccination, and with intensive surveillance, the disease was considered eradicated. Continued serological surveillance was designed to avoid animals that might have had vaccine-derived or maternal antibody titers. The pilot

project was successful, and the zone was officially declared free from CSF in 1997.

An interesting result of the project was that the swine population increased 53% by the end of the program (Rooijakkers 1999). The reasons for this increase are threefold: (1) there was a substantial reduction in mortality due to the control and eradication of CSF; (2) improved general husbandry practices meant that swine received better care, resulting in a lower overall mortality rate; and (3) as pigs died less frequently, more people became involved in swine production. Also, the price of swine from Rivas was higher than in the rest of the country. Buyers preferred them because they were perceived to be healthier.

The pilot project proved the feasibility of CSF eradication in small-scale subsistence holdings. In 1998, a second project based on the same strategic principles began with a large serological survey in the department of Rio San Juan in Nicaragua. At the end of the project, a large area for intensive vaccination was established and an area along the rest of the border between Nicaragua and Costa Rica was identified as CSF-free (OIRSA 2000).

CONCLUSIONS

The experiences of Haiti and southern Nicaragua represent two examples of successful disease eradication with dramatically different social and economic outcomes. In Haiti, eradication clearly did not benefit Haitian peasants, whereas Nicaraguan farmers now enjoy a more profitable swine production system.

In situations where two systems of production coexist, as is the case in many developing countries, programs are often based on the interests of large-scale producers. Small-scale subsistence farmers are frequently regarded as a logistic problem and a risk. When planning any disease control program, the opinions and priorities of both large-scale and small-scale farmers have to be considered. To ensure success, program strategies need to be adapted to reflect the different production systems.

In addition to control and eradication programs, official programs should prevent the introduction of disease. Once a disease has been introduced, early detection is the most efficient way to prevent the devastating effects of an exotic disease. The producers' willingness to report disease occurrence is dependent not only on an adequate compensation but also on the level of trust they have in the official veterinary service. This is often affected by their previous experience with official programs and their own perception of risk.

An efficient veterinary service clearly is a prerequisite to prevent the introduction of disease and ensure prompt detection, control, and eradication. Unfortunately, most veterinary services in developing countries have faced enormous challenges. In the last several decades, international financial institutions supporting

the economies of these countries have demanded that government be reduced. Often, veterinary services have not been considered a high priority and have suffered severe budget cuts, resulting in the loss of operational capability and presence in the field (Zepeda 1998). On several occasions, this has resulted in the introduction of disease.

A successful animal disease control and eradication program depends on producers' participation and acceptance of the program's objectives. The producers' acceptance is dependent on their perception of the importance of disease. Therefore, the program design must include biological parameters and socioeconomic indicators to measure success.

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2 SWINE INFLUENZA VIRUS

2.1 Swine Influenza: Etiology, Epidemiology, and Diagnosis

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SUMMARY

Swine influenza virus is a common pathogen of swine with a complex epidemiological pattern. Influenza viruses infect a wide range of mammalian and avian hosts. Cross-species transmission, particularly among human, avian, and swine hosts, and the constant evolution of influenza A viruses through mutation and reassortment present a complex and dynamic picture.

ETIOLOGY

Swine influenza is one of the most common respiratory diseases of pigs caused by influenza viruses. Belonging to the family Orthomyxoviridae, influenza viruses are enveloped, single-stranded RNA viruses with segmented genomes. The virus core contains seven or eight genome segments bound to nucleoproteins (NPs). The envelope is covered with two types of glycoprotein spikes, which are anchored on the matrix (M) protein: hemagglutinin (H) and neuraminidase (N). The hemagglutinin is responsible for attachment of the virus to receptors on target host cells and fusion with the cell membrane. The neuraminidase functions in the release of progeny virus from the infected cell.

Family Orthomyxoviridae consists of four genera: *Influenzavirus A*, *Influenzavirus B*, *Influenzavirus C*, and *Thogotovirus*. The virus type (A, B, and C) is based on the NP and M protein antigens. Relative to influenza B and C, influenza A viruses are more important pathogens in veterinary medicine. Type A viruses infect a wide range of hosts, including bears, cats, cattle, chickens, equines, gibbons, goats, humans, mink, seals, pigs, water buffalo, whales, yaks, and at least 50 avian species. Natural infection with influenza A virus with significant clinical disease occurs most frequently in humans, swine, horses, and poultry.

Type A influenza viruses are further divided into subtypes. The subtype is based on HA and NA antigens. Fifteen H (H1 to H15) and 9 N (N1 to N9) antigens have been identified to date. Only viruses of the H1N1, H1N2, and H3N2 subtypes have been consistently recovered from swine although the presence of some other subtypes have been detected serologically or by intermittent isolation of virus without clinical disease. In the United

States, the primary cause of influenza in pigs was H1N1 swine influenza virus (SIV) prior to 1998. In mid- to late-1998, swine herds in North Carolina, Texas, Iowa, and Minnesota exhibited clinical signs of influenza that were subsequently determined to be caused by an H3N2 SIV. At present, both H1N1 and H3N2 subtypes of SIV coexist in US swine populations, suggesting that reassortment between these subtypes may occur in the future. And, in fact, this has already occurred in England and United States.

The Orthomyxoviridae, particularly the type A viruses, show a high degree of antigenic diversity. Subtypes of influenza A viruses are constantly undergoing small antigenic modifications (antigenic drift) due to the accumulation of point mutations in their genetic material. In addition, due to the segmented genome, genetic reassortment occurs periodically when H and N genetic material is exchanged between viruses, thereby causing major antigenic changes (antigenic shift). To reflect the high degree of antigenic diversity among influenza viruses, a system of designating virus isolates has been developed. The current system of nomenclature contains two parts: (1) type and strain designation and, for influenza A viruses, (2) a description of the antigenic specificity of the H and N antigens. The strain designation for influenza virus type A contains information on the nucleoprotein antigenic type, the host of origin if isolated from nonhuman species, geographical origin, strain number, and the year of isolation. For the influenza A viruses, the antigenic description indicating H and N antigens follows the strain designation in parentheses. Thus, the earliest isolate of swine influenza is designated A/Swine/Iowa/15/30 (H1N1). There is no provision for describing distinct subtypes of B and C viruses.

PATHOGENESIS AND CLINICAL MANIFESTATIONS

Replication of SIV is generally restricted to the respiratory tract of pigs. The primary area of infection is the respiratory epithelium lining the airways. Pigs infected with SIV show a variety of respiratory clinical signs, including hard “barking” coughing, nasal and/or ocular discharge, sneezing, lethargy, anorexia, dyspnea, and hyperthermia. It is not uncommon for

pigs to develop body temperatures of $\geq 41^{\circ}\text{C}$ (105°F). Adult animals (sows and boars) commonly lose appetite and young animals (nurseries) are often reluctant to move.

Reproductive performance in both males and females can be affected by SIV infections. In particular, the H3N2 strain introduced into the United States has been associated with reproductive losses. Elevated body temperature in boars can produce adverse effects on spermatogenesis, resulting in reduced fertility. Depending on the stage of gestation, affected females can show a variety of reproductive problems, including delayed return to estrus, abortions, decreased litter size, and reduced viability of piglets at birth. There are conflicting reports as to whether SIV can pass the placental barrier and infect fetuses. Abortions concurrent with epizootics have been reported, occasionally with reports of virus isolation from aborted fetuses. In humans, infection with influenza virus has been associated with abortion or premature delivery, and influenza virus has been recovered from the placenta, amniotic fluid, and fetal heart. Indirect field evidence in support of an association between influenza and reproductive inefficiency has also been reported. In a prospective study of 2709 sows, a statistically significant association between seroconversion to swine influenza virus in early gestation and poor reproductive performance was found. The experimental evidence, however, is inconsistent. In a few instances, SIV was recovered from stillborn pigs and from fetal tissues and fluids after experimentally exposing gestating sows or gilts to the virus. In other instances, researchers have been unable to duplicate transplacental transmission of SIV or cause fetal death by in utero inoculation. Thus, the direct role of swine influenza virus in pregnancy wastage and infertility is not clear. At present, it is commonly believed that reproductive problems caused by SIV infection are due to high fever. Milk production in lactating females may be reduced, resulting in adverse effects on the nursing piglets. Field observations also indicate that SIV infection increases the number of nonpregnant sows and sow mortality as compared with production records prior to the outbreak.

The classic perception of swine influenza in the United States was that of a highly seasonal disease, occurring primarily during fall and winter months. The concept of seasonality began to undergo modification when it was shown that clinical outbreaks occurred in Illinois swine herds every month of the year, with a peak in the fall months. Later, Japanese scientists showed that influenza virus could be isolated from swine throughout the year. Clinically, two forms of disease occur in swine: endemic or epidemic (epizootic). Sow herds with endemic SIV (any subtype) may have sporadic abortions (due to the high fever associated with acute infection) and/or decreased conception

rates due to first- and second-trimester abortions that are recorded as not-in-pig events. Aborting sows will usually be off feed for 2 to 3 days with a fever of $\geq 41^{\circ}\text{C}$. In the endemic form of influenza, clinical disease, particularly respiratory disease, is more apparent in young pigs in a herd. In many cases, the clinical picture is complicated by infection with other respiratory bacterial pathogens.

Epidemic influenza is readily apparent in all age groups. The onset of disease is acute and dramatic, particularly in the sow herd. In naive sow herds, abortion may be widespread, ranging from 5% to 10% of the sow herd. Unlike porcine reproductive and respiratory syndrome (PRRS), influenza-induced abortion storms resolve within 2 to 3 weeks. Spread is rapid, typically sweeping through a breeding/gestation facility within a week. Similarly, the respiratory disease associated with epidemic influenza in growing pigs is of rapid onset, and the initial viral pneumonia-induced coughing is of short duration due to rapid spread among susceptible pigs. Transmission is by direct contact and via highly infectious viral aerosols within infected facilities. Under appropriate environmental conditions, airborne spread may result in an explosive spread among farms within very dense swine production areas. Unless appropriate management decisions and interventions are made, herds with acute or epidemic disease very commonly have endemic disease thereafter.

Epidemic disease is seasonal in nature, although clinical outbreaks of influenza occur throughout the year and peak in periods of greatest environmental stress. Disease onset occurs due to extreme temperature fluctuations. In the United States, it is commonly associated with chilling (cold stress) that can occur in the US Midwest due to dramatic drops in outdoor temperature or loss of heating, and with heating in the US Southeast due to malfunctioning drip cooling systems (heat stress) that remain on into the evening in combination with the increased ventilation rates of summer. Consequently, influenza would be more common in the late fall and winter months of the Midwest or late summer in the Southeast.

Pigs infected with one subtype of SIV usually cannot be differentiated from pigs infected with other subtypes on the basis of clinical signs because the clinical picture is similar with both infections. When H3N2 SIV initially emerged in the United States, field reports suggested that H3N2 SIV infections were more severe than H1N1 SIV infections. This was probably because many pigs were vaccinated with H1N1 SIV vaccines or already had antibodies due to natural infection with H1N1 SIV and, therefore, pigs could be protected from severe clinical signs due to H1N1 SIV infection. Although field observations suggest that the severity of H3N2 SIV infection may depend upon strain, isolates have not been evaluated for differences in virulence.

EPIDEMIOLOGY

Swine influenza was first described in 1918 in association with outbreaks in pigs on farms in western Illinois. These outbreaks coincided with the human influenza pandemic of 1918–1919. The prototype strain of SIV was recovered in 1930, was characterized as a type A subtype H1N1 virus, and was genetically and antigenically similar to the type A influenza virus implicated in the human pandemic. To date, outbreaks of swine influenza have been reported in Belgium, Brazil, Canada, the People's Republic of China, Colombia, Czechoslovakia, Denmark, France, Germany, Great Britain, Hong Kong, Korea, Iran, Italy, Japan, Kenya, the Netherlands, Poland, the Soviet Union, Sweden, Taiwan, and the United States.

Although most influenza infections in swine have been associated with influenza A viruses, there are infrequent reports of influenza type B and C infections in swine, as well. In 1983, a serological survey in India reported that 1 of 520 swine sera collected was positive for B/Singapore/222/79. Undocumented serological studies in the People's Republic of China also suggested that influenza type B is infectious for swine, as well. The susceptibility of swine to influenza B virus and its transmissibility from infected animals to susceptible contact swine was demonstrated under experimental conditions. In addition, antibodies against influenza B were found in swine from herds exhibiting an influenza-like disease during, or subsequent to, type B epidemics in the human population. Influenza type C also has been documented in swine. Fifteen strains of influenza C virus were isolated from abattoir swine in the People's Republic of China in 1981, and antibody against influenza C virus was found in swine sera. Experimental infection of pigs with influenza C virus demonstrated that swine could be infected with influenza C virus and that the virus could be transmitted from pig to pig for up to 25 days after experimental exposure.

The cumulative evidence indicates that waterfowl were the reservoir species of the original prototype virus and are the primary reservoir for the creation of new influenza subtypes. Infection in birds represents a well-adapted host-virus relationship in the sense that most influenza viruses are nonpathogenic for wild birds and tend to persist in avian populations. Influenza virus is stable for at least 4 weeks in water at 4°C (39°F) and for 5 days in water at 20°C (68°F), properties that facilitate transmission among waterfowl and from birds to other species. All 15 H and all 9 N subtypes are found in birds, so the opportunity for reassortment is available. Furthermore, the genetic relatedness of avian virus strains to those appearing in other species supports the hypothesis that the influenza viruses in birds are introduced into other population groups. The H3 subtypes in humans and the avian-like viruses in pigs, mink, seals, and whales may have emerged from birds. Finally, migrating birds,

especially feral ducks, have the potential for disseminating influenza virus over large areas and across great distances.

The World Health Organization's international influenza A surveillance effort, begun in the late 1970s, accumulated information that changed the concept of the epidemiology of the influenza viruses. The information that emerged regarding the influenza A viruses presented an extremely complex epidemiological picture characterized by (1) varying degrees of antigenic relatedness among influenza subtypes infecting different species, (2) a varying degree of infectivity for different species and evidence for cross-species transmission, and (3) the documented observation that different subtypes of influenza infecting a single individual may recombine *in vivo* to form a "new virus" (reassortment).

In 1978, the isolation of viruses that were antigenically related to known human influenza viruses from wild animals in the Pacific, including whales, demonstrated the close antigenic relationship sometimes seen among influenza subtypes infecting very different species. The whale virus was similar to the human A/PR/3/34 (H1N1) virus except for one peptide. Other viruses isolated from animals have shown a high degree of association with strains isolated from humans. As reviewed by Lvov et al. (1978), a virus of the Hong Kong complex (H3N2) was isolated from a common murre (*Uria aalge*), and a virus of the Asian complex (H2N2) was isolated from pintail ducks (*Anas acuta*).

Besides its veterinary importance, influenza in swine poses a potential public health threat due to the possibility of cross-species transmission of influenza viruses. In 1976, an influenza virus derived from an endemic swine virus was isolated from five military recruits with acute respiratory illness, one of whom died, at Fort Dix, New Jersey. Adding to the concern, the subtype of SIV involved (H1N1) was closely related to "Spanish flu," the virus responsible for the 1918–1919 pandemic in which 21,000,000 people died. Serological evidence showed that about 500 recruits were infected with the virus in a 4-week period. A retrospective evaluation showed that there were a total of 10 isolations from humans from 1974 through 1977, and that antigenically similar viruses had been circulating in the swine population for at least 3 years prior to the Fort Dix incident. The human epidemic that was anticipated, however, did not occur.

The recovery of A/Swine/Taiwan/7310/70 (H3N2), a strain indistinguishable from human A/Hong Kong/68 (H3N2), from pigs in Taiwan in 1970 provided the first direct evidence of the interspecies transfer of influenza viruses. Pigs exposed experimentally to either human-origin or porcine-origin virus became infected and transmitted the virus to pen mates. It was shown that A/Swine/Taiwan/7310/70 (H3N2) readily infected human volunteers. The H3N2 swine virus was apparently derived from humans. The appearance of H3N2 in swine

subsequent to H3N2 human epidemics has been documented extensively. Interestingly, even after the A/Hong Kong/1/68 (H3N2) influenza virus was no longer circulating in the human population, it has been maintained in the swine populations.

Soon after the H3N2 subtype was recognized in US swine populations, concern for the risk of transmission of this new type of influenza virus to humans surfaced. However, this risk is considered minimal for the strains of H3N2 virus circulating in US swine at this time. The initial H3N2 SIV isolate (1998) from pigs in North Carolina was neutralized with hyperimmune sheep antiserum against a 1995 H3N2 human influenza virus isolate (Chinese strain). This implies that the human influenza virus was introduced into US swine herds in either 1997 or early 1998. Thus, it is very likely that most people in the United States are already immune to later (1996, 1997, or 1998) human strains of H3N2 that provide solid protection against the H3N2 influenza currently circulating in US swine.

Pigs are known to be susceptible to influenza viruses of both avian and mammalian origin because their tracheal epithelium contains virus receptors for both strains. As such, pigs have been implicated as the intermediate host for adaptation of avian viruses to mammals and as the “mixing vessels” in which reassortment between human and avian influenza viruses occurs.

It was proved that reassortment of influenza A viruses from humans and pigs and from chickens and turkeys could occur under conditions resembling natural transmission. In a series of experiments, influenza viruses were allowed to spread naturally from experimentally infected animals to contact animals. In all experiments, reassortant viruses were isolated from contact animals. In experiments with Hong Kong influenza (H3N2) and swine influenza virus (H1N1) in swine, both kinds of reassortant viruses were isolated (H3N1, H1N2), and both of the reassortant viruses were genetically stable and caused mild infections in pigs. These studies provided evidence that reassortment of influenza viruses could occur between different species and suggested a possible explanation for the origin of new strains of influenza viruses.

DIAGNOSIS

Swine influenza virus infections can be diagnosed based on a combination of clinical signs, typical gross and histopathologic lesions, diagnostic tests to detect live virus, viral nucleic acid or antigen, and serological assays.

Virus Isolation and Antigen Detection

Tissues, particularly lungs, can be evaluated for the presence of live virus and antigen by using virus isolation, immunohistochemistry (IHC), fluorescent antibody (FA) techniques, and antigen-capture enzyme-linked immunosorbent assay (ELISA) (Directigen Flu A; Becton/

Dickinson, Sparks, MD, USA). The presence of SIV or viral antigens can also be evaluated using nasal or lung airway swab samples for virus isolation and/or with the commercially available antigen-capture ELISA kit. Virus can be isolated in cell culture (MDCK cell line) or in 9- to 10-day-old embryonated chicken eggs. Virus isolates can be subtyped to determine the H and N components; this is important for determining the SIV vaccine(s) to use in a herd.

Nasal swabs from acutely affected pigs can be used for virus isolation or antigen-capture ELISA. Pigs with high fevers ($\geq 41^{\circ}\text{C}$; $\geq 105^{\circ}\text{F}$) and off feed with a clear nasal discharge should be selected for sampling. Most pigs will shed virus for only 5 to 7 days after infection because immunity develops rapidly. Swabs with synthetic fiber (rayon or Dacron) tips are preferred because the residual chlorine in bleached cotton swabs may inactivate virus. Moist collection systems, such as Culturette swabs (Baxter Healthcare Corporation, Deerfield, IL) also work well. Alternatively, dry-type swabs can be broken off into small vials or tubes containing physiological saline or cell culture media. Freezing may inactivate virus and make its isolation more difficult. Therefore, samples should be frozen only if swabs cannot be delivered to the laboratory in a timely manner. Excess mucus or blood on the swabs also can interfere with successful use of antigen-capture ELISA kits. One lab has reported that this test has not worked as well on nasal swabs as on swabs directly applied to small airways in lung tissue.

Fresh and formalin-fixed lung collected at necropsy from pigs with respiratory disease are the samples most commonly used for diagnosis. Swine influenza virus initially infects the epithelium lining the airways, and the resulting lesion is predominantly a bronchopneumonia characterized by multiple coalescing foci of lobular consolidation in the cranioventral portions of lungs. These areas should be submitted for diagnostic evaluation. Fresh tissue (chilled, not frozen) can be used for FA test and virus isolation studies, and formalin-fixed tissue for the IHC test and histopathologic examination. Experimental studies have indicated that peak virus load in the airways is present at 24 hours after infection—well before gross lesions develop. Virus usually can still be detected in bronchioles and alveoli 48 to 72 hours after infection. In many pigs, very little virus may be found by FA or IHC by 72 hours post infection (PI), and distribution is often quite focal.

Histopathologic examination can demonstrate lesions suggestive of SIV infection for about 2 weeks after infection. By 3 weeks PI, recovery is almost complete. The later lungs are examined after infection, the more difficult the lesion becomes to evaluate. Porcine circovirus can induce bronchiolar damage similar to that induced by SIV, and both *Mycoplasma hyopneumoniae* and SIV infections induce significant peribronchiolar and perivascular lymphocytic cuffing. Samples from more than one pig are recommended to address the

diagnostic difficulties imposed by the focal nature of the lesions and the pig-to-pig variation in timing of infection, especially in cases of endemic respiratory disease.

In cases of abortion, direct isolation of virus from nasal swabs of sows that are acutely ill (i.e., animals with fever) or comparing acute and convalescent serological results from affected groups are the preferred methods to determine whether SIV is involved in reproductive problems. Attempts to isolate SIV from, or detect antigens in, fetuses are likely to be unrewarding.

Molecular Diagnostics

The presence of SIV in diagnostic specimens can also be determined by polymerase chain reaction (PCR). PCR-based assays applied to nasal swabs (antemortem) and lungs (postmortem) showed excellent specificity and good sensitivity as compared with virus isolation and antigen detection assays such as antigen-capture ELISA or IHC. PCR diagnostic assays can also provide the capability of differentiating subtypes directly on clinical specimens, as well as on isolates. However, the overall technical demands and cost of PCR diagnostics are drawbacks for routine diagnostic use.

Serology

Swine influenza virus has the ability to cause red blood cells to agglutinate. When HA subtype-specific antibodies are present, hemagglutination is inhibited. This reaction is the basis of the hemagglutination inhibition (HI) serological assay for detecting SIV antibodies. Since hemagglutination is based on the HA component of the virus, a single HI test cannot be used to detect both H1 and H3 antibodies. Rather, the appropriate virus must be used in separate H1 and H3 assays. Additional assays for detecting H3N2 SIV antibodies include the ELISA, indirect fluorescent antibody (IFA), and immunodiffusion.

Serum samples for serological evaluation should be collected at least 1 week after infection is suspected to have occurred. The HI test is considered moderately sensitive but highly specific, and test results appear to have a good correlation with protective immunity. Using the HI test, antibody titers of 1:10 to 1:20 are considered suspect, with 1:40 or greater considered positive. Moderately high titers (1:80 to 1:160) will be present by 7 days PI. Peak HI titers of 1:320 to 1:640 will be present by 10 to 14 days (in some cases, it may take as long as 3 weeks) and persist at fairly high levels until at least 4 weeks PI before beginning to decline. Anti-SIV HI antibodies may persist for up to 18 months.

ELISA is considered to be the most sensitive serological assay, and an ELISA for differentiating antibodies to H1 and H3 has been developed, although it is not available commercially. At present, an indirect ELISA using antigens of H1N1 SIV (IDEXX Laboratories, Westbrook, ME, USA) is commercially available for serodiagnosis of SIV infection. In some diagnostic laboratories, the IFA

test is also being used for detecting SIV-specific antibodies. However, cross-reactivity among different subtypes of SIV is a concern with respect to using indirect ELISA or IFA, since type A influenza viruses share a common matrix protein and nucleoprotein.

SN antibody is detected at about 7 days PI. The kinetics of the SN antibody response is similar to the antibody response determined by the HI test. Immunoglobulin A (IgA) antibodies are also produced in the respiratory tract, appearing 8 days PI and reaching maximum titers a week later. IgA titers decrease more rapidly than do the titers of HI antibodies in serum.

Humoral immune response to vaccination is similar to that observed in infection, but vaccination appears to induce a lower level of antibodies than does natural exposure. Colostrum-derived antibodies protect newborn animals. Maternal antibody in pigs from unvaccinated sows is detectable until 6 to 8 weeks of age, and in pigs from vaccinated sows until 18 to 20 weeks of age. Longitudinal samples may be necessary to monitor SIV in vaccinated herds.

Subtyping

As genetic reassortment between type A influenza viruses with different H and N genetic materials can occur, the subtype of virus must be determined when SIV is isolated. Immunological inhibition assays, such as the hemagglutination-inhibition test and the neuraminidase inhibition test, is the standard procedure for subtyping the virus. A disadvantage of this technique is requirement of virus isolate with relatively high HA titer.

With advances in technology, molecular assays have been applied to subtyping. PCR-based assays have been proven useful. PCR can be used directly on clinical specimens without virus isolation attempts and provides a good turnaround and sensitivity. PCR, if multiplex format is used, can also provide the additional advantage of identifying coinfection of SIV with different subtypes in samples, one of which may outgrow the other during virus isolation attempts. In addition to PCR, sequencing can be used for the same purpose as a part of genetic characterization of isolates. However, its high cost, time-consuming nature, and technical demand hinder its practical use for subtyping.

TREATMENT, PREVENTION, AND CONTROL

Treatment for SIV infections during acute outbreaks is limited to supportive therapy, i.e., reducing stress, medication to lower body temperature, minimization of secondary bacterial infections (e.g., aspirin, antibiotics), and ensuring that the pigs are made as comfortable as possible. Prevention is based on management practices designed to reduce the risk of SIV entering the herd. This includes biosecurity measures such as limiting the access of people and vehicles to the premises, properly cleaning and disinfecting

transport trucks, and quarantining new pigs before placing them in the general pig population of the farm. To minimize the reproductive losses due to SIV infection, vaccine may be used in replacement gilts and boars, as well as pregnant sows and gilts before farrowing. Due to the antigenic differences between different subtypes, i.e., H1N1 versus H3N2, vaccines containing only one subtype are not expected to mount the protective immunity against infections with other subtypes. Vaccines currently available include autogenous or licensed vaccines. Preexisting SIV HI antibodies must be <1:40 for the vaccine to work. Maternal antibody in pigs from unvaccinated sows may last until 8 weeks of age and in pigs from vaccinated sows until 20 weeks of age. For that reason, producers should not consider vaccinating until pigs are at least 8 weeks of age.

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2.2 Influenza A Viruses in Pigs in Europe

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SUMMARY

In Europe, swine influenza virus (SIV) is one of the most important primary pathogens of swine respiratory disease and has serious economic implications for the industry. Influenza A viruses emerge, or modifications to existing strains can occur, by one of three mechanisms: interspecies transmission of virus; antigenic change or drift in the major viral antigens through mutation; or genetic reassortment. All three have occurred naturally in pigs in Europe resulting in the emergence of “new” influenza viruses that have contributed to changes in the epidemiology of SIV. Influenza A viruses of H1N1, H3N2, and H1N2 serotypes are endemic in European pig populations. The current predominant strains of H1N1 and H3N2 serotypes originated by transmission of whole virus from birds and humans, respectively, to pigs. Both viruses became established and cocirculated widely before acting as progenitor strains in genetic reassortment. Following these events, human-avian reassortant viruses of the H3N2 serotype replaced the progenitor H3N2 viruses that were derived entirely from human virus. More recently, novel H1N2 viruses, derived from a multiple reassortant event involving human and avian viruses, emerged and spread widely in European pigs. All of the newly introduced/emerged influenza viruses have undergone many pig-to-pig transmissions because of the continual availability of susceptible pigs. During this time, these viruses adapted to their new host, acquired pathogenic properties, and became increasingly associated with disease epidemics. In addition, genetic drift has occurred in the genes of established viruses, particularly those encoding the major viral antigens, allowing new virus variants to gain a selective advantage in the presence of herd immunity. The heterogeneity in SIV has implications for diagnosis and control. It is essential to ensure the strains used in serodiagnostic tests are well matched to the current epidemic viruses. Vaccination against SIV is not used widely in Europe, but inactivated vaccines comprising historical H3N2 and H1N1 viruses are available, although their efficacy against infection with current viruses is poorly known.

INTRODUCTION

Swine influenza is a highly contagious acute viral disease of the respiratory tract of pigs that is distributed worldwide. The disease is economically damaging, primarily due to weight loss and reduced weight gain. In the United Kingdom (UK) the financial loss resulting from reduced weight gain in finishing pigs alone due to swine influenza virus (SIV) has been estimated at approximately £7 per pig, equivalent to a total loss in the UK per annum of £60 million (about US \$85 million) (Kay et al. 1994). In Europe, SIV is considered one of the most important primary pathogens of swine respiratory disease. The characteristics of the viruses responsible share some similarities with counterpart strains in North America, but also possess a number of significant differences. The epidemiology of SIV in Europe has historically been different from other parts of the world and, together with approaches for control, remains distinct.

RESERVOIRS OF INFLUENZA A VIRUSES

Influenza A viruses infect a large variety of animal species (Alexander and Brown 2000; Webster et al. 1992) including humans, pigs, horses, sea mammals, and birds. Given the worldwide interaction between humans, pigs, birds, and other mammalian species, there is a high potential for cross-species transmission of influenza viruses in nature. The prevalence of interspecies transmission depends on the animal species (Webster et al. 1992), with some hosts, including pigs, better able to support the replication and spread of “new” viruses. Aquatic birds are known to be the source of all influenza viruses for other species. Pigs are an important host in influenza virus ecology because they are susceptible to infection with both avian and human influenza A viruses, often being involved in interspecies transmission, facilitated by regular close contact with humans or birds. Following the transmission to, and independent spread of, avian or human influenza A viruses in pigs, these viruses are generally referred to as “avian-like” swine or “human-like” swine, reflecting their previous host. Furthermore, because the influenza genome contains eight segmented genes, it is possible for these genes to be

exchanged when two influenza viruses coinfect the same host. This process is referred to as genetic reassortment and can result in new genotypes of the virus becoming established in a particular host. Once a virus becomes established in a host and is able to spread readily among that species, it acquires certain characteristics in the viral genes that are common to influenza viruses from the same host, thereby resulting in species-specific evolution. Pigs are frequently involved in interspecies transmission and genetic reassortment of influenza A viruses, and as a result there are opportunities for “new” or previously unrecognized viruses to emerge.

VIRUS CHARACTERISTICS

Influenza viruses are grouped into types A, B, and C on the basis of the antigenic nature of the core proteins. The two surface glycoproteins of the virus, hemagglutinin (HA) and neuraminidase (NA), are the most important antigens for inducing protective immunity in the host and therefore show the greatest variations. For influenza A viruses, 15 antigenically distinct HA and 9 NA subtypes are recognized at present; a virus possesses one HA and one NA subtype, apparently in any combination. In addition, there can be significant variation antigenically in viruses of the same HA serotype that is usually related to the host species. Pigs are known to be susceptible to influenza A viruses of all HA serotypes (Kida et al. 1994) although only three serotypes—H1N1, H3N2, and H1N2—predominantly infect pigs.

HISTORY

Swine influenza was first observed in 1918 in the United States, Hungary, and China (Beveridge 1977; Chun 1919; Koen 1919). It coincided with an influenza pandemic in humans, which was the most severe of modern times, accounting for at least 20 million human deaths worldwide. It is now known that the causative agent of both infections was an H1N1 influenza A virus that was possibly derived from a common ancestor (Gorman et al. 1991; Kanegae et al. 1994; Reid et al. 1999). Descendants of these viruses continue to persist in pigs worldwide. That the virus most probably spread from humans to pigs is supported by observations from veterinarians who did not describe the disease in pigs until just after its appearance in humans. Although the disease in pigs was described during the following years (Dorset et al. 1922; McBryde 1927), it was not until 1930 that the virus was isolated and identified in North America (Shope 1931).

In Europe, swine influenza was observed later when virus isolations were made in the UK (Blakemore and Gledhill 1941a,b) and Czechoslovakia (Harnach et al. 1950), while at this time antibodies to H1N1 influenza viruses were demonstrated in pigs in the Federal Republic of Germany (Kaplan and Payne 1959). After these

episodes, the virus apparently disappeared from these countries, and there was no evidence of infections in Europe for nearly 20 years. It is not clear why viruses descended from the 1918 human pandemic strain (and now referred to as classical swine influenza virus) did not persist in pigs in Europe, in contrast to North America, but serosurveillance studies in several European countries failed to detect any antibodies to classical SIV. There was no evidence of infection of pigs in Europe until 1976, when classical SIV was isolated from disease outbreaks in northern Italy. The strains isolated were closely related to classical SIV from the United States (Nardelli et al. 1978), and the virus was probably introduced via imported pigs from the United States. The infection was limited to northern Italy until 1979, when swine influenza caused by classical SIV was reported from Belgium (Biront et al. 1980; Vandeputte et al. 1980) and France (Gourreau et al. 1980). The virus spread rapidly to pigs in all parts of Europe, and the disease became endemic.

OUTBREAK COURSE AND CLINICAL SIGNS

Swine influenza is related to the movement of animals from infected to susceptible herds, and clinical disease generally appears with the introduction of new pigs into a herd. Once a herd is infected, the virus is likely to persist through the production of young, susceptible pigs and the introduction of new stock. Outbreaks of disease occur throughout the year, but usually peak in the colder months. After an incubation period of 1 to 3 days, disease signs appear suddenly in all or a large number of animals of all ages within a unit. An acute, febrile, respiratory disease is characterized by fever, apathy, anorexia, coughing, sneezing, nasal discharge, conjunctivitis, a low mortality rate, and a rapid recovery. Secondary bacterial infections can often increase the severity of the illness and may result in complications, such as pneumonia. Subsequent to an influenza outbreak in a herd, there may be reduced reproductive performance through increased infertility, abortion, and stillbirth.

EPIDEMIOLOGY

Influenza A viruses of subtypes H1N1 and H3N2 have been reported widely in pigs, associated frequently with clinical disease. These include classical swine H1N1, “avian-like” H1N1, and “human-like” H3N2 viruses. These viruses have remained largely endemic in European pig populations and have been responsible for one of the most prevalent respiratory diseases in pigs. Although usually regarded as an endemic disease, epidemics may result when influenza infection occurs in an immunologically naive population (which can be linked to significant antigenic drift) or through exacerbation by a variety of factors such as poor husbandry, secondary

bacterial or viral infections, and cold weather. Serosurveillance results in Great Britain indicated that more than half of adult pigs in the national population had been infected with one or more influenza A viruses during their lifetime, including 14% of pigs that had been infected with influenza viruses of both human and swine origin (Brown et al. 1995b). This provides some indication of the risk of genetic reassortment of influenza A viruses in pigs (see the section on genetic reassortment).

Classical H1N1

This virus became endemic in pigs throughout Europe, with a seroprevalence of 20% to 25% (Brown et al. 1995b; Zhang et al. 1989), but following the emergence of “avian-like” H1N1 virus its continued circulation throughout Europe is uncertain. These viruses remained relatively stable both antigenically and genetically, with no evidence of involvement in virus reassortment (Brown et al. 1997).

“Human-like” Viruses

Infections of pigs with the prevailing human subtypes also occur under natural conditions. Shope (1938) presented serological evidence that human-to-pig transmission could occur, but it was not until the isolation of Hong Kong H3N2 virus from pigs in Taiwan in 1970 (Kundin 1970) that investigations began to examine the potential transmission of human strains to pigs. Although no disease was reported among infected pigs, in the next several years H3N2 viruses were isolated regularly from pigs (Ottis et al. 1982; Tumova et al. 1976) and/or antibody was demonstrated (Harkness et al. 1972; Tumova et al. 1976) in European swine populations. Since 1984, these viruses have been associated with outbreaks of clinical influenza in pigs throughout Europe (Aymard et al. 1985; Haesebrouck et al. 1985; Pritchard et al. 1987), with infections frequently characterized by high seroprevalence (Lange et al. 1984; Roberts et al. 1987; Tumova et al. 1980). The apparently high level of H3N2 infections in Europe was, until recently, in sharp contrast to the low prevalence in pigs in North America that suggested that these viruses were not established in the US swine populations, but occurred only by infrequent introduction from infected humans (Easterday 1980). Human H1N1 viruses can also infect pigs, but although pig-to-pig transmission has been demonstrated under experimental conditions (Kundin and Easterday 1972), most strains are not readily transmitted among pigs in the field (Hinshaw et al. 1978). Although there is serological evidence they are present in European pigs (Brown et al. 1995b; Roberts et al. 1987), most likely they occur only through frequent transmissions of the prevailing strains from humans and are not apparently maintained in pigs independently of the human population.

“Avian-like” Viruses

Since 1979, the dominant H1N1 viruses in European pigs have been “avian-like” H1N1 viruses that are antigenically and genetically distinguishable from classical swine H1N1 influenza viruses, but related closely to H1N1 viruses isolated from ducks (Pensaert et al. 1981; Scholtissek et al. 1983). These “avian-like” viruses appear to have a selective advantage over classical swine H1N1 viruses that are related antigenically, since in Europe they have replaced classical SIV (Brown 2000; Campitelli et al. 1997). Within 2 years of the introduction of “avian-like” viruses into pigs in Great Britain, classical swine H1N1 apparently disappeared as a clinical entity. In Europe, avian H1N1 viruses were transmitted to pigs, became established, and have subsequently been reintroduced to turkeys from pigs, causing economic losses (Andral et al. 1985; Ludwig et al. 1994; Wood et al. 1997).

H1N2 Viruses

Influenza A H1N2 viruses, derived from classical swine H1N1 and “human-like” swine H3N2 viruses were isolated in France in the late 1980s (Gourreau et al. 1994). These viruses inherited the HA gene from classical swine H1N1 and the NA gene from the swine-adapted human virus; however, although they were associated with clinical disease, they did not appear to spread widely. Since 1994, H1N2 influenza viruses (see the section on genetic reassortment) related antigenically to human and “human-like” swine viruses have emerged and become endemic in pigs in Great Britain (Brown et al. 1995a), frequently in association with respiratory disease. Subsequently, these viruses have spread to pigs in the rest of Europe (Marozin et al. 2000; Van Reeth et al. 2000a), suggesting that this relatively new virus subtype has become established.

Emergence and Reemergence of Influenza Viruses in Pigs

Emergence of new strains or modifications to existing viruses can occur by three methods. Firstly, an influenza A virus from another species can transmit *in toto* to pigs. Secondly, an influenza virus may undergo antigenic change or drift as a result of accumulating mutations with time in the genes encoding the major viral antigens. Finally, coinfection of a pig with two unrelated influenza A viruses can result in the production of a new virus derived by genetic mixing of the progenitor strains, leading to the potential emergence of a new virus with different antigenic and genetic characteristics. If this process involves a change in the HA and/or NA serotype, it is referred to as antigenic shift.

There is good current evidence that all three mechanisms have occurred naturally in pigs in Europe and will almost certainly continue to pose a threat to the industry as well as having implications for public health. Transmission of influenza A viruses from pigs to humans and

birds has been reported widely, contributing to the pig being considered a potential intermediate host for the reassortment of influenza A viruses, which may lead to the generation of a pandemic strain for the human population. The potential for avian and human influenza viruses to infect pigs is well established, and experimental infections of pigs have demonstrated that pigs are susceptible to infection with strains representative of all serotypes of influenza virus (Kida et al. 1994).

INTERSPECIES TRANSMISSION OF VIRUSES TO PIGS

In Europe, avian H1N1 viruses transmitted to pigs (see the discussion on “avian-like” H1N1 viruses) in the late 1970s established a stable lineage and spread widely, causing significant economic losses. All of the gene segments of the prototype viruses were of avian origin (Schultz et al. 1991), indicating that transmission of a whole avian virus into pigs had occurred. As a result, this has been implicated as the possible source of precursors of the next human pandemic virus (Ludwig et al. 1995). Phylogenetic analysis of the genes of these viruses has revealed that they have retained an entirely avian genetic composition throughout their maintenance in pigs.

Influenza viruses of subtype H3N2 are ubiquitous in animals and became endemic in the European pig population following the first transmissions from humans in the early 1970s. Influenza A viruses of H3N2 subtype, including those related closely originally to a human strain from 1973, continue to circulate widely in pigs long after their disappearance from the human population (Brown et al. 1995b; Haesebrouck et al. 1985; Wibberley et al. 1988). There is no apparent evidence of pigs being infected with this subtype prior to the pandemic in humans in 1968. Indeed, the appearance of an H3N2 subtype variant strain in the pig population of a country appears to coincide with the epidemic strain infecting the human population at that time (Aymard et al. 1980; Brown et al. 1995b; Nerome et al. 1981). The prevailing strains of H3N2 virus in the human population have been frequently transmitted to pigs since the early 1970s (Brown et al. 1995b), but do not usually persist independently, although viruses closely related to a human strain from 1997 may have become established in pigs in some areas, including Europe (Marozin et al. 2000). Transmission of human H1N1 influenza viruses to pigs has also been demonstrated and, although these viruses appear unable to persist independently, they are able to donate genes through genetic reassortment with other influenza viruses that are well adapted to pigs. Despite failing to establish a stable lineage in pigs, human H1N1 virus was one of the progenitor strains for the newly established H1N2 viruses (see below).

GENETIC REASSORTMENT

Continued cocirculation of influenza A viruses in pigs can result in the production of new reassortant viruses. Many herds are infected endemically, often with more than one serotype, and this provides a moderate risk of genetic reassortment. This is an ongoing process with frequent genetic exchange between cocirculating variants of the same virus. Rarely, genetic reassortment occurs between viruses of different serotypes and leads to the production of viable virus that either has a modified genotype or a “new” phenotype.

Evidence for the pig as a mixing vessel of influenza viruses of nonswine origin has been demonstrated in Europe by Castrucci et al. (1993), who detected reassortment of human and avian viruses in Italian pigs. Phylogenetic analyses of human H3N2 viruses circulating in Italian pigs revealed that genetic reassortment had been occurring between avian and “human-like” viruses since 1983 (Castrucci et al. 1993). All of these viruses retained an H3N2 serotype, but inherited avian (replacing human) virus genes that encode the “internal” proteins of the virus.

Further evidence for the emergence of new strains that can spread widely in pigs following genetic reassortment was the appearance of H1N2 virus in Great Britain in 1994 (Brown et al. 1995a), before apparent spread to the rest of Europe. The H1N2 viruses derived from a multiple reassortant event over a number of years involving human H1N1, “human-like” swine H3N2, and “avian-like swine” H1N1 (Brown et al. 1998). These viruses are genetically and antigenically very distinct from viruses of the same serotype that had been present in pigs in France briefly in the late 1980s.

The appearance of new serotypes for pigs does not always result in them becoming established in an immunologically naive population. An H1N7 virus isolated from pigs in Great Britain in 1992 was antigenically unique, being derived from human and equine viruses (Brown et al. 1994), but apparently failed to spread (see the section on virus adaptation) within pigs or to other species. However, the emergence of new viruses in pigs, even if the viruses fail to become established, may have implications for other hosts such as humans.

GENETIC DRIFT

Genes that code for the surface proteins HA and NA are subjected to the highest rates of change. Current epidemic strains are clearly distinguishable from the prototype strains. The HA gene of both the classical and “avian-like” swine H1N1 viruses is undergoing genetic drift, which is more marked in the latter. However, genetic drift in the HA gene of swine H1N1 viruses is confined generally to regions that do not influence the antigenic properties of the virus (Brown et al. 1997;

Luoh et al. 1992). This is in marked contrast to genetic drift in the HA gene of human H1N1 viruses (Xu et al. 1993). The more limited antigenic variation in the HA gene of swine viruses is probably due to the lack of significant immune selection in pigs because of the continual availability of nonimmune pigs.

In Europe, influenza viruses of H3N2 subtype are, antigenically, related closely to early human strains such as A/Port Chalmers/1/73. The limited immune selection in pigs facilitates the persistence of these viruses, which may in future transmit to a susceptible human population. However, some viruses, although related closely to the prototype human viruses, have antigenic differences in the surface glycoproteins and may cocirculate with the former strains (Brown et al. 1995a; Haesebrouck and Pensaert 1988; Kaiser et al. 1991). Recently, there has been considerable antigenic variation in the HA gene of “human-like” H3N2 viruses due to marked genetic drift, and this has led to an apparent increase in epidemics attributable to this virus (De Jong et al. 1999). These recent viruses appear to be only distantly related antigenically to the early prototype strains.

VIRUS ADAPTATION AND PATHOGENESIS

Following transmission of an influenza virus to pigs from birds or humans, the virus needs to adapt to the new host before becoming pathogenic for pigs. The available evidence suggests that this process can take many years after the initial introduction of virus. The newly introduced/emerged influenza virus may undergo many pig-to-pig transmissions because of the continual availability of susceptible pigs. The mechanisms whereby an avian virus is able to establish a new lineage in pigs are not fully understood, although following the introduction of an avian virus into European pigs in 1979 the virus was relatively unstable genetically for approximately 10 years (Ludwig et al. 1995). The continual genetic exchange between influenza viruses is likely to result in the emergence of “genetic variants” (within a virus strain or between viruses of different serotype) with a higher fitness, which can therefore gain a potential selection advantage.

It would appear that the adaptive processes took several years following transmission of both avian H1N1 and human H3N2 viruses to European pigs. Following new introductions of influenza A virus to pigs, close monitoring of the epidemiology of SIV in a population is essential to determine the rate of change, which, if elevated, may facilitate further transmissions across the species barrier, with potential implications for disease control in a range of other species, including humans. In future studies of the epidemiology of influenza viruses in pigs, it would be desirable to characterize all of the gene segments of viruses isolated in order to detect changing genotypes with potential implications for pathogenicity to pigs and/or other species.

It is possible that, following the transmission of an avian H1N1 virus to pigs in continental Europe in 1979 (Pensaert et al. 1981), subsequent infection of pigs was usually subclinical, since the virus was not well adapted to its new host. It would appear that the introduction from continental Europe of an “avian-like” swine H1N1 virus well adapted to its new host (Brown et al. 1997) into an immunologically naive pig population, such as found in Great Britain in 1992, may partly explain the rapid spread of the virus and its widespread association with disease outbreaks (Brown et al. 1993). This was consistent with the epidemiology of the virus in pigs in Europe as a whole. Interestingly, immunity to the antigenically related classical swine H1N1 viruses of widespread prevalence in pigs in Great Britain (Brown et al. 1995b) and continental Europe (Bachmann 1989) at the time apparently failed to prevent infection with the newly emerged “avian-like” swine H1N1 viruses.

The evolution and adaptation of human H3N2 viruses in pigs following transmission in the early 1970s appeared similar to that of avian H1N1 viruses. In Europe, the presence of these human H3N2 viruses in pigs was for at least 10 years based on antibody detection, and it was not until 1984 that the virus was first directly associated with outbreaks of respiratory disease in pigs (Haesebrouck et al. 1985). Such occurrences became increasingly more frequent thereafter (Castrucci et al. 1994; Wibberley et al. 1988). Locally in many parts of Europe “swine adapted” human H3N2 viruses became the predominant epidemic strain and still remain so, for example, in the Low Countries (De Jong et al. 1999; K. Van Reeth personal communication). Interestingly, H3N2 viruses circulating in pigs in Italy since 1983 all contain internal protein genes of avian origin, having replaced H3N2 viruses whose genes were derived entirely from human virus (Campitelli et al. 1997), suggesting that the acquisition of internal protein genes from an avian virus adapted to pigs afforded a selection advantage to these reassorted viruses.

DIAGNOSIS

Clinical diagnosis of infection with influenza virus is only presumptive because there are no pathognomonic signs. In addition to acute disease, there may be subclinical infection or atypical courses of infection, such as in a partially immune population. A definite diagnosis is possible only in the laboratory, either through isolation of virus or by demonstration of specific antibodies. Interpretation of serological data for viruses of the same HA serotype needs to take into account the presence of cross-reactive antibodies to related virus strains in order to identify the probable infecting strain.

Pigs infected with human H1N1 or H3N2 influenza virus readily develop specific antibodies to these viruses. As a result, the transmission of human influenza viruses

to pigs has been studied widely and monitored using serosurveillance methods. However, it has been shown that pigs infected with some avian influenza viruses may not always produce a detectable antibody response due to the resulting transient infection inducing no or low levels of humoral antibody (Hinshaw et al. 1981; Kida et al. 1994). These findings are important in studying the epidemiology of influenza virus in pigs, suggesting that serosurveillance may not be suitable for the detection of some reassortant or "new" influenza viruses in pigs. Natural and experimental infection of pigs with an H1N7 human-equine reassortant virus did not induce detectable humoral antibody, but the virus was able to transmit between pigs (Brown et al. 1994). These findings demonstrate the potential value of monitoring pigs for influenza viruses by using virus isolation and/or using molecular methods for detection.

In contrast to classical H1N1 viruses, the "avian-like" H1N1 and "human-like" H3N2 viruses from European pigs have changed significantly over a period of 20 years due to antigenic drift (Brown et al. 1997; De Jong et al. 1999). This information is useful for control purposes and for ensuring that the strains used in serodiagnostic tests are well matched to the current epidemic viruses.

CONTROL

The introduction of SIV into a pig herd can be prevented by applying standard sanitary measures to a herd of susceptible animals by eliminating contact with infected animals. Movement of pigs presents the greatest hazard to prevention and control of the disease. The occurrence in other hosts of influenza A viruses that may infect pigs requires that producers should ideally take measures to minimize contact between these species and pigs. The extent to which recovered pigs are resistant to reinfection is not known, but substantial levels of antibody are found for at least 6 months after infection. However, there is considerable variation in the antibody response of individual pigs following exposure to influenza virus.

Vaccines currently available for use in Europe against swine influenza viruses contain inactivated H3N2 (A/Port Chalmers/1/73) and H1N1 (European "avian-like") viruses in an oil-in-water adjuvant. They are used on the mainland of Europe, but generally their use is localized, and often they are administered as a combination vaccine with *Actinobacillus pleuropneumoniae* and Aujeszky's disease virus. Animals vaccinated with two injections 4 weeks apart are protected against experimental challenge via the intratracheal route. Antibodies persist for at least 9 months (Haesebrouck and Pensaert 1986). The suitability of these vaccines for protection against the newly emerged H1N2 viruses is relatively unknown, although the results of preliminary in vivo studies using "avian-like" H1N1 and swine H1N2 viruses indicated that exposure to the former did not provide

protective immunity to the latter (Van Reeth et al. 2000b). This was probably due to the antigenic heterogeneity in the HA. The efficacy of vaccines currently available (which contain historical strains) for protection against the prevailing epidemic strains is poorly known and is the subject of new initiatives supported by the European Union for better definition.

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2.3

Emergence of Novel Strains of Swine Influenza Virus in North America

Christopher W. Olsen

SUMMARY

Influenza virus infection is an important cause of respiratory disease in pigs throughout the world and an important public health concern. In North America, classical H1N1 subtype viruses remained the predominant cause of swine influenza from the time of their first isolation in 1930 through the late 1990s. Since 1997, however, this epidemiological picture has changed dramatically. H3N2 viruses of three different genotypes have appeared in the United States and Canada, causing a widespread epidemic of swine influenza. Subsequently, H1N2 viruses have been isolated from pigs in six states, and an H4N6 virus emerged among pigs in Canada. Taken together, these viruses pose significant challenges for the control of swine influenza, including the need to develop new diagnostic reagents and assays, and new vaccines. In addition, the appearance of these viruses emphasizes the need for continual influenza surveillance among pigs so as to rapidly recognize new threats to both the swine industry and human health.

INTRODUCTION

Influenza is a common and important respiratory disease among pigs throughout the world. Swine influenza is also unique in the sense that it has been both a historically significant and an ever-present disease, and yet it also presents newly emerging disease problems. This chapter traces the history and evolution of swine influenza viruses in North America since the initial appearance of the classical H1N1 subtype viruses in the early 1900s through the emergence of H3N2 and other subtypes of viruses in the 1990s.

INFLUENZA VIRUS BACKGROUND

Influenza Virus Structure and Nomenclature

Influenza viruses are enveloped, single-stranded, negative-sense RNA viruses in the family Orthomyxoviridae (Murphy and Webster 1996). Three *types* of influenza viruses exist: A, B, and C. Type C viruses are genetically

distinct from the A and B viruses and are only infrequently encountered. Humans are commonly infected with both type A or B viruses, but, with rare exceptions, only type A viruses are of significance in pigs and other animals. Type A viruses are, therefore, the focus of this chapter.

Influenza A viruses encode 10 viral proteins. These include the two large surface glycoproteins, the hemagglutinin (HA or H) that mediates virus binding to cell receptors and contains epitopes inducing neutralizing antibody response, and the neuraminidase (NA or N); the structural proteins, nucleoprotein (NP) and matrix proteins (M1 and M2); the nonstructural proteins (NS1 and NS2); and the PB1, PB2, and PA polymerase proteins (Lamb and Krug 1996). A unique aspect of influenza virus structure is that these viral genes are encoded on eight independent segments of RNA. It is the segmented nature of the influenza virus genome that enables these viruses to undergo genetic reassortment, which is an important mechanism for virus evolution and antigenic change (Murphy and Webster 1996; Webster et al. 1992).

Type A and B influenza viruses are defined by antigenic and genetic differences in their internal structural proteins: NP and M1. In contrast, *subtypes* of influenza A viruses are defined by antigenic and genetic differences in their HA and NA proteins. To date, 15 different subtypes of HA and 9 different subtypes of NA have been described. Influenza A viruses are thus designated as H1N1 subtype, H3N2 subtype, and so on (Murphy and Webster 1996; Webster et al. 1992).

Clinical Manifestations of Influenza Virus Infections in Pigs

Swine influenza can occur in explosive, rapidly progressive epidemics. Affected pigs present with fever, lethargy, anorexia, nasal and ocular discharge, coughing, and dyspnea (Easterday and Hinshaw 1992) lasting 7 to 10 days. The concomitant reductions in weight gain can add as much as 2 weeks to the time it takes hogs to reach market weight (B. C. Easterday personal communication). Swine influenza viruses are also one of the contributing factors (along with primarily porcine reproductive and respiratory syndrome virus, *Mycoplasma hyopneumoniae*,

and bacterial pathogens) to the porcine respiratory disease complex (PRDC) (Halbur 1996; Janke 1998). In either form, however, influenza virus infections represent an economically very important concern to the swine industry.

Public Health Implications of Influenza Virus Infections in Pigs

Beyond the impact on animal health, infection of pigs with influenza viruses also poses human public health risks. Most directly, swine influenza viruses are infectious to people as zoonotic agents. Zoonotic infections have been documented in the United States (Dasco et al. 1984; Hinshaw et al. 1978; Wentworth et al. 1997), Europe (De Jong et al. 1988), New Zealand (Eason and Sage 1980), and Hong Kong (Gregory et al. 2001), in some cases resulting in the death of the people infected (Kimura et al. 1998; Patriarca et al. 1984; Rota et al. 1989; Smith et al. 1976; Top and Russell 1977; Wentworth et al. 1994). On a broader scale, pigs are susceptible to infection with influenza viruses of both avian and mammalian origin because their tracheal epithelial cells contain virus receptor sialyloligosaccharides with both 2,3- (preferred by avian influenza viruses) and 2,6- (preferred by mammalian influenza viruses) *N*-acetylneuraminic acid-galactose linkages (Ito et al. 1998a). As such, pigs have been implicated as the intermediate host for adaptation of avian influenza viruses to mammals (Campitelli et al. 1997) and as the *mixing vessel* hosts in which human-avian influenza virus reassortment occurs (Scholtissek and Naylor 1988; Scholtissek et al. 1985; Webster et al. 1992). In this regard, it is important to realize that waterfowl provide a massive global reservoir of influenza viruses since they can be subclinically infected with viruses of all 15 HA and 9 NA subtypes. The viruses responsible for the human influenza pandemics of 1957 and 1968 were reassortants between human and avian viruses and are thought to have developed in pigs (Webster et al. 1992). More recently, human-avian influenza virus reassortants have been isolated from commercially raised pigs in Europe (Castrucci et al. 1993) and subsequently from children in the Netherlands (Claas et al. 1994). Finally, and of particular significance to this chapter's topic, it has become clear since 1998 that genetic reassortment in pigs can also produce novel viruses of clinical significance to the pigs themselves.

THE EPIDEMIOLOGY OF SWINE INFLUENZA IN NORTH AMERICA

The Predominance of Classical H1N1 Viruses from 1918 to 1997

Influenza viruses were first isolated from pigs in 1930 (Shope 1931), but influenza-like illness was recognized among American pigs as early as 1918 (Easterday and

Hinshaw 1992; Koen 1919). This was the same year that the *Spanish flu* pandemic killed 20 to 40 million people worldwide (Murphy and Webster 1996; Taubenberger et al. 1997, 2000). Recent evidence indicates that the early swine and human isolates were closely related H1N1 viruses that had recently emerged from an avian source (Reid et al. 1999, 2000; Taubenberger et al. 1997, 2000). It remains unclear whether a progenitor virus appeared first in people and then spread to pigs, or vice versa. In either case, however, surveillance data have demonstrated that these *classical* H1N1 influenza viruses remained the predominant cause of influenza among pigs in the United States through the 1990s. Specifically, studies conducted in 1976–1977 (Hinshaw et al. 1978), 1988–1989 (Chambers et al. 1991), and 1997–1998 (Olsen et al. 2000) documented widespread exposure of pigs in the north-central United States to H1 influenza viruses, with H1 seropositivity rates of 47%, 51%, and 28%, respectively, during these years.

Antigenic Drift Among Classical H1N1 Swine Influenza Viruses

The predominance of H1N1 viruses as the cause of swine influenza in North America throughout most of the 1900s should not be interpreted as indicating that these viruses never evolved and changed. In fact, a number of antigenic drift variants of H1N1 viruses have been isolated from North American pigs. Antigenic drift refers to the gradual accumulation of mutations in the HA (and NA) proteins in response to immune pressure in the population. Through this process, viruses evolve to evade neutralization by the host's immune responses. In 1991, a classical H1N1 virus with an antigenically and genetically distinctive HA was isolated from pigs in Quebec (Dea et al. 1992; Rekik et al. 1994). In this case, infected pigs also developed an atypical proliferative and necrotizing pneumonia rather than the bronchopneumonia that most commonly typifies swine influenza. In 1992, another classical H1N1 virus with an antigenically and genetically divergent HA was isolated from pigs in Nebraska (Olsen et al. 1993). These pigs exhibited atypical clinical disease, presenting with persistent, high fevers, but relatively little in the way of respiratory signs. Finally, analysis of a group of 26 classical H1N1 viruses isolated from pigs at slaughter in the north-central United States in 1997–1998 revealed extensive evidence of antigenic drift (Olsen et al. 2000). Differences in reactivity in hemagglutination-inhibition (HI) assays to four monoclonal antibodies (Mabs) demonstrated that these viruses could be divided into seven distinct antigenic groups. In fact, the only antigenic pattern not represented among these viruses was that of the reference virus isolated in 1988. Thus, antigenic drift clearly has occurred among the classical H1N1 swine influenza viruses. An obvious question is whether this antigenic drift has progressed to the point that the available swine influenza

virus vaccines will not provide cross-protection. There are no published data describing vaccination and heterologous challenge studies at this time. However, all 26 of the viruses isolated in the 1997–1998 study, although antigenically divergent by Mab analysis, reacted to identical titer as the 1988 reference virus in HI assays using polyclonal sera from pigs vaccinated with a commercially available swine influenza virus vaccine (Olsen et al. 2000).

The Emergence of H3N2 Influenza Viruses Among Pigs in North America

In Europe and Asia, human H3N2 influenza viruses have been isolated repeatedly from pigs (Campitelli et al. 1997; Castrucci et al. 1994; Mancini et al. 1985; Nakajima et al. 1982; Ottis et al. 1982). This was not, however, the case in North America throughout most of the 1900s. In the surveillance studies conducted in 1976–1977 (Hinshaw et al. 1978) and 1988–1989 (Chambers et al. 1991), seropositivity to H3 subtype viruses was very limited (1.4% and 1.1%, respectively). And, consistent with these serological data, only three H3 viruses were isolated from North American pigs during these time periods (Bikour et al. 1994, 1995; Hinshaw et al. 1978). Since 1997, however, the epidemiological pattern of swine influenza in North America has changed dramatically (Olsen 2001).

The serological survey conducted in 1997–1998 detected a distinct increase in H3 virus infections, with a seropositivity rate among pigs in the north-central United States of 8% (Olsen et al. 2000). This early serological evidence for H3 virus emergence among North American pigs was subsequently confirmed by the occurrence of a widespread epidemic of H3N2 influenza throughout the North American swine population. The initial report of H3N2 virus infection in pigs described an outbreak on a farm in North Carolina in August 1998. These pigs exhibited both influenza-like respiratory disease as well as abortions in 7% of the pregnant sows and a 2% mortality rate in the sows (Zhou et al. 1999). The latter presentations were extraordinary for swine influenza. Infection of pigs with classical H1N1 viruses rarely causes death and, with the exception of a few reports (Madec et al. 1989; Woods and Mansfield 1974; Young and Underdahl 1949), swine influenza viruses are not thought to target directly the reproductive tract of pigs. Subsequently, H3N2 virus isolates have been reported from pigs in Texas, Minnesota, Iowa, Nebraska, Colorado, Oklahoma, Wisconsin, Illinois, and Ontario (Karasin et al. 2000c; Webby et al. 2000; Zhou et al. 1999), with infections retrospectively identified as early as January 1997 in Ontario and March 1998 in Nebraska (Karasin et al. 2000c).

Genetic analyses of the H3N2 viruses isolated from pigs in North America clearly showed that, although these were all of the same overall subtype, they were not

genetically identical. In fact, at least three different genotypes of H3N2 viruses have appeared among North American pigs. The initially reported virus from North Carolina was a reassortant virus containing HA, NA, and PB1 genes from a human influenza virus, and M, NP, NS, PA, and PB2 genes from a classical swine virus (Zhou et al. 1999). All of the remaining H3N2 viruses isolated in the United States have been *triple-reassortant* viruses containing genes from three different influenza virus lineages. Once again, the HA, NA, and PB1 genes were of human influenza virus origin, and the NP, M, and NS genes were of classical swine virus origin, but the PA and PB2 genes in these viruses were of avian virus origin (Karasin et al. 2000c; Webby et al. 2000; Zhou et al. 1999). Finally, the H3N2 virus isolated in Ontario in January 1997 was of a third genotype. All eight RNA gene segments of this virus were of human influenza virus origin. As such, this virus was most likely an example of *reverse zoonosis* in which an influenza virus crossed the species barrier directly from a human being to infect a pig (Karasin et al. 2000c).

Beyond their overall genotypic differences, analyses of these H3N2 viruses revealed several other interesting facets. First, although all of the initially described triple-reassortant viruses (Karasin et al. 2000c; Zhou et al. 1999) were isolated in 1997–2000, their HA genes were phylogenetically most closely related to the 1994 and 1995 lineages of human influenza viruses. In fact, they completely lacked all of the *signature sequences* of the more contemporary 1997 human virus lineage (Karasin et al. 2000c). This suggests that the progenitor human influenza virus that gave rise to these swine viruses most likely entered the swine population several years before the emergence of the reassortant viruses. More recently, one virus isolated in Colorado in 1999 appeared, by phylogenetic analysis, to contain an HA gene derived from a 1997 lineage human virus. This might suggest that this virus resulted from a new reassortment event separate from that which initially gave rise to the other H3N2 viruses (Webby et al. 2000). However, its HA gene contained only a subset of the 1997 lineage signature mutations (C. W. Olsen unpublished data), so this isolate may also simply represent drift from the original triple-reassortant lineage.

A second notable finding among the H3N2 viruses is that the initially described triple-reassortant viruses (Karasin et al. 2000c; Zhou et al. 1999) contained specific amino acid differences in their HA proteins compared with the double-reassortant North Carolina virus and the wholly human virus isolated in Ontario (Karasin et al. 2000c). These differences are of interest because, although host-range restriction among influenza viruses is thought to be a polygenic trait, the HA has been strongly implicated as an important determining factor (Murphy and Webster 1996; Webster et al. 1992). In this regard, it is noteworthy that the triple-reassortant genotype

viruses have spread throughout the swine population of the United States, while the double-reassortant North Carolina virus, as of December 2000, has not reappeared elsewhere in the population (G. Erickson personal communication). Similarly, the wholly human H3N2 virus isolated in Ontario was recovered only once from a single piglet on one farm (Karasin et al. 2000c). These infection patterns suggest that the HA differences in the triple-reassortant viruses may have provided a replication advantage for these viruses in pigs (Karasin et al. 2000c). Additional factors that may influence the ability of the triple-reassortant viruses to efficiently replicate in pigs include the presence of avian polymerase genes (Webby et al. 2000; Zhou et al. 1999) or the effect of having the specific constellations of human, swine, and avian virus genes functioning in concert. As such, beyond being an important clinical concern, these H3N2 viruses may also serve as powerful research tools. Using recently developed reverse genetics systems (Fodor et al. 1999; Hoffmann and Webster 2000; Neumann and Kawaoka 1999; Neumann et al. 1999, 2000), it may be possible to use these viruses to elucidate the genetic controls of host-range restriction and replication efficiency of influenza viruses in pigs.

The emergence of the H3N2 viruses has posed significant challenges for the control of swine influenza, including the need to develop new diagnostic reagents and assays, and new vaccines. However, their emergence also emphasizes the need for continual influenza surveillance among pigs so as to be able to rapidly recognize new threats to both the swine industry and, potentially, human health. Heightened surveillance activities and more complete genetic characterization of virus isolates have already demonstrated that swine influenza virus evolution in North America has not stopped with the H3N2 viruses.

Second-Generation H1N2 and H1N1 Viruses

Two unique viruses isolated in 1998–1999 were directly derived from the triple-reassortant H3N2 swine viruses. These were the products of *second-generation reassortment* between a triple-reassortant swine virus and a classical H1N1 swine virus. The first virus was an H1N2 subtype virus that was isolated from pigs in Indiana in 1999 (Karasin et al. 2000a). Phylogenetic analyses revealed that this isolate's HA gene was derived from a virus of the classical H1 swine lineage, whereas all of its other genes were most closely related to those of the triple-reassortant H3N2 viruses recovered from pigs in the United States in 1998–1999. Clinically, influenza-like illness spread throughout the farm of origin of this H1N2 virus over a 6-week period beginning in November 1999. The affected pigs exhibited fevers, lethargy, lack of appetite, dyspnea and, once again, 20 of 600 sows aborted.

This was the first reported isolation of an H1N2 virus in North America, but H1N2 reassortants have been re-

covered previously from pigs in France (Gourreau et al. 1994), Belgium (Van Reeth et al. 2000), the United Kingdom (Brown et al. 1995, 1998) and Japan (Ito et al. 1998b; Nerome et al. 1982, 1985; Ouchi et al. 1996; Sugimura et al. 1980; Yasuhara et al. 1983). The H1N2 viruses in Japan and the United Kingdom caused large-scale outbreaks of disease in the regional pig populations (Brown et al. 1995, 1998; Ito et al. 1998b; Ouchi et al. 1996). Likewise, since the initial H1N2 virus isolation in Indiana, additional H1N2 viruses of the same genotype have been isolated from pigs in six states in the United States (Karasin et al. 2001), suggesting that H1N2 viruses are now circulating along with H1N1 and H3N2 viruses in the US swine population.

Another example of a second-generation reassortant virus was recovered from a 57-year-old man in Wisconsin (Cooper et al. 1999). Phylogenetic analyses revealed that, in this case, a virus of the triple-reassortant genotype acquired both H1 HA and N1 NA genes from a contemporary classical swine virus, yielding an H1N1 subtype virus. This man did not raise pigs, but he routinely butchered a hog every 1 to 2 months for food consumption. He presented clinically with chills, fever, and cough, was treated with amantadine, and recovered uneventfully.

The Isolation of H4N6 Influenza Viruses from Pigs in Canada

The most recently described virus to emerge within the North American pig population emphasizes the extensive reservoir of influenza viruses that exists among waterfowl. In October 1999, an H4N6 subtype virus was isolated from pigs on a farm in Ontario, Canada (Karasin et al. 2000b). Genetic analyses demonstrated that this was a non-reassortant, wholly avian influenza virus that crossed the species barrier in toto to infect pigs. In Europe in the late 1970s and 1980s, an avian H1N1 virus entered the swine population and eventually became the dominant cause of swine influenza in Northern Europe (Pensaert et al. 1981; Scholtissek et al. 1983; Webster et al. 1992). However, this H4N6 virus represents the first documented isolation of a wholly avian influenza virus from North American pigs, and the first time that an H4N6 subtype virus has been isolated from naturally infected pigs anywhere in the world.

Clinically, approximately 5% of the 2600 grower/feeder pigs and young boars in one unit of the farm of origin of this H4N6 virus exhibited coughing, dyspnea, and weight loss. The specific mechanism by which this virus entered the pig population on the farm is unclear, but may be related to the farm being adjacent to a lake on which large numbers of waterfowl congregate each fall before beginning their southward migrations.

Interestingly, H4 and N6 viruses are among the most common influenza viruses in the Canadian waterfowl population (Sharp et al. 1993). Serological testing

revealed spread of this H4N6 virus within several units of the farm of origin that had received pigs from the initially affected unit, thus confirming the ability of this virus to spread from pig to pig following initial introduction from waterfowl (Karasin et al. 2000b). Finally, although there were no reports of human infection among the farmworkers at the time of the outbreak, it should be noted that the HA protein of this virus contained specific amino acids in the positions previously suggested to confer binding to the 2,6 receptors available in human beings for influenza virus infection (Karasin et al. 2000b).

CONCLUSIONS

The epidemiological picture of swine influenza in North America has changed dramatically in recent years (Olsen 2001). After a nearly 80-year period during which classical H1N1 viruses were virtually the sole agent of swine influenza, an antigenic shift has occurred since 1997–1998. Reassortant H3N2 viruses have emerged and spread throughout the swine population, and subsequently a second-generation reassortant H1N2 virus and a wholly avian H4N6 virus have appeared. These events have posed new challenges for the veterinary and swine production communities. However, they should also serve as a reminder of the important role that pigs play in the overall epidemiology and ecology of influenza viruses and the necessity to be vigilant in detecting and characterizing unique viruses among pigs. Intensive surveillance efforts will ultimately benefit both the swine population and our world's human population.

ACKNOWLEDGMENTS

This work was supported in part by grants from the USDA Agricultural Experiment Station, the USDA National Research Initiative, and the University of Wisconsin-Madison.

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3 PORCINE PARAMYXOVIRUSES

3.1

Blue Eye Disease: Clinical Signs and Lesions

Alberto Stephano

SUMMARY

Originally reported and generally restricted to central Mexico, blue eye disease of swine is caused by a rubulavirus in family Paramyxoviridae. Infection may result in encephalitis, reproductive failure, and corneal opacity. Clinical signs are variable and primarily depend on the age of the animal, type of herd, production system, management, and presence of other infections.

INTRODUCTION

In 1980 in central Mexico, numerous outbreaks of a disease in nursing piglets characterized by central nervous system (CNS) signs, corneal opacity, and a high mortality were reported in La Piedad, Michoacán (Stephano et al. 1981). Later, similar outbreaks were observed in the states of Jalisco and Guanajuato.

Initial studies revealed a nonsuppurative encephalitis in affected animals, and a hemagglutinating virus was isolated from them (Stephano and Gay 1983; Stephano et al. 1981). The physicochemical, morphological, and culture characteristics, as well as serological studies, showed that the disease was caused by a new virus that was subsequently identified as a member of family Paramyxoviridae and designated blue eye paramyxovirus (BEP). The disease was experimentally reproduced in swine inoculated with BEP, thus fulfilling Koch's postulates (Stephano and Gay 1983, 1984, 1985a; Stephano et al. 1986a). This chapter provides clinical and pathological aspects of blue eye disease in pigs.

EPIDEMIOLOGY

In 1980, clinical signs (i.e., CNS signs and a high mortality) were seen primarily in nursing piglets. In pigs over 30 days of age, signs were less frequent, more subtle, and caused little or no death (Stephano et al. 1981). In 1983, severe outbreaks of encephalitis, respiratory disease, and up to 30% mortality were reported in some herds in pigs weighing 15 to 45 kg (33 to 99 pounds). Typically, these herds mixed pigs from several sources, were not particularly well managed, and had concurrent disease problems, particularly respiratory (Stephano and Gay 1985b, 1986a). Additional studies revealed the effects of

the infection on reproductive performance (Stephano and Gay 1984, 1985a). Affected parameters included an increase in repeat breeding, an increase in stillbirths and mummies, and a small increase in abortions. In 1988, orchitis, epididymitis, testicular atrophy, and a severe decline in semen quality were also associated with BEP infection (Campos and Carbajal 1989; Stephano et al. 1990).

Clinically, BED is frequently associated with other infections. Recently, outbreaks of BED with concurrent porcine reproductive and respiratory syndrome virus (PRRSV) infection were reported in which clinical signs were more severe than generally associated with either pathogen by itself (Stephano 1998).

Since 1980, BED has been restricted almost exclusively to the central region of Mexico. In the first few years following its recognition, the virus was isolated from outbreaks in the states of Michoacán, Jalisco, and Guanajuato. Subsequently, the virus was disseminated to the neighboring states of Querétaro, the state of México, and the Federal District. As a result of an active market in pigs during this period, they were transported from La Piedad, Michoacán, to various states, where BED was subsequently diagnosed: Nuevo León, Hidalgo, Tlaxcala, Tamaulipas, Puebla, Yucatán, and Campeche (Stephano et al. 1986b, 1988b). Serological evidence showed antibodies against BEP in at least 16 states (Fuentes et al. 1992). However, since clinical BED was not reported nor cases diagnosed in these states, it is considered to be a disease of central Mexico.

A number of porcine viruses in family Paramyxoviridae have been identified, but, up to the present, BED and the etiologic agent of BED have been found only in Mexico (Stephano et al. 1982, 1999). Recently, family Paramyxoviridae was reorganized, and BEP was placed in subfamily Paramyxovirinae, genus *Rubulavirus* (type species: mumps virus) (Rima et al. 1995). Information can be found elsewhere on the extensive molecular studies to characterize BEP (Berg et al. 1991, 1992, 1997; Reyes-Leyva et al. 1993; Sundqvist et al. 1990, 1992).

CLINICAL SIGNS

Clinical signs in farrow-to-finish herds may start in any group of swine, but are most commonly first observed in

farrowing facilities. In this circumstance, CNS signs and a high mortality are seen in nursing piglets, some of which develop corneal opacity. At about the same time, corneal opacity may become apparent in older pigs (Stephano and Gay 1985a; Stephano et al. 1988a). The mortality rate increases rapidly and then quickly declines. Once the outbreak is over, new cases are not seen unless susceptible animals are introduced into the herd, as may occur in continuous-flow operations.

Clinical signs are variable and primarily dependent on the age of the animal, type of herd, production system, management, or presence of other infections. Piglets 2 to 15 days of age are the most susceptible, and clinical signs appear acutely. A few healthy piglets may suddenly exhibit lateral recumbency or progressive CNS signs. Most frequently, clinical signs start with fever, rough hair coat, and an arched back posture, sometimes accompanied by constipation or diarrhea. These are followed by progressive nervous signs, i.e., ataxia, weakness, stiffness—primarily in the rear legs—muscular trembling, and abnormal postures such as dog sitting. Anorexia is not seen as long as piglets can move. Some piglets are hyperexcitable and paddle or squeal when touched. Other signs include lethargy with involuntary movements, dilated pupils, apparent blindness, and occasionally nystagmus. Conjunctivitis, swollen eyelids, and lacrimation are also present. Frequently, eyelashes are pasted closed with exudate and, in 1% to 10% of affected piglets, unilateral, and occasionally bilateral, corneal opacity develops. Frequently, corneal opacity occurs in the absence of other apparent signs. In most cases, opacity resolves and disappears spontaneously. In the first cases observed in 1980, most piglets died within the first 48 hours of the appearance of clinical signs. In later years, death frequently occurred after 4 to 6 days (Stephano and Gay 1985a; Stephano et al. 1988a).

Of piglets farrowed during an outbreak, 20% to 65% are affected. Morbidity within litters is typically 20% to 50%, with 87% to 90% of affected piglets dying. Mortality increases for 2 to 9 weeks after the first case, primarily depending on the management system and the type of facilities.

Most sows with affected litters do not show clinical signs, but some become moderately anorexic for 1 or 2 days prior to the appearance of clinical signs in their piglets. Corneal opacity is also seen in some sows in the breeding herd during the outbreak. Clinical signs are also mild in gestating females and generally limited to transient anorexia or corneal opacity. Replacement animals and other adult swine in the herd also occasionally develop corneal opacity.

Reproductive parameters are generally affected for 4 months (from 2 to 11 months). During an outbreak, there is an increase in the number of returns to estrus, a decline in the farrowing rate, an increase in the wean-to-service interval, and an increase in nonproductive sow

days. Abortion is not typical of BED, but may be seen in a variable percentage of sows in acute outbreaks. There is also a marked increase in stillbirths and mummified fetuses, and, as a result, a reduction in the number of pigs born live and total pigs per farrowing.

Boars frequently show no clinical signs, although corneal opacity and moderate anorexia are occasionally seen. However, libido may decline either transiently or permanently. One field study found that 29% to 73% of boars were affected. Semen quality may also change, with a moderate to severe decline in spermatozoa concentration, motility, and viability. Azoospermia or necrospemia may develop, in which case the ejaculate appears either abnormally clear or brown and cloudy, occasionally with blood. Swelling of the testicles and epididymis is seen in some boars, in which cases the tissues become turgid and edematous. Occasionally, this condition progresses and the epididymis develops a granular texture. This is frequently followed by testicular atrophy (usually unilateral), with the testicle becoming small, soft, and flaccid. Boars with epididymal and testicular lesions frequently lose libido during the acute phase of the disease (Campos and Carbajal 1989; Stephano et al. 1990).

Swine over 30 days of age in farrow-to-finish herds show moderate, transient signs, such as anorexia, fever, sneezing, and coughing. CNS signs are less common and subtler. When present, they consist of listlessness and lethargy, marked incoordination, circling, head movements, and prostration. Mortality is usually low. Corneal opacity is unilateral or occasionally bilateral, but only 1% to 4% of pigs are affected. Opacity and conjunctivitis may continue to appear in the herd for a month without the appearance of any other clinical signs (Stephano 1984).

Beginning in 1983, severe outbreaks were seen in finishing herds, with 5% to 20% mortality. Severe CNS and respiratory signs were seen in pigs weighing 15 to 45 kg (33 to 99 pounds), with corneal opacity in 10% to 30%. Prominent signs included lethargy, incoordination, circling, lateral recumbency with paddling, walking without stopping, walking into objects or walls of pens—even to the point of injury—abnormal gait, such as high stepping, and abnormal postures, such as dog sitting and head pressing. Posterior paresis was seen occasionally. Dyspnea, coughing, and sneezing were also present, with gross lesions apparent in pulmonary tissues on necropsy. A study of 22 finishing farms in which outbreaks occurred suggested common factors: pigs from various sources were mixed, swine were of different ages and weights but usually weighed 10 to 25 kg (22 to 55 pounds), and pigs were introduced into the herd every week. Management of these farms was generally deficient, and pigs were overly stressed. Clinical signs generally were precipitated by stress or associated with respiratory disease. A number of pathogens were associated with BED, including Aujeszky's disease virus, *Actinobacillus pleuropneumoniae*, and mycoplasma (Stephano and Gay 1985b).

In 1998, severe cases of BED were reported in association with PRRSV and larger herd size. Reproductive parameters were more severely affected than with either BED or PRRSV alone. Under these circumstances, the rate of pigs born alive fell and the rate of mummies rose; the mortality was higher, and the number of pigs weaned fell dramatically. Severe clinical signs were also seen in replacement gilts, including death, and many pigs were sent to slaughter because of their poor condition (Stephano 1998).

In well-managed herds with all-in, all-out flow, the disease is controlled by closing the herd to susceptible animals and applying standard control measures, including washing and disinfection of facilities and equipment.

PATHOLOGY

Macroscopic Lesions

If lesions are present, conjunctivitis and varying degrees of corneal opacity are characteristic of BED. As already discussed, lesions may be seen in a variable number of animals. The cornea may be one to three times normal thickness. Occasionally, other ocular lesions are present, such as vesicles, ulcers, and keratoconus of the cornea and exudate in the anterior chamber (Stephano and Gay 1985a, 1986b; Stephano et al. 1988a).

In boars, inflammation of the testicles and epididymis is observed, with a marked increase in diameter and weight as a result of edema in acute cases. These changes are frequently unilateral. As the disease progresses, orchitis and epididymitis develop, and the testicle and epididymis become turgid. The epididymis becomes granular and firm due to the formation of spermatic granulomas. Subsequently, testicular atrophy with testicular fibrosis and adherence to the tunica albuginea develops. Occasionally, hemorrhages in the tunica albuginea, epididymis, and testicle are present (Campos and Carbajal 1989; Ramírez et al. 1995; Stephano et al. 1990).

In piglets, moderate pneumonia may be observed, usually affecting the ventral portion of the cranial lobules of the lung. Moderate gastric distention may be seen and semidigested milk may be present in the stomach. There may be moderate distention of the urinary bladder due to the accumulation of urine and a slight accumulation of fluid and fibrin tags in the peritoneal cavity. The brain may be congested, and cerebrospinal fluid may be increased. Occasionally, pericardial and renal hemorrhages may be found. In finishers, renal hemorrhages and pulmonary lesions are common (Stephano and Gay 1985a, 1986b).

Microscopic Lesions

Histological changes in the brain and spinal cord are compatible with a nonsuppurative encephalitis primarily involving the gray matter of the thalamus, middle

brain, and cerebral cortex, and is accompanied by a diffuse and multifocal gliosis. There is perivascular infiltration with lymphocytes, plasma cells, and reticular cells, with neuronal necrosis, neuronophagia, meningitis, and choroiditis. Intracytoplasmatic inclusion bodies within the neuronal cells have also been observed. The severity and extension of the lesions vary among pigs (Allan et al. 1996; Pérez et al. 1988; Ramírez and Stephano 1982; Stephano and Gay 1986b; Stephano et al. 1988a).

The lungs may show multifocal areas of interstitial pneumonia, characterized by thickening of the septae and mononuclear infiltration. Many affected pigs have a moderate tonsillitis with epithelial desquamation and inflammatory cells in the crypts.

The histopathology of the eyes is primarily limited to the cornea. The opacity and thickening are associated with edema and anterior uveitis. The cornea may become several times its normal thickness. Varying degrees of neutrophil and mononuclear infiltration are seen in the endothelial vasculature and adjacent tissues of the iridocorneal angle, corneoscleral angle, and cornea. In cells of the external layer of the cornea, cytoplasmic vesicle formation is observed, occasionally with separation of the layers from which the vesicles originate (Pérez et al. 1988; Stephano and Gay 1986b; Stephano et al. 1988a).

In affected boars, testicular lesions are of different types and degrees, depending on the clinical course of the disease. The germinal epithelium may show areas of degeneration and necrosis. The interstitial tissue may show hyperplasia of Leydig's cells, mononuclear cell infiltration, hyalinization of vascular walls, and fibrosis. The epithelial cells of the epididymis show vesicle formation and loss of cilia. Rupture of the epithelial walls leads to leakage of spermatozoa into the intertubular spaces, infiltration with inflammatory cells and macrophages, and phagocytosis of spermatozoa. This leads to fibrosis, spermatic granulomas, and testicular atrophy (Ramírez et al. 1995, 1997; Stephano et al. 1990).

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3.2 New Approaches in the Diagnosis of Porcine Rubulavirus (LPMV)

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SUMMARY

A blocking enzyme-linked immunosorbent assay (ELISA) was developed to detect antibodies to porcine rubulavirus [La Piedad Michoacán virus (LPMV) strain] in serum samples from pigs. The test, based on a monoclonal antibody against the LPMV hemagglutinin-neuraminidase glycoprotein, had a sensitivity of 99% and a specificity of 97%. The results of this test agreed with those obtained by an indirect ELISA, hemagglutination inhibition, indirect immunofluorescence, and virus neutralization tests. The blocking ELISA is considered the most suitable test for routine screening for antibodies against LPMV.

INTRODUCTION

The porcine rubulavirus was originally isolated from the brain of a piglet showing signs of central nervous system disorders, pneumonia, and corneal opacity (Moreno-Lopez et al. 1986). The disease was first observed in 1980 during an outbreak of encephalitis in piglets on farms around the town of La Piedad, in the state of Michoacán, Mexico (hence the abbreviation to LPM virus). Since the report of the initial outbreak of the disease, the virus has spread throughout Mexico and is now endemic in the country (Stephano et al. 1988).

Rubulaviruses, belonging to the Paramyxoviridae family, are large and enveloped. The virion is composed of an internal nucleocapsid containing the single-stranded RNA genome and associated proteins, such as the nucleoprotein (NP), the large (L) protein, and an outer lipoprotein envelope containing viral peplomers consisting of proteins such as the hemagglutinin-neuraminidase (HN) and the fusion (F) proteins. The HN and F proteins are both glycosylated transmembrane proteins involved in attachment of virus to the cells and the fusion of the cell membranes. During infection by members of the Paramyxoviridae family, antibodies are produced against both internal and external proteins. However, only antibodies to the HN and F proteins have been shown to be important in eliciting a virus-neutralizing response. The HN (or H) protein induces the most efficient neutralizing antibodies, although the

best protective response is achieved if antibodies toward both proteins are produced (Norrby et al. 1975; Paterson et al. 1987; Spriggs et al. 1988).

Clinical signs are variable, depending on the age of the pig. Piglets 2 to 15 days of age are most susceptible and usually succumb to nervous system disorders within 48 hours after onset of clinical signs, with a mortality of up to 90%. Up to 10% of infected piglets develop corneal opacity. Mortality is low in pigs older than 30 days, and nervous disorders are scarce, although respiratory illness is more common in such cases. Sows may return to estrus more frequently or experience abortions, stillbirths, or mummified fetuses. Boars can develop orchitis, epididymitis, and testicular atrophy (Stephano et al. 1988).

SEROLOGICAL DIAGNOSIS OF LPMV

Due to the variability in symptoms associated with LPMV infection, it is difficult to diagnose the disease clinically. Evidence exists that LPMV infection is widespread in Mexico. However, the exact extent of the infection and the distribution throughout pig herds in Mexico is still vague. Therefore, an accurate and rapid laboratory diagnosis of the infection is particularly important to confirm LPMV as the causal agent of a disease outbreak. Procedures currently in use for the diagnosis of LPMV infection in Mexico are limited to retrospective demonstration of seroconversion to LPMV and/or isolation of the virus in cell culture and subsequent serological typing. For serological diagnosis, the most commonly used test is the hemagglutination inhibition (HI) test. Other serological techniques frequently used for serological diagnosis of LPMV are indirect fluorescent antibody (IFA), virus neutralization (VN), and indirect enzyme-linked immunosorbent assay (ELISA). A comparative study of these four techniques for screening antibodies to LPMV has been performed (McNeilly et al. 1997). All four tests have advantages, as well as disadvantages. The HI test is cheap and quick but gives false-negative results at low dilutions. The serum has to be pretreated with heparin/MgCl₂ to obtain reliable results. The IFA and VN tests are both quite laborious and require a trained person to

perform them and analyze the results. The indirect ELISA has the advantage of being cheap, quick, and easy to interpret, but antiswine antibodies frequently bind nonspecifically in the reactions, which complicates the interpretation of results.

One way to overcome the problem of nonspecific reactions is to use a blocking ELISA (B-ELISA) format. In a B-ELISA, sera are added to microtitration plate wells coated with viral antigen. If antibodies to the virus are present in the sample (i.e., the animal was infected), they bind to viral antigens. Next, enzymatically labeled monoclonal antibody (Mab) directed to an epitope on the virus is added to the well. If the epitope for which the Mab is specific is occupied by antiviral serum antibodies, the Mab cannot bind to the site. But if the site is unoccupied (i.e., the serum was free of antibodies), the labeled Mab binds to the site. The reaction is visualized by adding a substrate that will develop a color. Thus, wells with the enzyme-labeled Mab (i.e., wells with a negative sample) change color. The binding of a Mab to a single epitope on the virus gives a very specific reaction and causes no nonspecific reactions, as occur in the indirect ELISA. The Mabs used in B-ELISAs developed for diagnostic purposes are usually directed against a protein eliciting protective immunity.

DEVELOPMENT OF A BLOCKING ELISA

A B-ELISA against LPMV was developed to facilitate screening of large numbers of swine sera (Nordengrahn et al. 1999). The assay is based on a Mab against HN glycoprotein, which was shown to be one of two proteins important for protective immunity against LPMV. For this purpose, stable hybridoma clones secreting Mabs were selected and antibodies further characterized by immunoprecipitation. The results indicated that the majority of Mabs were directed either to the HN glycoprotein or to NP of the LPMV. A Mab was selected for its ability to block LPMV-specific sera in a B-ELISA. The Mab was verified by radiolabeling, HI, and IFA tests.

The results of a B-ELISA can be interpreted by calculating the percent inhibition (PI) according to the following equation:

$$PI = [(negative\ control\ OD - sample\ OD)/negative\ control\ OD] \times 100$$

where *negative control OD* represents the optical density result of a known antibody-negative serum sample run on the same microtitration plate as the sample.

The LPMV B-ELISA cutoff values, expressed as PI, were determined on a number of negative, positive, and weak-positive serum samples. The results of the test were verified by comparison to those of indirect ELISA and HI and VN tests (McNeilly et al. 1997). Sera from experimentally infected piglets were used in the

study. In an experiment carried out in Belfast, 3-day-old pigs were inoculated with LPMV-85 strain. Blood was sampled on days 0, 5, 8, 11, and 14 after inoculation and serum collected for use in serological studies. In a second experiment in Mexico (Ramirez-Mendoza et al. 1997) for studies of the reproductive tract, 9-month-old boars were inoculated with porcine rubulavirus strain PARC-3. Blood was sampled on days 0, 9, 12, 15, 19, 32, and 38 after inoculation, and selected serum samples from the boars were tested by B-ELISA. The results agreed with those from previous studies (McNeilly et al. 1997), i.e., all piglets showed antibody response by days 8 to 11 after infection. All boars were positive on B-ELISA on day 9, which also agreed with the results of the first experiment. From these data, a PI value for cutoff was set: all sera with a PI over 35 were considered true positive, and all with a PI under 25 were considered true negative.

The B-ELISA was further evaluated on a large number of field sera from Mexico. A total of 597 serum samples were randomly collected from pig herds in different parts of Central Mexico (states of Guanajuato, Michoacán, and Querétaro). From the 597 sera tested, 10 clear positives, 10 clear negatives, and the 40 sera that produced an intermediate response (PI 25 to 35) were selected and further analyzed by VN and IFA tests. The results from the different tests agreed with the B-ELISA except for five false-negative sera with PI values of 21 to 34 and five false-positive sera where four samples had PI ranging from 37 to 42 and one with a PI of 84.

Based on the aforementioned results, the diagnostic sensitivity and specificity of the B-ELISA were calculated:

$$sensitivity = [true\ positive / (true\ positive + false\ negative)] \times 100$$

$$specificity = [true\ negative / (true\ negative + false\ positive)] \times 100$$

Thus, the B-ELISA was shown to have a sensitivity of 99% and a specificity of 97%.

CONCLUSIONS

The B-ELISA based on a Mab against the HN protein was robust, highly specific, and sensitive, and detected infection at an early stage in pigs. It is, therefore, suitable for screening a large number of sera. It is recommended that a B-ELISA system be used in epidemiological studies in Mexico and considered for use in outbreaks of undiagnosed diseases in pigs in other Central and South American countries or in the United States. This assay will also be practical for monitoring the results of vaccination programs, which in the near future will be routinely practiced in intensive pig-production units in Mexico.

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3.3 Comparison of Tests for Serological Diagnosis of Blue Eye Disease

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SUMMARY

To evaluate the performance of serological assays used for diagnosis and in seroepidemiological surveys for blue eye disease (BED), the sensitivity, specificity, and agreement (κ statistic) was estimated for the immunoperoxidase (IPX), enzyme-linked immunosorbent assay (ELISA), and hemagglutination inhibition (HI) tests. Data for sensitivity and agreement estimates were obtained by testing 495 sera from five farms in a blue eye disease-infected area by using ELISA as the gold standard. The sensitivity of IPX relative to ELISA was 68.4%, the specificity was 96.5%, and the κ was 0.60; the sensitivity of HI test relative to ELISA was 76.0%, the specificity was 83.4%, and the κ was 0.58; HI relative to IPX had a sensitivity of 66.2%, a specificity of 92.7%, and a κ of 0.62. The specificity of the tests was 100%, based on results of testing 400 sera from farms in a BED-free area of the country. By means of a stratified (age and farm) serological survey, it was determined that the ELISA recognized more infected animals than did the other assays. It was concluded that ELISA, IPX, and HI could be used for herd diagnosis and to evaluate the pattern of infection within herds. However, for the evaluation of individual animals, a combination of the three tests should be used.

INTRODUCTION

The hemagglutination inhibition (HI) test has been widely used for diagnosis and in seroepidemiological studies of blue eye disease (BED) in Mexico because it is easy to perform and the results are reproducible (Martínez et al. 1986; McNeilly et al. 1997). However, alternate assays are becoming available. In pigs experimentally inoculated with BED virus, antibodies were detected by 8 days after inoculation by HI, virus neutralization (VN), indirect fluorescent antibody (IFA), and the indirect enzyme-linked immunosorbent assay (ELISA) tests (McNeilly et al. 1997; Pallares et al. 1995). Hernández-Jáuregui et al.

(1992) compared indirect ELISA with VN and HI and found that both VN and ELISA had a sensitivity of 89.1% compared with a sensitivity of 84% by HI. The authors concluded that the most sensitive tests were VN and ELISA followed by HI; however, HI was useful as a herd test because it was highly specific. Recently, a blocking ELISA based on a specific monoclonal antibody (Nordengrahn et al. 1999) and an immunoperoxidase (IPX) test have been developed (Gay 1989), and both are available as kits.

The aim of this study was to compare the HI test, ELISA, and IPX test in regard to diagnostic sensitivity, specificity, and observed proportion of agreement (κ statistic) in order to evaluate their utility as herd and individual animal tests.

SAMPLES AND ASSAYS

Four farrow-to-finish farms from a BED-free area (state of Sonora) and five farms from a BED-endemic area were selected. Within herds, sampling was stratified by age, and 10 pigs were sampled from each age group: 15 days and 1, 2, 3, 4, 5, and 6 months of age. In breeding herds, five gilts were sampled, as well as five sows with each parity for parities 1 to 5 (Morilla 1997). A total of 895 sera was collected, i.e., 400 from the four farms in the BED-free area and 495 from the five farms in the BED-endemic area.

The HI procedure described by McNeilly et al. (1997) was followed using guinea pig red blood cells. A positive response was considered $\geq 1:80$, and the antibody titer was the maximum dilution of the serum where hemagglutination inhibition occurred. A commercial IPX test was used following the procedures suggested by the manufacturer (Laboratorios Pronabive, Mexico City). The test was read with an inverted microscope. A commercial blocking ELISA kit was used following the protocol of the manufacturer (Svanova Biotech, Uppsala, Sweden). Samples with a percentage of inhibition $>50\%$ were

considered positive for antibodies to BED virus. The ELISA was used as a gold standard (Hernández-Jáuregui et al. 1992), and ELISA results were used to obtain the relative sensitivity and specificity of the IPX and HI tests. The observed proportion of agreement between tests or κ statistic was obtained according to the method described by Thrusfield (1995).

RESULTS AND DISCUSSION

All of the 400 sera from the pigs from BED-negative farms were negative by the HI, ELISA, and IPX tests, therefore the specificity of the tests was 100%. These results agreed with those obtained through the official serological surveys done by the Animal Health Authorities using HI and IPX tests in the BED-free area of the country, i.e., the states of Sonora and Yucatán.

The results of the comparison of the three tests using sera from farms with infected animals were as follows: of the 495 sera tested by ELISA, 288 (58.18%) were positive; of the 495 sera tested by the IPX test, 218 (44.04%) were positive; and of the 483 sera tested by the HI test, 268 (55.49%) were positive. Using the ELISA as the gold standard, the relative sensitivity of IPX was 68.4%, the specificity was 96.5%, and the κ was 0.60; the sensitivity of HI relative to ELISA was 76.0%, the specificity was 83.4% and the κ was 0.58; the HI relative to IPX had a sensitivity of 66.2%, a specificity of 92.7%, and a κ of 0.62.

The ELISA was used as the gold standard because it has a sensitivity similar to VN (Colmenares et al. 1995; Hernández-Jáuregui et al. 1992); IPX followed in sensitivity and specificity. Agreement (κ) with ELISA was classified as “substantial” (Thrusfield 1995).

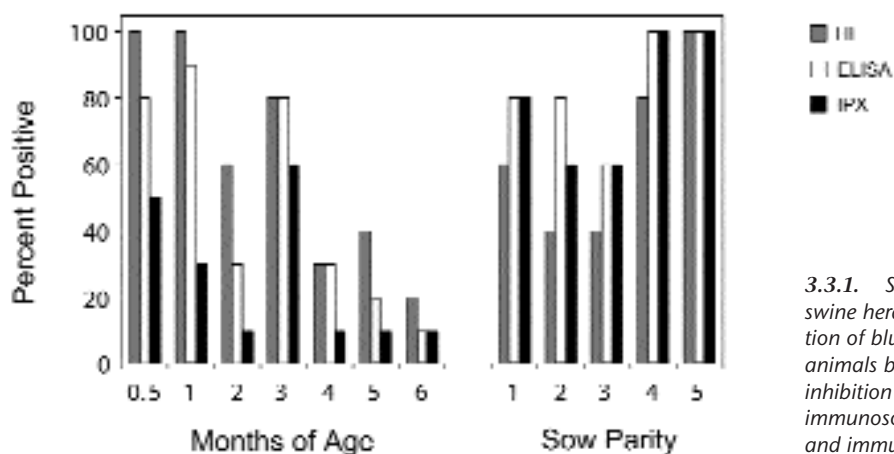
HI recognized anti-BED antibodies satisfactorily, but it had a certain degree of lack of sensitivity. The

level of agreement (κ) with ELISA was classified as “moderate” (Thrusfield 1995). All sera with high HI titers (1:320 to 1:2560) were also positive by ELISA and IPX, but less agreement was found when titers were 1:160 or lower. Antibody titers $\leq 1:40$, considered negative by HI, were positive by ELISA and the IPX test. The discrepancy among tests could be due to the presence of nonspecific substances in serum that interfere with the HI test (McNeilly et al. 1997), greater sensitivities of ELISA and IPX, or perhaps each assay recognizes antibodies against different viral antigens and these antibodies may appear and disappear during the course of an infection, as was demonstrated by Hernández et al. (1998).

The serological profile of the five farms by using the three tests was similar, although there was some discrepancy among the tests in the different age groups, as is exemplified in one farm in Figure 3.3.1. The overall infection pattern was that, in all farms, sows had antibodies regardless of parity. In four farms, weaners, growers, and finishers were also seropositive. In the fifth farm, maternal antibodies were found until month 3 of age, but finishers were seronegative, indicating that there was no active infection in that age group.

CONCLUSIONS

It was concluded that any of the three tests could be used diagnostically at the herd level and to evaluate virus circulation in the herd. For diagnosis of individual animals, however, the ELISA was best, though it would be advisable to use it in conjunction with the IPX test or the HI test if gilts and/or boars are going to be introduced in BED-free farms.



3.3.1. Serological profile of a swine herd comparing detection of blue eye disease-positive animals by hemagglutination inhibition (HI), enzyme-linked immunosorbent assay (ELISA), and immunoperoxidase (IPX).

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3.4 Seroepidemiology of Blue Eye Disease

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SUMMARY

Blue eye disease (BED) virus infection is common in swine herds in some areas of Mexico, where it causes reproductive and respiratory disease and mortality of piglets with neurological signs. Three serological studies were carried out to determine the infection pattern within farms, the serological association with other swine pathogens, and the distribution of the disease in the country. The serological profile of infected herds showed that sows act as virus reservoirs and weaners, growers, and/or finishers, depending on the farm management, as virus amplifiers. Swine that had antibodies against BED virus (BEDV) were also more likely to be seropositive to porcine reproductive and respiratory syndrome virus, Aujeszky's disease virus, swine influenza virus, *Leptospira interrogans* serovar *icterohaemorrhagiae*, and *L. interrogans* serovar *bratislava*, compared with swine seronegative to BEDV. These results suggested that when BEDV was present, other pathogens were also present, but probably due to the level of hygiene in BEDV-infected herds rather than synergism between the virus and other pathogens. A serological survey done during 1999–2000 showed that BEDV was present mainly in the highly pig-dense areas of the states of Jalisco, Michoacán, Guanajuato, Tlaxcala, Estado de México, Hidalgo, and Querétaro. It was concluded that BED was an endemic disease that affected herds mainly in the central part of the country.

INTRODUCTION

In Mexico in 1982, a new swine disease appeared that was characterized by encephalitis, pneumonia, reproductive disorders, orchitis, conjunctivitis, and corneal opacity. It was named blue eye disease (BED). Morbidity was variable and death occurred mainly among suckling pigs with neurological clinical signs. A hemagglutinating virus was isolated and classified in the family Paramyxoviridae, genus *Rubulavirus* (Stephano et al. 1988).

Since the original report, outbreaks of the disease have occurred mainly in the central area of the country, which is considered endemically infected, although seropositive animals have occasionally been found in other areas (Fuentes et al. 1992; Mercado et al. 1998). Serology has been used

for epidemiological studies, most frequently using the hemagglutination inhibition (HI) assay because it is easy to perform and has good sensitivity and specificity when compared with serum neutralization, enzyme-linked immunosorbent assay (ELISA), and immunoperoxidase (Colmenares et al. 1995; Hernández-Jáuregui et al. 1992; Martínez et al. 1986; McNeilly et al. 1997)

The aim of this project was to determine by HI test the pattern of BED virus (BEDV) infection in herds, the serological association of BEDV with other swine pathogens commonly found in sows, and the distribution of the disease in Mexico.

SEROLOGICAL PATTERN OF HERD INFECTION

Serological profiling of swine herds has been a useful method for determining the pattern of infection within herds and the effect of specific interventions on the circulation of the virus. Within herds, the profile is done by stratifying pigs by age and sampling 10 pigs from within each age group: 15 days and, 1, 2, 3, 4, 5, and 6 months of age. Within breeding herds, sows are stratified by parity and five animals sampled within each strata: gilts and parities 1, 2, 3, 4, and 5 (Martínez et al. 1986; Morilla 1997; Rosales et al. 1987). Following this procedure, animals from 300 herds within the BED-endemic area were bled and the sera tested by HI.

The results showed three general patterns: (1) Most commonly, sows were infected, maternal antibodies were present until 1 month of age, and pigs became infected after weaning, as shown by an increase in the seropositive pigs beginning at month 2 of age (Figure 3.4.1). (2) On some farms, nearly 100% of the pigs were seropositive. (3) In the third pattern, sows were infected, and maternal antibodies were found until month 3 of age, after which growers and fatteners remained seronegative, indicating that there was no active infection in those age groups. These serological patterns were similar to those seen with Aujeszky's disease virus (Morilla et al. 1995) and, more recently, with another recently identified paramyxovirus, Menangle virus (Love et al. 2000). The results suggested that sows are likely the reservoirs of the virus and that pigs during the growing period become the amplifiers of the virus.

Table 3.4.1. Summary of blue eye disease virus serological surveys done in Mexico

States	Positive/Tested	%	References
Aguascalientes	3/47	6.3	Correa et al. 1998
	0/24	0	(survey 1999-2000)
Baja California Norte	0/27	0	Carreón and Fuentes 1991
	0/14	0	Fuentes et al. 1992
	0/34	0	Correa et al. 1998
Baja California Sur	0/43	0	Correa et al. 1998
Campeche	95/107	88.7	Fuentes et al. 1992
	0/48	0	Correa et al. 1998
	0/31	0	Mercado et al. 1998
Coahuila	0/36	0	Carreón and Fuentes 1991
	0/53	0	Fuentes et al. 1992
	0/9	0	Correa et al. 1998
	0/298	0	Mercado et al. 1998
Colima	8/35	22.8	Fuentes et al. 1992
	0/48	0	Correa et al. 1998
	0/85	0	(survey 1999-2000)
Chiapas	0/20	0	Fuentes et al. 1992
	1/59	1.7	Correa et al. 1998
	0/190	0	Mercado et al. 1998
	12/878	1.4	(survey 1999-2000)
Chihuahua	0/42	0	Carreón and Fuentes 1991
	0/67	0	Fuentes et al. 1992
	0/82	0	Correa et al. 1998
Distrito Federal	0/8	0	Fuentes et al. 1992
	0/38	0	Mercado et al. 1998
	7/109	6.4	(survey 1999-2000)
Durango	0/32	0	Fuentes et al. 1992
	0/51	0	Correa et al. 1998
	0/32	0	Mercado et al. 1998
Guanajuato	23/48	47.9	Carreón and Fuentes 1991
	37/107	34.6	Fuentes et al. 1992
	2/46	4.3	Correa et al. 1998
	125/466	26.8	Mercado et al. 1998
	1915/6438	29.7	(survey 1999-2000)
Guerrero	45/78	57.7	Fuentes et al. 1992
	0/59	0	Correa et al. 1998
	0/1	0	Mercado et al. 1998
Hidalgo	0/42	0	Fuentes et al. 1992
	0/38	0	Correa et al. 1998
	0/31	0	Mercado et al. 1998
	34/184	18.5	(survey 1999-2000)
Jalisco	52/63	82.5	Carreón and Fuentes 1991
	302/1015	29.7	Fuentes et al. 1992
	0/133	0	Correa et al. 1998
	701/6705	10.4	Mercado et al. 1998
	1647/8375	19.7	(survey 1999-2000)
Estado de Mexico	26/128	20.3	Fuentes et al. 1992
	0/62	0	Correa et al. 1998
	159/1594	10.0	Mercado et al. 1998
	185/2427	7.6	(survey 1999-2000)
Michoacán	25/35	71.4	Carreón and Fuentes 1991
	28/66	42.4	Fuentes et al. 1992
	1/35	2.8	Correa et al. 1998
	21/57	36.8	Mercado et al. 1998
	128/756	16.9	(survey 1999-2000)
Morelos	3/34	8.8	Fuentes et al. 1992
	13/329	3.9	Mercado et al. 1998
Nayarit	0/69	0	Correa et al. 1998
	0/10	0	Mercado et al. 1998
	0/18	0	(survey 1999-2000)
Nuevo León	0/44	0	Fuentes et al. 1992
	0/75	0	Correa et al. 1998
	0/12	0	Mercado et al. 1998
	0/1401	0	(survey 1999-2000)

Table 3.4.1. (continued)

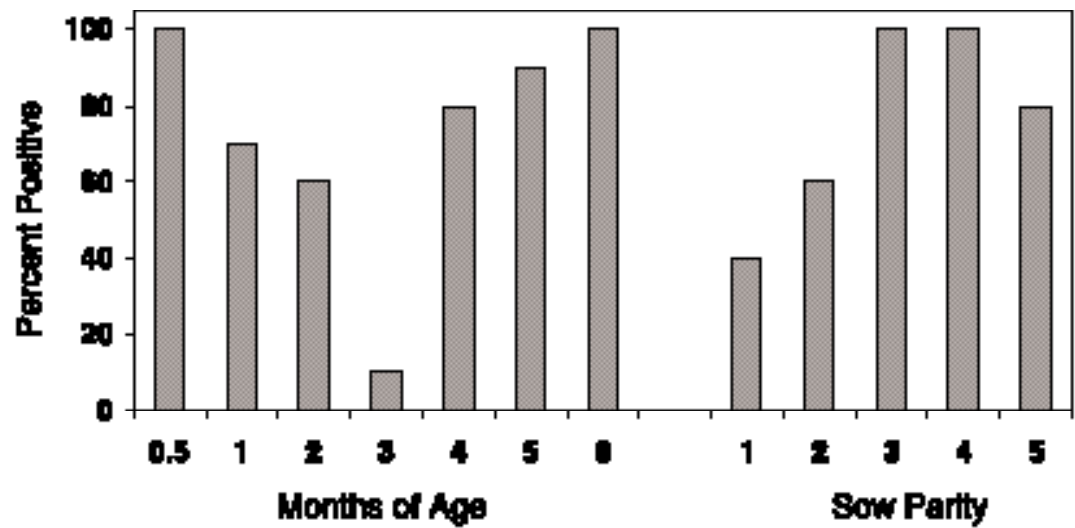
States	Positive/Tested	%	References
Oaxaca	1/29	3.4	Correa et al. 1998
	0/2	0	Mercado et al. 1998
Puebla	32/218	14.6	Fuentes et al. 1992
	0/124	0	Correa et al. 1998
	11/448	2.4	Mercado et al. 1998
	1/1189	0.1	(survey 1999-2000)
Querétaro	20/42	47.6	Carreón and Fuentes 1991
	131/526	24.9	Fuentes et al. 1992
	2/66	3.0	Correa et al. 1998
	31/557	5.6	Mercado et al. 1998
	440/3517	12.5	(survey 1999-2000)
Quintana Roo	21/35	60.0	Fuentes et al. 1992
	0/42	0	Correa et al. 1998
	0/33	0	Mercado et al. 1998
San Luis Potosí	0/28	0	Fuentes et al. 1992
	0/28	0	Correa et al. 1998
	0/56	0	Mercado et al. 1998
	0/192	0	(survey 1999-2000)
Sinaloa	0/59	0	Fuentes et al. 1992
	0/44	0	Correa et al. 1998
	0/5	0	Mercado et al. 1998
	0/336	0	(survey 1999-2000)
Sonora	0/69	0	Carreón and Fuentes 1991
	128/201	63.7	Fuentes et al. 1992
	0/201	0	Correa et al. 1998
	0/132	0	Mercado et al. 1998
	0/634	0	(survey 1999-2000)
Tabasco	17/48	35.4	Correa et al. 1998
	0/95	0	(survey 1999-2000)
Tamaulipas	0/37	0	Correa et al. 1998
	0/55	0	Mercado et al. 1998
Tlaxcala	13/50	26.0	Correa et al. 1998
	0/3	0	Mercado et al. 1998
	84/121	69.4	(survey 1999-2000)
Veracruz	0/67	0	Fuentes et al. 1992
	0/80	0	Correa et al. 1998
	0/115	0	Mercado et al. 1998
	?0/504	0	(survey 1999-2000)
Yucatán	0/29	0	Fuentes et al. 1992
	0/43	0	Correa et al. 1998
	0/404	0	Mercado et al. 1998
	0/236	0	(survey 1999-2000)
Zacatecas	0/30	0	Fuentes et al. 1992
	31/50	62.0	Correa et al. 1998
	0/30	0	Mercado et al. 1998
TOTAL (by reference)	120/362	33.1	Carreón and Fuentes 1991
	868/3011	28.8	Fuentes et al. 1992
	71/1780	4.0	Correa et al. 1998
	1061/11466	9.2	Mercado et al. 1998
	4453/27733	16.0	(survey 1999-2000)

In about 70% of the seropositive herds, no clinical signs were reported in pigs, indicating that the infection was subclinical. However, it is not uncommon to find animals with corneal opacity and orchitis that are seronegative for BEDV. These results support the observation made by Rosales et al. (1987) that animals on some seropositive farms did not show clinical signs, whereas other farms with pigs showing clinical signs had no seropositive ones. For this reason, HI should not be used

to determine the infection status of individual animals, although it has been very useful for profiling herds.

SEROLOGICAL ASSOCIATION BETWEEN BEDV AND OTHER SWINE PATHOGENS

It is unknown why BED outbreaks occur in only a specific region of the country. Clinical disease is unknown outside this area, even though infected animals have been



3.4.1. Serological profile (hemagglutination inhibition assay) of a blue eye disease virus-infected herd.

introduced into other swine-producing areas. To explore a possible association between BEDV and other pathogenic viral and bacterial microorganisms in swine, a model described by Diosdado et al. (1999) was followed. For this, a serological survey was done on 100 farrow-to-finish farms, sampling 10 sows from each farm and collecting a total of 1000 sera. Each serum sample was tested for antibodies against the most common swine pathogens:

1. Porcine reproductive and respiratory syndrome virus (PRRSV) by using a commercial ELISA (IDEXX Laboratories, Westbrook, MA, USA)
2. Swine influenza virus (SIV) by using an agar gel immunodiffusion (AGID) test with group A nucleocapsid antigen
3. Aujeszky's disease virus (ADV) by using a commercial ELISA (IDEXX Laboratories, Westbrook, MA)
4. Transmissible gastroenteritis virus (TGEV) of swine and porcine respiratory coronavirus (PRCV) by using a commercial differential ELISA (Svanova Biotech, Uppsala, Sweden)
5. BEDV by using an HI assay
6. Porcine parvovirus (PPV) by using an HI assay
7. *Leptospira interrogans* (12 serovars) by using a microagglutination test

The strength of the association between BEDV and other pathogens was evaluated by calculating the odds ratio (OR) and 95% confidence intervals (CI) (Thrusfield 1995). Significant associations were seen between BEDV and ADV (OR = 4.11, 95% CI = 2.64, 6.41); SIV (OR = 2.55, 95% CI = 1.02, 6.43); PRRSV (OR = 2.31, 95% CI = 1.45, 3.69); *L. icterohaemorrhage* (OR = 2.28,

95% CI = 1.16, 4.5); and *L. bratislava* (OR = 2.1, 95% CI = 1.11, 4.03). There was not a significant association between BEDV and PRCV, TGEV, or the 10 other serovars of *L. interrogans*. The association between BEDV and PPV could not be evaluated because all sows had antibodies against parvovirus.

The serological association between BEDV and ADV, SIV, PRRSV, *L. icterohaemorrhage*, and *L. bratislava* probably was not due to synergism among BEDV and other pathogens, but was a reflection of the sanitary status of the farms where BED was present. It was therefore concluded that BEDV infection was not exacerbated by the presence of other pathogens.

BEDV DISTRIBUTION IN MEXICO

BED outbreaks were first reported in the highly pig-dense area of the state of Michoacán in 1982 and subsequently spread from there. However, BEDV infection was evident before 1982 as 16% (58 of 356) of sera collected between 1972 and 1980 from swine in the state of México had antibodies against BEDV (Rosales et al. 1988). Serological surveys using the HI test during the 1980s and 1990s showed that seropositive animals were present in 16 of the 32 states (Table 3.4.1), but BED outbreaks were reported in only the central area of the country—mainly in the states of Michoacán, Guanajuato, and Jalisco. Occasionally, seropositive animals were found in other areas, but no clinical cases (Carreón and Fuentes 1991; Correa et al. 1998; Fuentes et al. 1992; Mercado et al. 1998). A serological survey based on convenience sampling during 1999–2000 in farrow-to-finish farms located in several states found a range of seroprevalence of 0.08% to 69%, with a mean of 26% (Table 3.4.1). The infection was concentrated in the pig-dense areas of the country, i.e., the



3.4.2. States where blue eye disease virus antibodies were found in swine in the 1999–2000 serological survey.

states of Michoacán, Guanajuato, Jalisco, Tlaxcala, and Hidalgo, where there is extensive trade in swine and outbreaks are known to occur (Figure 3.4.2). Occasionally, seropositive animals were found in other states, but there were no reports of clinical BED.

The wide distribution of seropositive animals (16 states) seen at the beginning of the 1990s was due to trade in swine. There was a reduction of pig movement as a result of controls imposed in the eradication area by the national Classical Swine Fever campaign. In addition, the states of Sonora and Yucatán did not allow the introduction of pigs from states within the classical swine fever eradication area. Official serological surveys in the states of Sonora and Yucatán have demonstrated that they are free of BEDV, but there are also other swine areas in the country that have remained free.

It may be concluded that BEDV is endemic in the central part of the country because of the density of swine herds in the region, and because farms have low-to-medium sanitary status, few biosecurity measures, and constantly introduce animals without evaluating their disease condition.

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3.5 Vaccination Against Blue Eye Disease

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SUMMARY

Although blue eye disease (BED) has only been diagnosed in Mexico, it is considered one of the five most important diseases of swine in the country because of its economic effects. Generally, infection causes respiratory and nervous signs in young pigs and reproductive problems in adults. Occasionally, infection produces a bluish corneal opacity. Up to now, no serological cross-reactions have been found between BED virus and other pig paramyxoviruses. Transmission occurs both horizontally and vertically and, experimentally, by the oral route. The disease is established in several states (Guanajuato, Jalisco, and Michoacán) and, periodically, outbreaks appear in other areas. For BED prevention and control, an experimental, inactivated (gamma irradiated), oil-adjuvanted vaccine has been developed. When tested under controlled conditions in BED-susceptible seronegative pigs, it proved to be safe and more antigenic than two commercial vaccines. An efficacy test was developed, and 100% of piglets from vaccinated sows survived although the challenge aerosol dosage killed all of the controls. The vaccine has been tested at several commercial farms and again proved to be safe and antigenic. Two acute outbreaks of BED were controlled by vaccinating all swine on the pig farms. Clinical cases and deaths ended at 6 and 8 days after vaccination, respectively.

INTRODUCTION

Blue eye disease (BED) is a viral infection originally identified in La Piedad Michoacán (LPM), Mexico. Under natural conditions, the pig is the only susceptible species, and infection produces respiratory and neurological signs in suckling and weanling pigs and reproductive failure in sows and boars. In some pigs, a white-to-bluish corneal opacity is present in one or both eyes; this is the clinical sign for which the disease was named (Campos 1981; Stephano et al. 1981).

The disease was first observed in Mexico in 1980 (Campos 1981; Campos et al. 1982; Stephano et al. 1981), and Mexico is the only country in which BED has been reported. Initially, a hemagglutinating virus was isolated from one of the outbreaks (Stephano et al. 1981). The BED virus (BEDV) was determined to be a

paramyxovirus and named LPM virus (Correa-Girón et al. 1986; Moreno-López et al. 1986) or porcine paramyxovirus (PPMV) (Berg et al. 1991). More recently, this virus has become known as porcine rubulavirus (PRuV) (Murphy 1996; Murphy et al. 1999; Rima et al. 1995).

PREVENTION AND CONTROL

Prevention and control of BED in infected herds are based on maintaining standards of sanitation and hygiene. In addition, the following types of vaccines have been used: (1) Formalinized vaccines prepared from the brains of pigs dying acutely from the disease. The effectiveness of these initial vaccines is unknown. (2) Inactivated, experimental vaccines prepared with virus cultivated in continuous cell lines. Under experimental conditions, one of these vaccines demonstrated good antigenicity in pigs of different ages (Zamora et al. 1990). Two other vaccines, according to the literature, have shown good results under field conditions against natural viral challenge (Hernandez-Jáuregui et al. 1990, 1994; Iglesias et al. 1994). There is also information about the efficacy of two additional vaccines in which gestating sows were vaccinated and their passive immunity protected 71% and 81.5% of their suckling piglets, respectively (Fuentes 1993; Stephano 1992).

EXPERIMENTAL OIL-ADJUVANTED, INACTIVATED, BED VACCINE

An experimental, inactivated (gamma irradiated), oil-adjuvanted vaccine was developed in 1990 through the Porcine Paramyxovirus Project of the Centro Nacional de Investigaciones Diciplinarias en Microbiología Veterinaria (CENID-MV), Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (SAGARPA).

ANTIGENICITY AND SAFETY OF VACCINE

The antigenicity of the vaccine was evaluated by intramuscular (IM) vaccination of a group of six PRuV serologically negative adult pigs. Serum samples were collected on days 0, 7, 13, 42, 115, and 144 after vaccination. The vaccine

stimulated average hemagglutination inhibition (HI) antibody titers of $10^{0.69}$ to $10^{1.9}$ and average serum-virus neutralization (SVN) antibody titers of $10^{0.30}$ to $10^{2.10}$. Both types of antibodies were detected on day 13 and persisted until the end of the experiment; another type of experimental vaccine was produced and studied simultaneously that produced lower antibody titers (Zamora et al. 1990).

The vaccine was also tested under controlled conditions in a group of seven 16-day-old piglets farrowed to a dam from a specific pathogen-free, BED-free farm. Prior to the experiment, the sow was tested and determined to be free of PRuV antibodies. Five piglets were vaccinated three times with 4 ml of the experimental BED vaccine on days 0, 10, and 21, and two were controls. In vaccinated animals, the levels of HI- and SVN-detectable antibodies were periodically evaluated during the 140 days of the experiment. The HI \log_{10} mean antibody titers were 0.84, 2.24, 1.77, and 1.41 on days 21, 63, 97, and 140 after vaccination; SVN titers were 1.20, 2.72, 2.38, and 2.58 on the same days. It was determined that the vaccine was effectively inactivated and vaccinated pigs remained healthy, with only a transitory inflammatory reaction observed at the injection site. At 160 days, the vaccinated pigs were killed, and slight macroscopic and microscopic lesions were found within muscle tissue at the injection site. A commercial vaccine against porcine parvovirus, used as a control, produced the same type of lesions (Martínez 1996; Martínez et al. 1995a).

Potency of Vaccine in Young Pigs

A procedure for testing vaccine potency was developed. For this purpose, it was necessary to determine the challenge dose of virulent PPMV-LPM that would be fatal for at least 80% of susceptible suckling piglets. Groups of five susceptible 4- to 8-day-old piglets were challenged with different dilutions of virus. Each group was exposed to virus in a chamber equipped with three nebulizers that dispersed the inoculum by producing particles of 0.5 to 5.0 microns in diameter. The aerosol for the first group was produced from 27 to 28 ml of a PPMV-LPM suspension with a virus titer of $10^{7.9}$ 50% cell culture infectious dose (CCID₅₀) per milliliter. \log_{10} dilutions of the initial viral concentration were used for the aerosol inoculation of subsequent groups of piglets. Thus, the second group received an aerosol generated from 27 to 28 ml of a virus suspension containing $10^{6.9}$ CCID₅₀/ml and the third group with 30 ml of $10^{4.35}$ CCID₅₀/ml. A fourth group (control) was inoculated with 30 ml of supernatant fluid from uninfected porcine kidney (PK-15) cell cultures. Each group of piglets was maintained for 30 to 42 minutes inside the aerosol chamber. The undiluted dosage produced clinical signs and death in 80% of the piglets, the first dilution killed 60%, and the lowest dosage also killed 60% of the group. The five non-challenged control piglets and all piglets from the

BED-vaccinated sows survived the challenge without showing either clinical signs or dying (Martínez 1996; Martínez et al. 1994, 1995c; Pallares et al. 1995, 1997).

Based on previous work, 7-day-old piglets were found to be the most likely to exhibit high morbidity and mortality after challenge (Galina et al. 1989, 1992). The more recent aerosol exposure study confirmed that suckling piglets at 4 to 7 days of age were highly susceptible.

In summary, the components of a PPMV-LPM challenge model capable of inducing fatal infection in 80% of susceptible suckling piglets were established, including the challenge dose, aerosol exposure time, piglet age, and number of piglets per treatment. The aerosol chamber used in these experiments has been described (Colmenares 1990), and all tests were performed in isolation units.

Efficacy and Potency of Vaccine in Breeding Animals

To evaluate vaccine potency in gestating sows under controlled conditions, sows were vaccinated in the last third of gestation, and assays were performed to (1) demonstrate that they were negative for anti-PRuV serum antibodies before vaccination, (2) measure their serum antibody response to vaccination, (3) demonstrate maternal antibodies in the dam's colostrum, (4) demonstrate transfer of passive antibody to the offsprings, (5) determine the duration of maternal antibody titers in piglets, and (6) evaluate the correlation between the presence of maternal antibody in piglets and the piglet's resistance to BEDV challenge.

To evaluate the protection of piglets born to vaccinated sows and protection conferred through passive immunity under experimental conditions, two gestating sows serologically negative for anti-BEDV antibodies were vaccinated IM (4 ml) at approximately 81 and 95 days of gestation. A third gestating sow received a placebo inoculum and served as an unvaccinated control. When their piglets were 4 to 7 days of age, they were challenged following the procedure previously described.

All sows and piglets were bled immediately prior to challenge and periodically after challenge in order to follow serum antibody titers in the vaccinated sows and their piglets, as well as to monitor the serological status of the unvaccinated control sow and her piglets.

At parturition, the colostrum from vaccinated sows had HI antibody titers of $10^{0.69}$ to $10^{1.9}$ and SVN antibody titers of $10^{0.3}$ to $10^{2.1}$. On the day of challenge, their piglets had HI antibody titers of $10^{0.69}$ to $10^{1.6}$ and SVN antibody titers of $10^{1.2}$ to $10^{2.1}$. Following challenge, all piglets from vaccinated sows survived and showed no clinical signs of BED. In contrast, the unvaccinated control sow and her piglets remained negative for detectable anti-PRuV serum antibody titers and, when challenged, all piglets presented 100% morbidity and mortality (Martínez 1996; Martínez et al. 1994, 1995b).

VACCINE VALIDATION IN FIELD CONDITIONS BEFORE BED OUTBREAKS

In the second stage of the research, the experimental oil-adjuvanted PRuV vaccine was tested under field conditions on several pig farms—one in the state of México (Zumpango) and five in the state of Guanajuato—that had experienced BED outbreaks. On these farms, safety was validated by clinical observation of the vaccinated pigs, antigenicity was evaluated by serological testing, and efficacy was assessed by comparing morbidity and mortality rates before and after vaccination.

The antigenicity of the experimental BED vaccine was evaluated in 36-day-old piglets during a field outbreak in Zumpango. Five piglets received one dose of 2 ml IM, and serum samples were collected for serological evaluation. On days 0, 14, and 28, the average HI titers (\log_{10}) obtained were 1.18, 2.86, and 2.98. When using a commercial vaccine (designated *Vaccine 1*) for comparison, the results were 1.17, 1.9, and 1.42 and, in the controls, 1.05, 2.02, and 1.04. On the other hand, when applying twice the experimental oil-adjuvanted vaccine, at 36 and 50 days of age, the HI antibody titers were higher: 1.53, 3.46, and 3.7 \log_{10} (Correa-Girón et al. 2000).

The PRuV vaccine has been found safe for gestating sows, weaned piglets, and growing pigs. However, when 2 ml of vaccine were administered IM to 12- to 14-day-old suckling piglets, a strong local inflammatory reaction was observed. For this reason, we studied the effect of reduced vaccine dosages (0.01, 0.1, and 1 ml) on the development of injection site reactions and antibody responses. Conventional 28-day-old suckling piglets were vaccinated during a BED outbreak that occurred in Uriangato, Guanajuato. One group had average maternal HI antibody titers of $10^{0.89}$ on day 0. After being vaccinated with 0.01 ml of the inactivated vaccine, these animals did not develop detectable HI antibody titers when compared with the control group. A second group, initially with HI antibody titers of $10^{0.77}$, received 0.1 ml and developed antibody titers (\log_{10}) of 0.17, 0.59, and 0.17 at 37, 76, and 101 days after vaccination, respectively. The third group, vaccinated with 1 ml, had no detectable HI antibody titers on day 0 and developed antibody titers (\log_{10}) of 1.37 and 1.52 at 37 and 76 days after vaccination, respectively. Controls had HI antibody titers of 0, 0, and 0.84 \log_{10} on the same respective days. The response on day 101 appeared to correspond to the field virus activity on the farm, i.e., the BED field outbreak. The 1-ml dosage has been used successfully in suckling piglets from two other farms without producing injection site inflammatory reactions. Instead of the gluteal muscular region, it is currently recommended that the vaccine be administered in the neck muscles.

A similar experiment was performed using groups of sows during a BED outbreak in Zumpango. On vaccination day 0, four groups had average HI antibody titers of

1.4 to 1.8 \log_{10} . Following administration of 2 ml of the experimental oil-adjuvanted vaccine to one group, antibody titers increased to 2.26 to 2.27 \log_{10} on postvaccination days 43 and 78. Using 1 ml of vaccine, antibody titers of 2.32 to 2.50 \log_{10} were obtained. And, when using 0.25 ml, antibody titers were 1.98 \log_{10} on day 43. In the controls, antibody titers did not increase and actually declined from 1.6 \log_{10} on day 0 to 1.3 \log_{10} on day 43. Since this trial, 1.0 ml of vaccine has been used routinely.

Based on the serological evaluation of the immune response in sows during a BED outbreak in Zumpango, the oil-adjuvanted vaccine has shown excellent antigenicity when administered at the rate of 2 ml IM in each leg, with the vaccination repeated 14 days later. In a group of sows with an average HI antibody titer of 1.48 \log_{10} on the day of vaccination, mean antibody titers were 2.65, 2.95, 2.55, 3.04, and 2.2 \log_{10} on days 14, 28, 63, 92, and 127 after vaccination, respectively. Commercial Vaccine 1 was used concurrently for comparison by administering 2 ml IM, as per the manufacturer's instructions. Following administration of Vaccine 1 in sows with average HI antibody titers of 1.2 \log_{10} , mean antibody titers of 1.48, 2.08, 0.918, 1.5, and 1.8 \log_{10} were observed on days 14, 28, 63, 92, and 127 after vaccination, respectively. Mean HI antibody titers in sows with virulent field PRuV infection were 1.9, 1.48, and 1.96, on days 0, 14, and 28, respectively (Martínez et al. 2000). Thus, high doses of the experimental BED vaccine produced a serum antibody response that was much higher than antibody titers stimulated by commercial Vaccine 1 or by field virus.

Taking into account this experience, another experiment was performed with three groups of 12 to 13 sows on an infected farm from Valle de Santiago, Guanajuato. This time, the first group was vaccinated IM with 1 ml of the experimental BED vaccine on day 0, when they had mean HI antibody titers of 1.18 \log_{10} , with this dosage repeated 10 days later. Mean antibody titers rose to 1.92 \log_{10} on days 71 and 113 after vaccination. The dams vaccinated with commercial Vaccine 2, following the same vaccination schedule, had mean HI antibody titers of 1.57 \log_{10} on day 0 that rose to 1.63 and 1.65 \log_{10} on days 71 and 113, respectively. In contrast, the controls had values of 1.42, 1.54, and 1.43 \log_{10} on days 0, 71, and 113, respectively. This demonstrated that 1 ml of the experimental BED vaccine was more immunogenic than commercial Vaccine 2.

CONTROL OF BED OUTBREAKS BY VACCINATION

The efficaciousness of the experimental BED vaccine for controlling acute clinical outbreaks of virulent PRuV was studied in a small (350 head), "backyard" herd in Uriangato, Guanajuato. Clinically, the disease caused central nervous system involvement, corneal opacity, and the death of 100 suckling piglets in the previous month and

scrotal inflammation in one of the three boars. PRuV was isolated in the laboratory to confirm the diagnosis, and all pigs in the herd were vaccinated. Six days after vaccination, the next sow farrowed. No signs of BED were observed in this litter, and no further clinical cases appeared during the next 12 months. For the first month after the whole-herd vaccination, newborn piglets received 1 ml of vaccine during the first days of life. Then the vaccination schedule was changed, and piglets were vaccinated at 1 month of age. Although no vaccine has been used during the last 12 months, no clinical cases have been observed. After the outbreak was controlled, newborns had a much better appearance. On vaccination day 0, the sows of this farm had an average HI antibody titer of $1.69 \log_{10}$, which rose to 2.39, 2.20, 2.23, 1.84, and 2.20, on days 37, 76, 101, 140, and 190, respectively.

In another backyard farm, also from Uriangato and close to the aforementioned farm, the acute clinical outbreak resolved 8 days after vaccination of all swine following approximately the same vaccination regime.

BOAR VACCINATION

Safety studies in boars are still in progress, and vaccination of boars is not recommended until the research is complete. At this time, caution is advised because scrotal region inflammation has been observed in some animals a few days after administration of vaccine. According to the producers' observations, however, no alterations in the boars' reproductive and productive parameters have been observed. This remains to be corroborated scientifically.

CONCLUSIONS

Based on these results, the experimental BED vaccine can be considered to be highly safe and immunogenic. When administered at high dosage (2 ml in each gluteal region, twice), the experimental vaccine produced higher HI antibody titers than the response produced by either of the two commercial vaccines or field virus infection.

In addition, we successfully developed a protocol for testing PRuV vaccine potency. After the procedure has been corroborated, it would be advisable to use it for evaluating the potency of other inactivated PRuV vaccines, particularly those recommended for use in gestating sows for the purpose of providing passive protection to suckling piglets via the colostrum. Using this protocol, the inactivated, experimental, oil-adjuvanted PRuV vaccine was shown to provide excellent protection.

In conclusion, these experiments are providing important assistance to pork producers in the prevention and control of BED in the affected areas of Mexico.

ACKNOWLEDGMENTS

This work was supported in part by the Consejo Nacional de Ciencia y Tecnología (CONACYT: Project K0022 B) and the Fundación Guanajuato Produce, A.C. (Project 17-00).

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3.6

The Molecular Biology of Porcine Rubulavirus (La Piedad Michoacán Virus, LPMV)

Mikael Berg and Jorge Moreno-López

SUMMARY

La Piedad Michoacán virus (LPMV) is the causative agent of “el síndrome del ojo azul” or blue eye disease of pigs, which was first reported in 1981. Since then, the virus has been isolated, its genome sequenced, some of the viral proteins studied in detail, and some aspects of its replication cycle studied. In addition, diagnostic tools have been developed. Although LPMV causes a severe disease in its natural host, there is still much to learn about LPMV. This chapter summarizes what is known of the molecular aspects of the virus.

INTRODUCTION

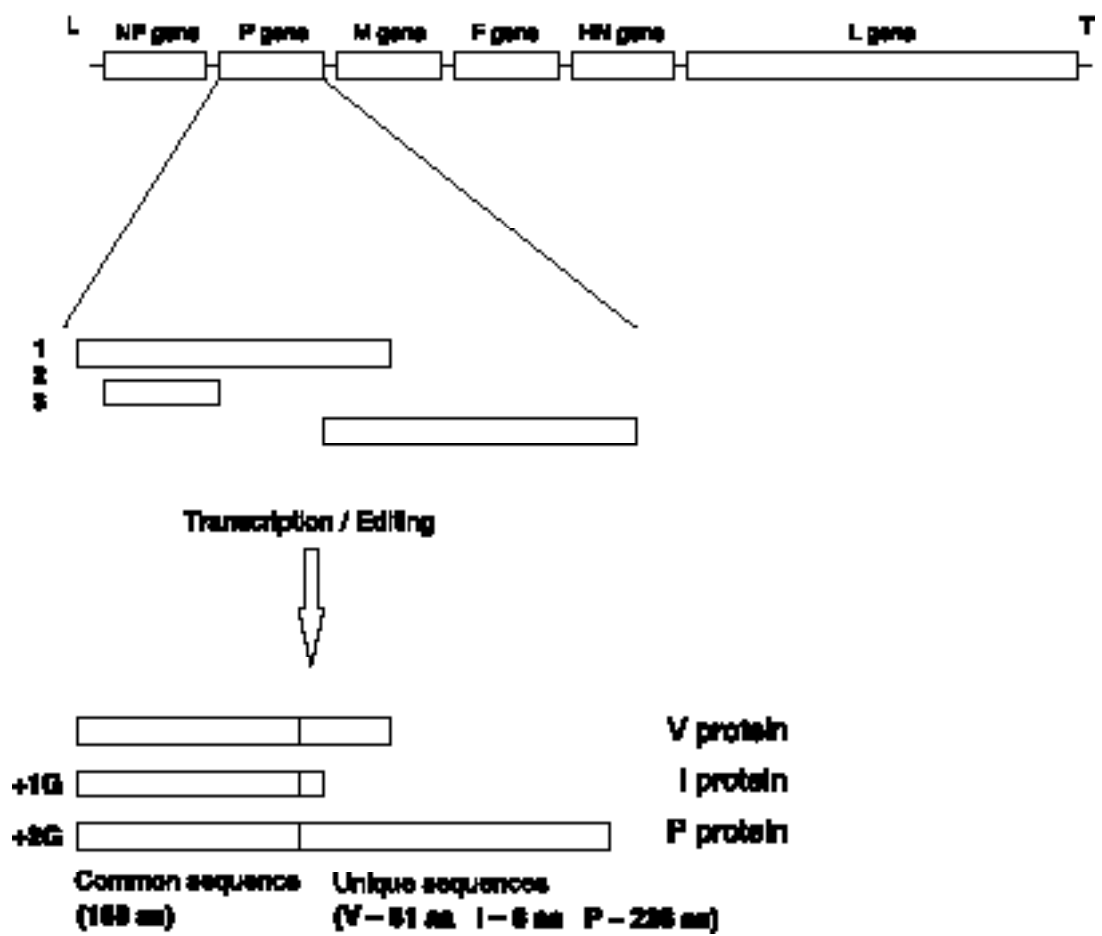
Once LPMV was isolated, the first goal was to establish its genetic relationship with other paramyxoviruses. That it was a paramyxovirus, or a related virus, was established early in the research on the basis of electron microscopy and protein-profiling comparisons (Moreno-López et al. 1986; Sundqvist et al. 1990). However, not until the first gene was molecularly cloned and its sequence determined could the true relationship of LPMV to other viruses established. Berg et al. (1991) described the cloning of the matrix (M) protein gene and comparative sequence analysis with other paramyxoviruses. On the basis of the M protein gene, mumps virus and simian virus 5 were determined to be LPMV's closest relatives. The identity on the amino acid level was 46% and 36%, respectively. Shortly afterward, the hemagglutinin-neuraminidase (HN) gene (Sundqvist et al. 1992) and the phosphoprotein (P) gene (Berg et al. 1992) were cloned and sequenced. Comparative sequence analysis of these genes confirmed the close relationship of LPMV to mumps virus and simian virus 5. Amino acid homology of the HN gene was 41% for mumps virus and 43% for simian virus 5. In addition, other paramyxoviruses were compared: parainfluenza type 2 (38%), parainfluenza type 4 (35%), Newcastle disease virus (32%), Sendai virus (26%), parainfluenza type 3 (28%), and measles virus (16%). Comparing the P gene is somewhat more complicated because it encodes several protein products, the P protein and the V protein, which

share their N-terminal 168 amino acids (Berg et al. 1992). The analysis of the P-unique segment revealed the following identity rates: 43% (mumps virus), 35% (simian virus 5), 38% (parainfluenza type 2), and 31% (parainfluenza type 4). On the other hand, the V-unique segment was more conserved (60%, 56%, 49%, and 51%, respectively) relative to the aforementioned viruses. In addition, the general organization of the P gene and editing pattern was similar to these four viruses, but differed significantly from the other viruses (Sendai, parainfluenza type 3, measles). The editing and functioning of V and P was studied more in detail, as discussed below. Later, more detailed phylogenetic studies of the LPMV fusion (F) protein gene (Berg et al. 1997), large (L) protein gene (Svenda et al. 1997), and nucleocapsid protein (NP) (Svenda 1998) confirmed these earlier papers. This classified LPMV as a rubulavirus (Rima et al. 1995). Interestingly, as discussed by Svenda (1998), it was found that one of the closest relatives of LPMV is Mapuera virus, a paramyxovirus recovered from bats (Zeller et al. 1989).

GENETIC ORGANIZATION OF LPMV

Except for the extreme 5' or 3' ends, the complete nucleotide sequence of the viral genome has been determined. The genome organization is similar or identical to other viruses within the genus *Rubulavirus* (Figure 3.6.1). It is organized as a 3' leader sequence, NP gene, P gene, M gene, F gene, HN gene, L gene, and finally a 5' trailer sequence (Figure 3.6.1). The complete genome is 15,193 nucleotides long. The figure is based on a leader and trailer sequence of 40 nucleotides each, although we do not know their exact length and sequence. The intergenic sequences, described in an earlier article (Linné et al. 1992), vary markedly both in length and sequence. Noteworthy is that no open reading frame (ORF) for a small hydrophobic (SH) protein was found.

It appears, as far as we have been able to determine, that all of the viral proteins of LPMV have functions identical to those of corresponding proteins of more extensively studied paramyxoviruses. A summary of the known functions of the gene products of LPMV follows.



3.6.1. The genomic organization of La Piedad Michoacán virus. F, fusion; HN, hemagglutinin-neuraminidase; I, I protein; L, large; M, matrix; P, phosphoprotein; NP, nucleocapsid protein; V, V protein.

Nucleocapsid (NP) Gene and Protein

The complete NP gene spans 1783 nucleotides with 88 nucleotides at 5' terminus and 60 nucleotides at the 3' terminus of the translated region. The ORF encodes a protein of 545 amino acids. A comparison of this protein to NP proteins of other paramyxoviruses revealed conserved regions interrupted with less conserved regions (Svenda 1998; Svenda et al. 2002). There are several lines of evidence that NP has many functions in the paramyxovirus replication cycle. First of all, NP builds the core structure of the nucleocapsid, together with viral RNA, P protein, and L protein (Svenda 1998). It appears that the V protein may also be a component of the nucleocapsid. We have, for example, demonstrated that NP and V proteins interact in glutathione-S-transferase (GST)-pulldown experiments (Svenda et al. 2001). This, however, does not necessarily mean that the V protein is a part of the nucleocapsid. The NP has other soluble forms that take part in replication and transcription of the viral genome.

When expressed alone, NP forms granular structures in the cytoplasm. This is similar to what has been observed in infected cells (Svenda 1998). The nature of the structure and the specific proteins composing the latter NP complex have not been determined.

In summary, the LPMV NP protein is a protein of 545 amino acids forming the viral nucleocapsid. In addition, it interacts with other viral proteins, although the significance of the interaction is not yet clear. Its interaction with the P protein has been shown for other viruses, as well, and is probably due to their common function as components of the viral RNA machinery. The significance of its interaction with V protein is not clear.

Phosphoprotein (P) Gene and Proteins

The complete P gene is 1373 nucleotides long (Berg et al. 1992). It is unique among the genes in its capacity to encode several proteins. In the case of LPMV, the gene can encode the P protein, the V protein, the I protein, and the C protein (Berg et al. 1992). The possibility of encoding

a fourth protein (C) in another ORF makes LPMV unique among the rubulaviruses.

P, V, and I proteins share 168 amino acids at the N terminus, the rest of the proteins being unique. The P protein has 236 C-terminal-unique amino acids, the V protein has 81, and the I protein has only 6. This ability to encode three different proteins with the same N terminus but different C terminus is due to RNA editing; that is, during transcription, extra G bases are added at a certain site in the transcript. This site is similar to gene ends/polyadenylation sites. In positive-sense RNA, the editing site of LPMV is UUUAAGAGGGGGG. When transcripts are unedited, the V protein is translated. The addition of one extra G (UUUAAGAGGGGGG) leads to translation of I protein, and the addition of two extra Gs (UUUAAGAGGGGGGG) leads to translation of P protein. The putative C protein could be translated from all transcripts if the second AUG is used as initiator codon instead of the first one.

The function of the different proteins is not known, but, if analogous to other paramyxoviruses, it is likely that the P protein is a factor for the polymerase L protein, and V protein may be an inhibitory factor for replication. No function has yet been proposed for the I protein. The P protein can interact with both the NP and the L protein (Homann et al. 1991; Horikami et al. 1992; Parks 1994). In Sendai virus, one of the functions of the P protein is to act as a chaperone to keep the NP in the cytoplasm as monomers and, later, as encapsidation substrate (NP-P) (Curran et al. 1995). In the rubulavirus simian virus 5, the V protein has been suggested to play a similar role in sequestering the NP in the cytoplasm (Precious et al. 1995). Some reports indicate that Sendai virus V protein can function as a negative regulator of RNA genome synthesis (Curran et al. 1991) or as a factor involved in pathogenesis (Delenda et al. 1997; Kato et al. 1997; Schneider et al. 1997). It has been shown that V protein can also interact with NP in case of simian virus 5, parainfluenza type 2, or Sendai virus (Horikami et al. 1996; Precious et al. 1995; Watanabe et al. 1996). The V protein appears to be dispensable, both in cell culture and in vivo. For example, parainfluenza type 1 does not have an ORF for V and still replicates efficiently (Mat-suoka et al. 1991).

Recently, several of the protein products from the P gene have been implicated in the viral defense against the action of type I interferon (IFN) (García-Sastre 2001). The Sendai virus C protein appears to target the signal transducers and activators of transcription (STATs) and Tyk2 molecules, thereby disrupting the IFN signaling (Garcin et al. 1999; Gotoh et al. 1999). Similarly, the V protein of simian virus 5 and parainfluenza type 2 targets the STAT1 and STAT2, respectively, disrupting the IFN signaling (Didcock et al. 1999; Young et al. 2000).

Thus, the proteins from the P gene appear to be multifunctional, being involved both in viral RNA synthesis

and in the viral defense against IFN. Concerning the function of the corresponding proteins of LPMV, it is known that the P and NP proteins interact with each other, and the V and NP proteins interact with each other (Svenda et al. 2001). It seems that this interaction is mediated via their unique C terminuses (Svenda et al. 2001). Also, the L protein appears to interact with these proteins, forming a larger protein complex (Svenda et al. 2001).

In virions, the P protein is highly phosphorylated. When analyzed by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis technique, two forms of the P protein are present. It appears that these are differentially phosphorylated (Sundqvist et al. 1990). The relevance of this observation is not clear to us. An explanation may be that the two forms interact with different proteins in the viral capsid.

It is still unknown whether the C protein is expressed. There are some indications that two versions of the C protein are expressed in infected cells (M.B. unpublished observations). However, immunofluorescence and immunoprecipitation have failed to demonstrate conclusively that the C protein is expressed.

The ratio between the P, V, and I proteins is controlled by the RNA editing. The editing ratio has been determined in two ways: First, by amplification of the transcripts over the editing site, cloning, and then sequencing a fair number of clones and determining the ratio. Second, the editing ratio was studied by a primer elongation method (Berg et al. 1992) and quantification of the products. During normal lytic infection of porcine cells, the ratio between P, V, and I is 33%, 51%, and 5%, respectively. Other versions were also evident, which should give proteins with an extra amino acid. This transcript ratio was also seen when infected organs were tested (Hjertner et al. 1998). During persistent LPMV infection, though, the ratio is different. There is much more V than P, 80% compared with 14% (Hjertner et al. 1998). This may have interesting implications, since it has been suggested that the V protein has a negative effect on viral genome replication, as well as disrupts IFN signaling (Curran et al. 1991; García-Sastre 2001). This observation is discussed at greater length in the section on LPMV persistent infection.

In summary, some functions of the P gene products are known from observations from the study of other paramyxoviruses, but additional functions remain to be elucidated. Concerning the viral defense against the type I IFN system, LPMV appears to have two proteins dedicated to this problem: the V and the C. This makes LPMV unique among the rubulaviruses. The relevance of this observation is at present purely speculative.

Matrix (M) Gene and Protein

This was the first LPMV gene to be cloned and sequenced (Berg et al. 1991). The gene itself is 1376

nucleotides long, with relatively long untranslated sequences. The ORF is 1107 nucleotides long, encoding a protein of 369 amino acids. It is somewhat unusual in that its initiator codon is the second of the gene. Not much is known about the protein. If analogous to other paramyxoviruses, the M gene product is assumed to be a matrix protein lining the inner section of the viral membrane. The M gene may have other functions, but except for the sequence, there are no reports of studies concerning the LPMV M protein.

Fusion (F) Gene and Protein

The F gene of LPMV is 1845 nucleotides long and encodes a protein of 541 amino acids. The fusion protein is one of the viral surface glycoproteins. The F protein needs to be cleaved in two subunits to be active. From the deduced nucleotide sequence of LPMV F gene and the protein sizes of the virion, we assume that the F protein of LPMV is cleaved into F1 and F2 at the cleavage site common to paramyxovirus F proteins. In the case of LPMV, the site is His-Arg-Lys-Lys-Arg (Berg et al. 1997). This sequence should render the protein easily cleaved by a ubiquitously expressed proteinase, such as furin. The protein is glycosylated, based on labeling experiments (Sundqvist et al. 1990). According to the deduced amino acid sequence, there are four putative glycosylation sites in F1 and one in F2.

Hemagglutinin-Neuraminidase (HN) Gene and Protein

The other surface glycoprotein is the HN protein. This gene and the protein is one of the most extensively studied of LPMV, probably due to its potential role in vaccine development. In LPMV, the HN gene is 1906 nucleotides long and encodes a protein of 576 amino acids (Sundqvist et al. 1992). As its name suggests, it has two activities: binding to sialic acid residues on cellular receptors (hemagglutination) and cleaving sialic acid (neuraminidase) from the same, or similar, receptors. Hemagglutinating activity was identified shortly after the virus was identified (Moreno-López et al. 1986; Stephano et al. 1988). The HN protein binds specifically to NeuAc α 2,3 lactose residues on susceptible cells (Reyes-Leyva et al. 1997, 1999). The same researchers purified the protein and determined that the glycanic portion consisted of mannose, galactose, GlcNAc, GalNAc, and Neu5Ac in 3:3:4:1:1 molar ratios (Reyes-Leyva et al. 1999). Earlier labeling experiments demonstrated that the HN protein was glycosylated (Sundqvist et al. 1990). Based on its amino acid sequence, there are four potential glycosylation sites (Sundqvist et al. 1992).

Zenteno-Cuevas et al. (1998) studied the secondary structure of the hemagglutination protein by using computer-aided analysis. As postulated by Sundqvist et al. (1992), the protein was determined to consist of a short signal peptide, an intracellular domain, a transmem-

brane domain, and an extracellular portion possessing hemagglutinating and neuraminidase activity. The secondary structure of the extracellular segment was predicted to be organized in a β -loop- β alternating with a few α helices (Zenteno-Cuevas et al. 1998). The regions where the enzymatic activities were predicted to reside are well conserved among rubulaviruses and paramyxoviruses. The relationship of these predictions to the actual structure of the protein remains to be determined.

Large (L) Gene and Protein

The large protein is, as the name suggests, the largest protein expressed from the genome. The gene constitutes about 45% of the entire genome and is postulated to carry all polymerase functions, i.e., initiation, elongation, methylation, editing, polyadenylation, etc. The complete L gene of LPMV is 6786 nucleotides long, encoding a protein of 2251 amino acids (Svenda et al. 1997). By aligning various L proteins, several highly conserved regions have been identified (Svenda et al. 1997). The L protein of paramyxoviruses has been very difficult to study, and the L protein of LPMV is no exception. We know from various GST-pulldown experiments that it interacts directly with P, or NP, or possibly with both (Svenda et al. 2001). Based on our unpublished observations and the comprehensive sequence alignment found by Svenda et al. (1997), there is no reason to believe that the L protein of LPMV differs in any aspect from L proteins of other paramyxoviruses.

REPLICATION AND EDITING OF THE VIRAL GENOME

Not much is known about viral transcription and replication, and only one serious study has been published in this area. Hjertner et al. (1998) studied certain aspects of the viral replication cycle by comparing lytic infection versus two passages of persistently infected cells. A comparison of these two types of infection suggested that the so-called transcription gradient was different in the L gene such that persistently infected cells had lower levels of L protein and released lower levels of virus. A decrease was also seen in the amount of the P protein, another protein of the polymerase complex. As mentioned earlier, one particularly interesting finding from this study was that the editing ratio, that is, the ratio between P, V, and I transcripts, was quite different when comparing lytic infection to persistent infection (Hjertner et al. 1998). One can only speculate as to the significance of these observations, but some viral product that normally stabilizes the long L transcript might be missing or not as abundant in persistently infected cells. In addition, production of defective interfering particles seems to be a common feature of persistent infections *in vitro*. The relevance of this for the state of viral persistence is purely speculative (Hjertner et al. 1998).

PERSISTENCE IN VITRO AND IN VIVO

Paramyxoviruses are well known for their ability to establish persistent infection in cell cultures. As mentioned earlier, Hjertner et al. (1997) established a porcine kidney cell line persistently infected with LPMV. This cell line has an expression bias toward more V than P transcripts, 80%/14% compared with 51%/33%. This may be relevant to the establishment and maintenance of the persistent state, since it has recently been shown that the V protein disrupts IFN signaling, rendering IFN less potent. More V protein might be needed to circumvent the action of the IFN system. This hypothesis remains to be proven, but seems to hold potential.

Whether the virus can persist in vivo is of great interest. Wiman et al. (1998) investigated this possibility. Pigs were infected, and 53 days after infection the pigs were killed and tested for the presence of virus. No virus was detected by virus isolation, but viral genomic RNA and mRNA were detected in the midbrain of convalescent pigs by using reverse transcriptase-nested polymerase chain reaction. The editing ratio was also assessed, but it was not possible to determine whether there was a different editing ratio when compared with normal lytic infection (Hjertner et al. 1998; Wiman et al. 1998). The discovery that LPMV can persist in recovered pigs may be important to the epidemiology and pathology of the disease.

STRAIN VARIATION

There have been several strains of porcine rubulavirus isolated since its initial recognition. The names of these strains are usually originated in the village from where they were isolated, i.e., Los Chopos and San Fandila. Apart from LPMV 1984, the only other strain that has been partially sequenced is San Fandila 1988. The complete F gene of San Fandila was sequenced and compared with that of LPMV 1984 (Berg et al. 1997). There were 16 nucleotide differences, but only four leading to amino acid substitution. The variation was 0.8%. This suggests, according to this very limited study, that the various isolates are relatively conserved. There are no conclusive studies demonstrating differences in pathogenicity between strains.

CONCLUSIONS

This chapter has summarized much of the current molecular information on LPMV. Certainly, much remains to be studied. Possibly, we will find that LPMV resembles closely related viruses, i.e., PIV2, PIV4, and MuV. However, LPMV may be unique in some aspects. We already know, for example, that LPMV has an additional ORF in the P gene called "C" and that LPMV does not have a so-called SH protein gene, as do some of the related viruses. In addition, it will be important and interesting to compare LPMV with

other newly emerged paramyxoviruses: Mapuera (Zeller et al. 1989), Nipah, Menangle (Philbey et al. 1998), Hendra, and Tioman viruses (Chua et al. 2001).

ADDENDUM

GenBank accession numbers: NP (no number as yet), P: AF416650, M: AJ278914, F: Y10803, HN: S77541, and L: X98125.

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3.7 Porcine Rubulavirus (LPMV) Infection in PK-15 Cell Line

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SUMMARY

The morphogenesis of La Piedad Michoacán virus (LPMV) in infected porcine kidney (PK-15) cells was investigated using immunoassays and electron microscopy. LPMV infection in PK-15 cells caused cytopathic effects characterized by cytoplasmic vacuolation, formation of syncytia, and cytoplasmic inclusion bodies. At early stages of infection (5 to 60 minutes after inoculation), hemagglutinin-neuraminidase protein of LPMV was detected by immunofluorescence microscopy on cytoplasmic membranes and areas adjacent to the nuclear membrane. At later stages of infection (24 hours after inoculation), hemagglutinin-neuraminidase proteins were detected throughout the cytoplasm. In comparison, nucleoprotein of LPMV was mainly detected scattered in the cytoplasm of infected PK-15 cells during the early stages of infection and later became aggregated, forming inclusion bodies predominantly in the cytoplasm of syncytia. The same pattern of morphogenesis was also observed by immunohistochemical labeling and immunogold-labeling electron microscopy. Virus particles and nucleocapsids isolated from infected cells are morphologically similar to members of the Paramyxoviridae family.

INTRODUCTION

La Piedad Michoacán virus (LPMV) porcine rubulavirus is the causative agent of an endemic disease of pigs in Mexico that is characterized by pneumonia, encephalitis, conjunctivitis, and corneal opacity (Murphy et al. 1999). Piglets are most susceptible to the infection, but older animals can also be affected. In naturally or experimentally infected males, epididymitis and orchitis may develop. In affected pregnant sows, abortions and an increase in stillbirths and mummified fetuses are common clinical manifestations of the infection (Ramírez-Mendoza et al. 1997; Stephano et al. 1988).

The LPMV was initially isolated from the brain of a piglet with neurological disorders (Moreno-López et al. 1986). Since then, LPMV has been studied in detail at the molecular level (Berg et al. 1991, 1992, 1997; Hjertner et al. 1997; Linné et al. 1992; Sundqvist et al. 1990, 1992; Svenda et al. 1997, 1998; Wiman et al. 1998). However,

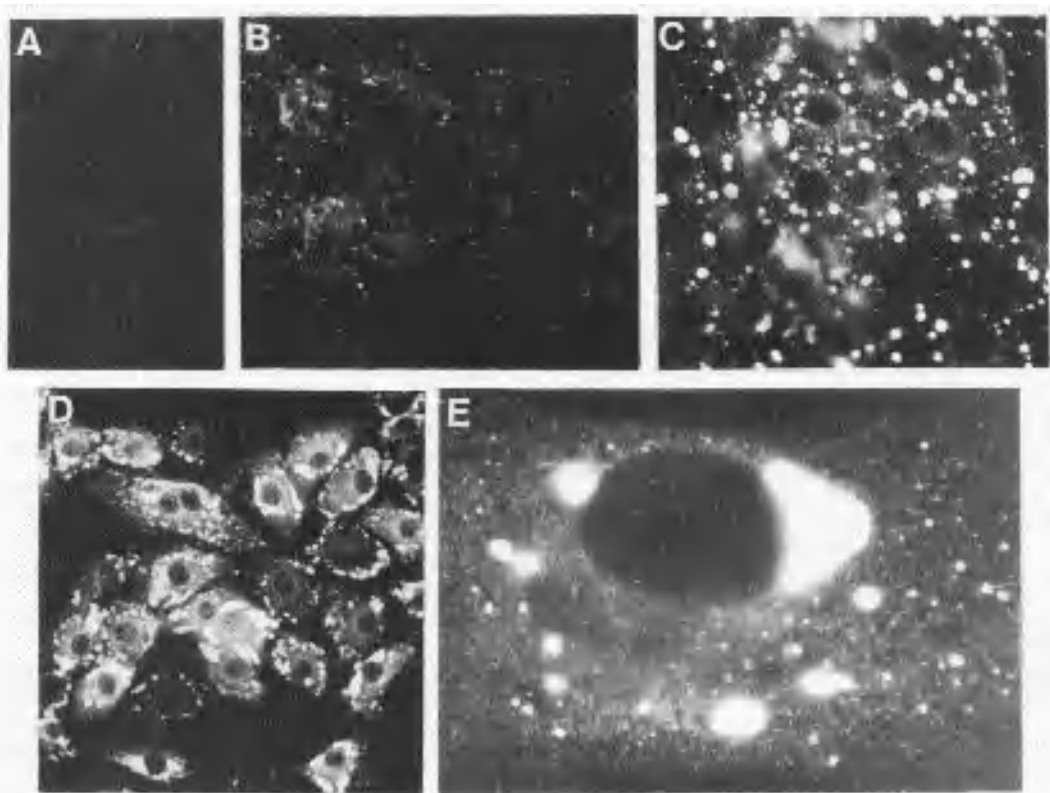
many questions regarding the pathogenesis of LPMV infection still remain unanswered.

To improve our understanding of viral pathogenesis, the morphogenesis of LPMV in PK-15 cells over time post inoculation (PI) was characterized using light microscopy, immunofluorescence, immunohistochemistry, electron microscopy (EM), and ultrastructural immunogold labeling. Specifically, transport of hemagglutinin-neuraminidase (HN) surface glycoprotein, and nucleoprotein (NP) within infected cells were assessed using monoclonal antibodies specific for each protein to identify sites for early and late internalization of LPMV. Morphological characteristics of virions were also examined

LPMV REPLICATION IN PK-15 CELLS

At 5 minutes PI, the HN protein, a surface glycoprotein of LPMV, was detected by immunofluorescence microscopy at the external surface of the cytoplasmic membrane, whereas the NP was detectable only in the cytoplasm of infected cells. At 60 minutes PI, the HN protein started to appear within the cytoplasm and adjacent to the nuclear membrane. Later, intense fluorescence for the HN protein was observed throughout the entire cytoplasm and on the cell surface. Similar observations were also made using immunogold-labeling EM. Dense aggregates of gold particles were apparent along the cytoplasmic membrane of PK-15 cells at 5 minutes PI. At 60 minutes PI, gold particles were also apparent throughout the cytoplasm.

In comparison, the NP protein was initially identified as a small pinpoint fluorescence structure in the cytoplasm of infected PK-15 cells, which became larger and more numerous at 60 minutes PI (Figure 3.7.1). By 12 hours PI, cytoplasmic granular fluorescent structures, suggestive of viral matrices, were evident. These structures were round to oval, occupied large areas of the cytoplasm, were particularly numerous in syncytia, and corresponded to inclusion bodies. No immunofluorescence for the NP protein was observed on the cell surface during *late infection*. Numerous cytoplasmic inclusions, frequently located close to the nuclear membrane, were observed at 24 hours PI. The same observations were made on EM with immunogold labeling.



3.7.1. Immunofluorescence of paraformaldehyde-fixed and Triton X-100-treated La Piedad Michoacán virus (LPMV)-infected porcine kidney (PK-15) cells incubated with a monoclonal antibody against the nucleoprotein (NP) of LPMV. **A:** Mock-infected cells. **B:** At 5 minutes post infection (PI), small discrete fluorescent spots are visible in the cytoplasm close to the cell membrane ($\times 200$). **C:** At 60 minutes PI, punctate fluorescent structures are more evident ($\times 300$). **D:** At 12 hours PI, abundant aggregates of fluorescent structures (inclusion bodies) are visible subjacent to the cytoplasmic membrane ($\times 200$). **E:** A single cell showing massive fluorescent perinuclear structures at 48 hours PI. Fluorescent foci of variable sizes are also seen in the cytoplasm ($\times 3000$). Adapted from Hernández-Jáuregui et al. (2001).

Severe cytopathic effects—i.e., massive syncytia formation, cell degeneration, and necrosis—were observed in LPMV-infected PK-15 cells examined at 48 hours PI. Syncytia frequently contained over 50 nuclei and multiple cytoplasmic inclusion bodies.

The EM examination of purified LPMV showed particles with typical paramyxovirus morphology. The virions were spherical or pleomorphic in shape, with a diameter of 150 to 170 nm. The envelope was clearly defined and peplomers, approximately 12 nm long, were distributed throughout the surface of the virion. The nucleocapsid showed helical symmetry with an outer diameter of approximately 15 nm. Purified, negatively stained nucleocapsids with typical herringbone morphology, similar to that reported for other paramyxoviruses, were observed. These nucleocapsids were labeled by the immunogold procedure using the monoclonal antibody (Mab) to the NP protein of LPMV.

DISCUSSION AND CONCLUSION

It is generally believed that fusion of paramyxoviruses with the host cell membrane is the first step of cell infection and occurs at neutral pH. After fusion of the viral envelope and cytoplasmic membrane of the host cells, the viral proteins are released into the cytoplasm. The coexpression of the HN and F proteins within the host cell membrane is needed for the initiation of cell fusion, which results in the formation of syncytia (Heminway et al. 1994; Tanabayashi et al. 1992). However, this mechanism may not be applicable to all paramyxoviruses. In the case of simian virus type 5 (SV-5) as a model to study transport of paramyxovirus surface proteins, Leser et al. (1996) reported that the HN protein was internalized to cells through receptor-mediated endocytosis pathway via clathrin-coated pits within 45 to 50 minutes. The F glycoprotein of SV-5, on

the other hand, remained on the cell surface. The method by which Newcastle disease virus inserts the HN glycoprotein into the cytoplasmic membrane is likely to be common to the attachment proteins of all paramyxoviruses (Wilson et al. 1987).

In our study, LPMV internalization appears to occur after attachment of the virion to the cell surface, which leads to the fusion of the virion with the cell membrane. These events were visualized by the presence of punctate immunofluorescence and aggregates of gold label on the surface of the cell membrane. During earlier stages of infection, virions were in close contact with cytoplasmic membranes. Immunogold particles were also on the surface of virions and within the cytoplasm. The HN glycoprotein did not appear to follow any particular endocytotic pattern, as the gold label was found on the cytoplasm in a random pattern. This label was concentrated in the perinuclear region during the first 30 to 60 minutes after inoculation, which was well correlated with the immunofluorescence microscopic observations.

Nucleocapsid aggregation was a prominent feature in LPMV-infected PK-15 cells and led to the formation of multiple cytoplasmic inclusion bodies, a feature that is a distinctive characteristic of paramyxoviruses. The Mab against the NP protein not only labeled the inclusion bodies, but also revealed distinct fine and coarse spots within the cytoplasm, especially in association with the plasma membrane. These features are in agreement with observations of the participation of the nucleoproteins with the cytoskeleton and the cytoplasmic membranes during the process of viral maturation and budding in related viruses.

In conclusion, our observations indicated that the mechanisms of LPMV replication in PK-15 cells are generally similar to those of other members of the Paramyxoviridae family.

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3.8

Carbohydrate Specificity and Porcine Rubulavirus Infectivity

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SUMMARY

We analyzed the interaction of porcine rubulavirus strain La Piedad Michoacán virus (LPMV) with specific saccharidic sequences to understand the pathogenicity of the virus. Sialyl- α 2,3-lactosamine (Neu5Ac- α 2,3-Gal β 1,4-GlcNAc) was identified as the main determinant for LPMV infection. Using inhibition assays and by modifying the glycosylation pattern of cells, it was confirmed that the susceptibility of cells and tissues to LPMV depends, in part, on the presence of specific saccharidic sequences. Histologically, newborn pigs were more susceptible to LPMV due to the wide distribution of sialyl- α 2,3-lactosamine in their central nervous system and respiratory tracts. Glycosylation, as well as susceptibility to viral infection, changed with physiological maturation of pigs, and the viral receptor was restricted to the respiratory system and sexual organs in adult animals. Our results confirmed the relevance of sialyl- α 2,3-lactosamine as a component of the viral receptor and a determinant for the cellular tropism shown by LPMV.

INTRODUCTION

A porcine rubulavirus is the etiologic agent of blue eye disease in pigs and a strain La Piedad Michoacán virus (LPMV) (isolated from pigs of La Piedad, Michoacán, Mexico) has been used extensively in experimental studies (Stephano et al. 1988). Along with the human mumps virus, LPMV is a member of the family Paramyxoviridae (Rima et al. 1995).

Clinically, LPMV infection of swine is characterized by neurological signs, respiratory and reproductive disease, and a characteristic corneal opacity (Stephano et al. 1988). Affected newborn pigs show ataxia, muscular tremors, and involuntary movements. Infected animals generally die within 48 hours of the onset of signs. In animals at 30 days of age and older, neurological signs are present in some animals, but few die due to the infection (Moreno-López et al. 1986; Stephano et al. 1988). In infected adult boars, orchitis, epididymitis, and testicular

atrophy are frequently observed (Campos and Carbajal 1992; Stephano 1999). Corneal opacity is found in 1% to 10% of the infected swine.

The natural LPMV infection pathway seems to be nasopharyngeal (Allan et al. 1996; Stephano and Gay 1985). Mucous cells, turbinates, and tonsils are postulated to be the site for initial virus replication. From these sites, the virus may be disseminated to the nervous system and lungs (Allan et al. 1996; Stephano and Gay 1985). The virus is shed through the respiratory and urinary tracts (Allan et al. 1996; Ramírez et al. 1997).

Two different proteins are expressed on the surface of LPMV: hemagglutinin-neuraminidase (HN) and fusion (F), which are assembled as homo-oligomers (Lamb and Kolakofsky 1996). As in other paramyxoviruses, the HN protein is anchored to the viral envelope by its N-terminal site (Thompson et al. 1988). The HN protein mediates the attachment of LPMV to the cytoplasmic membrane of target cells. This process has two distinct functions: virus binding to glycosylated receptors (Reyes-Leyva et al. 1993, 1997) through a lectin, and specific cleavage of sialic acid through neuraminidase activity (Moreno-López et al. 1986; Stephano et al. 1988). Consequently, the HN protein seems to be crucial in the replication cycle of LPMV, as in other paramyxoviruses.

It has been proposed that specific receptors for LPMV exist in the different tissues of the host, suggesting that expression of these receptors could be modified during physiological maturation and development. The difference in expression of the receptors might also explain the wide range of symptoms. In living organisms, the most common posttranslational modification of newly synthesized proteins is glycosylation (Lis and Sharon 1993). Glycosylation is species specific and cell specific and is determined by a covalent bond between carbohydrates and the peptide backbone. The synthesis of glycoproteins is codified genetically and involves *O*-[*N*-acetyl-D-galactosamine (GalNAc) to serine or threonine] and *N*-glycosidically linked glycans [*N*-acetyl-D-glucosamine (GlcNAc) to asparagine] (Lis and Sharon 1993). The *N*-glycosidically linked glycans are

transferred cotranslationally to a protein in the endoplasmic reticulum. Modification is completed in the Golgi apparatus by the addition of carbohydrates, such as sialic acid (Neu5Ac) and fucose (L-Fuc). Glycosylated structures are highly heterogeneous, dependent on the cellular and physiological states of organisms (Lis and Sharon 1993; Schulze and Manger 1992). In particular, Neu5Ac is a molecule found in almost all eukaryotic cells and frequently binds to the anomeric carbon by $\alpha 2,3$ or $\alpha 2,6$ to galactose (Gal) (Schauer 2000). It has been demonstrated that all of these modifications are regulated by cellular development (Alvarez et al. 1999; Vallejo et al. 2000). In this chapter, we present recent advances in determining the specificity of LPMV for saccharidic residues and explain how the pathogenesis of the disease is affected by maturation of the pig.

VIRUS

Virus strain LPMV used for the study (La Piedad, Michoacán, Mexico, 1985) was kindly provided by Dr. Jorge Moreno-López (University of Uppsala, Sweden). An African green monkey kidney cell line (Vero cells) was used for virus propagation. The cells were cultured in minimum essential medium (MEM, Gibco, Mexico City) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μ g/ml streptomycin (Sigma Chemical, St. Louis, MO, USA). Cultured cells were maintained at 37°C in a 5% CO₂ atmosphere and constant humidity. Virus concentration was determined by a plaque assay and expressed as plaque-forming units per milliliter (PFU/ml), as previously described (Hernández et al. 1998). Virus (1.3×10^{-6} PFU/0.1 ml) was propagated in confluent monolayers of Vero cells. Inoculated cells were monitored for cytopathic effects, such as syncytium formation and cellular lysis, for 72 to 96 hours after inoculation.

ASSESSMENT OF HEMAGGLUTININ ACTIVITY AND CARBOHYDRATE SPECIFICITY OF LPMV

Hemagglutination (HA) assays were performed to assess hemagglutinin activity of LPMV as previously described (Reyes-Leyva et al. 1993). The assay was conducted using fresh human erythrocytes and erythrocytes pretreated with neuraminidase from *Vibrium cholerae* (0.1 U of neuraminidase per 0.5 ml erythrocytes) or trypsin (100 μ g/0.5 ml). Erythrocytes were obtained from the Blood Bank of the Instituto Mexicano del Seguro Social and the National Institute of Respiratory Diseases, Mexico City. The HA activity of LPMV was titrated by serially diluting 25 μ l of the virus (1.3×10^{-6} PFU/0.1 ml) in 25 μ l of 0.1 M phosphate-buffered saline (PBS, pH 7.4) and adding a 2% suspension of erythrocytes-PBS. After 30 minutes of incubation at 25°C, the virus HA titer was recorded as

the reciprocal of the highest dilution showing HA activity. Sugar specificity of LPMV was determined by addition of different carbohydrates and glycoproteins to LPMV and comparing the hemagglutinating activity between treated and control groups. For the control group, PBS was added in place of the carbohydrate solution. Results were expressed as the minimum concentration of carbohydrates or glycoproteins that completely inhibited the HA activity of LPMV (Reyes-Leyva et al. 1993).

LPMV agglutinated erythrocytes from several species, but the optimal HA was observed with human type A2, rabbit, and chicken erythrocytes. Neuraminidase treatment of erythrocytes completely abolished the HA activity of the virus, whereas treatment with trypsin increased the HA titer by four to five times. These results suggested the participation of sialic acid residues in the virus-erythrocyte interaction, since the virus receptor was eliminated by neuraminidase. Trypsin treatment of erythrocytes may have unmasked specific viral receptors.

The HA activity of LPMV was specifically inhibited by Neu5Ac; *N*-glycolyl-neuraminic acid (Neu5Gc) and octulosonic acid (Kdo) also inhibited HA activity of the virus, but to a lesser extent than Neu5Ac. Previously α -anomeric derivatives of Neu5Ac were found to have a greater capacity to inhibit the virus HA activity (Reyes-Leyva et al. 1999). These results suggested that the *N*-acetylated groups [N-C(O)-CH₃] on C5 and the carboxyl group on C1 from Neu5Ac were determinants for the LPMV-cell receptor interaction. Structures lacking the *N*-acetylated group, such as Kdn and Neu5Gc, possessed a limited capacity to interact with the virus. Sialyl- $\alpha 2,3$ -lactosamine (Neu5Ac- $\alpha 2,3$ -Gal $\beta 1,4$ -GlcNAc) was better recognized by LPMV than sialyl- $\alpha 2,6$ -lactosamine. Carbohydrates that did not have an inhibitory effect on HA of LPMV were L- and D-fucose, D-mannose, GalNAc, GlcNAc, D-glucose, D-galactose, D-galactosamine, D-glucosamine, D-mannosamine, and lactose. In comparison, agglutination of bovine and horse erythrocytes by influenza viruses requires the recognition of NeuGc- $\alpha 2,3$ -Gal. Human and chicken erythrocytes have been reported to be agglutinated by several viruses that contain Neu5Ac- $\alpha 2,3$ -Gal and Neu5Ac- $\alpha 2,6$ -Gal bonds (Toshihiro et al. 1997).

EFFECT OF RECEPTOR CARBOHYDRATE MOIETY ON LPMV INFECTIVITY

An in vitro study using the Vero cell line and known glycosylation inhibitors was conducted to determine whether the glycosylation of receptors on host cells was a determinant for LPMV infection. Infectivity of the virus for Vero cells was assessed with and without pretreatment of the cells with tunicamycin (TUN), deoxynojirimycin (DNM), deoxymannojirimycin (DMM),

and dexamethasone. TUN interferes with the dolichol-phosphate transfer in the transport of oligosaccharides to be incorporated into newly synthesized glycoproteins in the endoplasmic reticulum. DNM and DMM interfere with the glycosidase activity at the endoplasmic reticulum and with a mannosidase found in the Golgi apparatus, respectively. These enzymes are known to participate sequentially in oligosaccharidic processing during protein glycosylation. Dexamethasone has the capacity to increase the expression of the β -galactosyl- α 2,6-sialyl transferase. The cells (5×10^4 cells/ml) were treated with different concentrations of TUN, DNM, or DMM prepared in MEM, and incubated for up to 8 hours at 37°C. Under similar conditions, cultured cells were treated with dexamethasone at various concentrations and incubated for 12 hours at 37°C. Then, treated and untreated cells were inoculated with LPMV at a rate of 1000 syncytia-forming units per milliliter (SFU/ml). After the inoculum was removed, the cells were further incubated in MEM supplemented with 2% FBS at 37°C for 96 hours. Virus infection was determined by the presence or absence of syncytium formation. When present, the number of SFU was calculated to determine a percent reduction of infectivity. TUN treatment of Vero cells completely inhibited LPMV infection at low concentrations (as low as 1.3 μ g/ml). DNM at 5 μ g/ml inhibited 75% of the LPMV infectivity. In contrast, no significant reduction in viral infectivity was observed with the DMM treatment at the same concentration (5 μ g/ml) after 4 hours of incubation. Dexamethasone at 1 to 5 μ M/ml inhibited the viral infection in Vero cells. According to our results, the increase in β -galactosyl- α 2,6-sialyl transferase activity yielded a decrease in β -galactosyl- α 2,3-sialyl transferase (Vandamme et al. 1993) in Vero cells, which in turn decreased the number of possible receptors to LPMV. Our results were also confirmed by histochemistry, as described below. Neu5Ac- α 2,3 and Neu5Ac- α 2,6 residues were identified with fluorescein isothiocyanate (FITC)-labeled *Sambucus nigra* (SNA) and *Maackia amurensis* (MAA) lectins, respectively, which are known to recognize the aforementioned saccharidic sequences (Broekaert et al. 1984; Wang and Cummings 1988).

HISTOCHEMICAL STUDY OF CARBOHYDRATE DETERMINANTS FOR LPMV INFECTIVITY

Specific lectins against sialic acid were conjugated to FITC, as previously described (Savage et al. 1992), and used to examine the presence of specific carbohydrate moieties on the surface of Vero cells. The same approach was also used to assess the effect of treatment of the cells with glycosylation inhibitors on the expression of carbohydrates on the surface. Vero cells fixed on coverslips were incubated with MAA and SNA lectins (10 μ g/ml)

conjugated with FITC for 30 minutes at 37°C. After washing with Tween 20-PBS (0.001%) with 0.1% bovine serum albumin, stained cells were examined using fluorescence microscopy. The specificity of the reaction in controls was determined using nonconjugated lectins at the same protein concentration. The presence of Neu5Ac in Vero cells was confirmed, which could be linked to Gal at α 2,3 (Neu5Ac- α 2,3-Gal) and recognized by MAA, or at α 2,6 (Neu5Ac- α 2,6-Gal) that were identified by SNA. Lectin histochemistry of cells treated with glycosylation inhibitors demonstrated the complete inhibition of sialylated oligosaccharide expression. DNM treatment reduced 70% the recognition of Vero cells by MAA and completely inhibited their SNA recognition. Interaction of Neu5Ac on Vero cells by both lectins was affected by DMM treatment. In contrast, dexamethasone increased 100% the capacity of Vero cells to interact with SNA, and reduced 70% their recognition by MAA. LPMV infectivity was abolished in all treated Vero cells, confirming the relevance of Neu5Ac-2,3 α -Gal as determinant for viral infectivity.

HISTOCHEMISTRY OF PUTATIVE LPMV RECEPTORS IN TISSUES

Histochemical tests using lectins specific for Neu5Ac enabled us to identify the presence of putative rubulavirus receptors in various tissues of both newborns and adult pigs. Our study revealed a higher proportion of Neu5Ac- α 2,3-Gal in the epididymal and lung tissues of adult pigs, whereas the highest concentration of Neu5Ac- α 2,3-Gal was found in the CNS of newborn pigs. These observations strongly indicated that potential receptors for LPMV are present in association with damaged tissue. Enzyme activity, such as that of β -galactosyl- α 2,6-sialyl transferase, was much lower in adult swine than in newborn pigs. These results resemble the regulation of these enzymes expression by dexamethasone, suggesting that several hormonal factors might participate in the regulation of transferases expression and could induce an adaptive effect in some pathogens.

CONCLUSIONS

The main goal of this study was to identify the mechanisms involved in the pathogenesis of the porcine rubulavirus, LPMV. Interactions of viral proteins with the cellular receptors play an important role in viral infections. It is well known that viral adsorption is necessary to initiate an infection (Tardieu et al. 1982). Several members of family Paramyxoviridae, including LPMV, share this characteristic, which is mediated by the HN. In other paramyxoviruses, on the other hand, the same process is regulated by G or H protein, depending on the subfamily (Rima et al. 1995). It is interesting to note that the HN protein of LPMV possesses two different

activities: (1) hemagglutinating activity, which enables the virus to recognize and adhere to the receptors localized in host cell surface; and (2) neuraminidase activity, implicated in the elution of virus from its receptor. In this regard, HN protein might also play an important role in interacting with the matrix protein during the release of the viral progeny (Garcia-Beato et al. 1996; Schulze and Manger 1992). Two domains have been predicted from sequence analyses of the HN protein of LPMV: an intracellular domain and a transmembrane domain in the N terminus. The transmembrane peptide possesses a hydrophobic region of 22 amino acids. This region shows high rigidity and inaccessibility to solvents. For this reason, it has been proposed as an anchor domain (Zenteno 1997). In addition, an extracellular domain was also identified in the HN protein and postulated to have some functions associated to hemagglutinin and neuraminidase activities in the carboxyl region, whose structural distribution in space remains a type with β -loop strands alternated by few α helices (Zenteno et al. 1998). Colman et al. (1993) made a similar proposal, comparing different HN proteins from several paramyxoviruses by using the neuraminidase from the influenza virus.

The HN protein is able to recognize saccharidic structures on the cellular surface corresponding to the anomeric C1 of Neu5Ac. It has been demonstrated that the hemagglutinins of influenza viruses A and B have a similar specificity for carbohydrates, i.e., sialic acid (Govorkova et al. 1996), as LPMV in Vero cells. This explains in part the susceptibility and permissibility of Vero cells for the infection. Modification in the expression of sugars on Vero cells, inhibited by glycosylation inhibitors as well as dexamethasone, demonstrated that neuraminic acid is necessary for LPMV recognition and determines viral infectivity. The hemagglutinin domain from the HN protein of the LPMV contains one of the single α -helix structures, suggesting that the α helix might participate in the saccharidic recognition of the cellular receptor (Zenteno et al. 1998).

The specificity of LPMV might be related to the clinical symptoms in both newborn and adult pigs. In young animals, the infection affects mainly the respiratory system and CNS, provoking death, whereas, in adult pigs, the damage is selective for the reproductive system. We speculate that the primary sites of replication are localized in the respiratory tract and in those places where it is possible to find sialyl- α 2,3-lactosamine containing oligosaccharides. In newborn pigs, the virus is localized in several regions of the CNS at 4 days post infection (PI). In pigs at the age of 17 days, the virus is localized mainly in the mesencephalic region, and in the olfactory bulb from 3 to 11 days PI (Allan et al. 1996). In adult pigs, invasion of LPMV may occur similarly as observed with Aujeszky's virus (Kluge et al. 1992). These reported differences in viral distribution in the CNS from the pigs

might indicate differences in the virus dissemination from the primary site of replication to the CNS (Allan et al. 1996; Stephano et al. 1988). The participation of saccharidic structures in HN protein recognition is relevant in identifying the main target in infected organisms. We determined that LPMV recognizes specifically sialyl- α 2,3-lactosamine on the surface of Vero cells and in the newborn pigs, but not the anomeric sialyl- α 2,6-lactosamine, which predominates in the organs of adult pigs. Variations in the saccharidic structures, in terms of age, resemble those found in tissue tropism of LPMV (Reyes-Leyva et al. 1999; Vallejo et al. 2000). Influenza A virus interacts specifically with oligosaccharides on the cellular surface, but shows some variations in their specificities, which seem to be modified according to the origin of the host cell (Toshihiro et al. 1997). Influenza A virus binds mainly Neu5Ac- α 2,3-Gal; however, the change in a single amino acid on the hemagglutinin sequence alters the specificity of the receptor to the influenza virus from NeuAc- α 2,6-Gal to NeuAc- α 2,3-Gal (Toshihiro et al. 1997).

The HN from LPMV is able to agglutinate erythrocytes from different animal species. The fundamental role of sialic acid has been evidenced in the recognition process by these cells. Enzymatic cleavage of this residue eliminates virion and HN glycoprotein recognition. Trypsin treatment of erythrocytes increased the hemagglutination capacity, which indicates that the HN receptor is not only hidden, but also becomes part of the glycolipids associated with the cellular membrane. We consider that, in these conditions, the HN protein is necessary for virus recognition by the host cell. Therefore, the HN protein should be taken into consideration when designing a putative vaccine against porcine blue eye disease.

ACKNOWLEDGMENTS

This work was supported in part by the Consejo Nacional de Ciencia y Tecnología (CONACYT Project 27609 M) and Dirección General del Personal Académico (DGAPA-PAPIIT-IN224598) Universidad Nacional Autónoma de México.

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3.9 Neuropathology of Porcine Rubulavirus Infection

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SUMMARY

Porcine rubulavirus (PRuV) infection is an important cause of economic loss in pig production in Mexico. Clinical signs include encephalomyelitis, pneumonia, reproductive failure and corneal opacity. Although gross lesions are not apparent in the central nervous system of affected pigs, lesions of nonsuppurative encephalomyelitis can be seen on microscopic examination. The results of experimental studies at our laboratories indicate that 3-day-old pigs are more susceptible to PRuV than 17-day-old pigs. They also indicate that, following intranasal and intraconjunctival inoculation, PRuV gains antero-grade access to the brain via the nasal mucosa and olfactory pathway. This finding may explain the rapid onset of nervous signs in young pigs during spontaneous outbreaks of PRuV infection. The epidemiological significance of persistence of PRuV in the central nervous system of young pigs is unknown.

INTRODUCTION

A novel disease of pigs characterized by encephalomyelitis, pneumonia, reproductive failure, and occasional corneal opacity was first reported from La Piedad, Michoacán, Mexico, in 1980 (Moreno-López et al. 1986; Stephano et al. 1988). A paramyxovirus isolated from affected pigs (Moreno-López et al. 1986; Stephano et al. 1988) was initially termed La Piedad Michoacan virus (LPMV) or “blue eye” paramyxovirus. Following molecular and biological characterization (Sundqvist et al. 1990, 1992; Svenda et al. 1997), LPMV has been classified as a member of the genus *Rubulavirus* within the family Paramyxoviridae. This new genus includes the prototype virus, human parotiditis virus, the simian parainfluenza viruses, SV5 and SV41, the human parainfluenza viruses 2, 4a and 4b, Newcastle disease virus, and avian paramyxoviruses 2 to 9 (Rima et al. 1995).

Piglets up to 2 weeks of age are most susceptible to PRuV infection (Moreno-López et al. 1986; Stephano et al. 1988). Typically, these young animals become prostrate and develop progressive nervous signs, including ataxia, weakness, rigidity (mainly of the hind limbs), muscle tremors and abnormal posture (including adop-

tion of a sitting position), involuntary movements, dilated pupils, apparent blindness, and hyperexcitability characterized by paddling movements and excessive squealing (Stephano et al. 1988). Morbidity of approximately 20% and around 90% mortality have been reported in piglets between 4 and 10 days of age (Moreno-López et al. 1986). Up to 10% of affected piglets develop corneal opacity. Nervous signs are much less frequent in older animals, but, when present, comprise ataxia, circling, and swaying of the head. Although corneal opacity in 1% to 4% of infected animals may be the only clinical sign in pigs over 30 days of age (Stephano et al. 1988), this lesion can occur in up to 30% of fattening pigs on some infected farms.

Microscopic brain lesions in affected pigs comprise a nonsuppurative encephalomyelitis affecting mainly the gray matter of the thalamus, midbrain, and cerebral cortex. These lesions are characterized by multifocal-to-diffuse gliosis, neuronal necrosis, neuronophagia, meningitis, and choroiditis (Stephano et al. 1988).

Whereas neurological lesions are rare in adult pigs, PRuV-infected sows can have increased numbers of mummified fetuses, stillborn piglets, abortions, and a reduced conception rate. Epididymitis and orchitis may develop in 25% to 30% of boars naturally or experimentally infected with PRuV (Ramírez-Mendoza et al. 1997).

We have previously reported the distribution of virus in tissues of young pigs experimentally infected with PRuV (Allan et al. 1996), and the pathogenesis of lesions in the reproductive tract of sexually mature boars has been described (Ramírez-Mendoza et al. 1997). A few reports have been published on the neural lesions in pigs infected with PRuV (Galina et al. 1989; Kennedy et al. 1994; Ramírez-Herrera et al. 1997; Stephano et al. 1988; Wiman et al. 1998). This chapter documents the results of recent investigations in our laboratories on the neuropathogenesis of PRuV infection.

MATERIALS AND METHODS

To investigate the pathogenesis of the neurological lesions, we examined central nervous system (CNS) tissues of seven pigs that had been inoculated intranasally and intraconjunctivally at 3 days of age with $10^{7.0}$ 50% tissue

culture infectious doses (TCID₅₀) of an isolate of PRuV and killed sequentially from days 1 to 8 post inoculation (PI) (Allan et al. 1996). The experiment was repeated on a group of ten 17-day-old pigs that were necropsied between days 1 and 14 PI. Samples of nasal mucosa, olfactory bulb, olfactory nerve, forebrain, midbrain, and hindbrain, optic nerve, eye, and cervical, thoracic, and lumbar regions of spinal cord from all infected pigs and from six uninoculated controls were collected for virus isolation (Allan et al. 1996) and into 10% neutral buffered formalin for processing for microscopic examination. An immunohistochemical technique was used to determine the cellular distribution of PRuV in selected paraffin sections (Table 3.9.1). Control procedures consisted of substitution of an inappropriate antiserum for the primary antiserum and application of the technique to tissue sections from uninfected control pigs. Selected sections were also examined for the presence of apoptotic cells by using the in situ end labeling technique for fragmented DNA (Gold et al. 1993).

RESULTS

Clinical Signs

Clinical signs were apparent from day 5 PI in all piglets infected with PRuV at 3 days of age (Allan et al. 1996). These signs included dullness, increased respiratory rate, muscle tremors, and incoordination. They became progressively more severe, and all remaining pigs had respiratory distress, muscle tremors, and arched back on day 7 PI. Several pigs adopted sternal recumbency at this time, with the head held forward and mouth open. Three pigs developed corneal opacity, and several pigs died. Mild nervous and respiratory signs were apparent from day 6 PI in approximately 30% of the piglets infected with PRuV at 17 days of age. These signs became moderate by day 8 PI in several pigs, but none died.

Histopathologic Lesions

Microscopic changes were seen in the nasal mucosa, brain, eye, and spinal cord of the piglets infected at 3 days of age. Neutrophilic and lymphocytic infiltration of nasal mucosa and adjacent olfactory neuropil was apparent from day 4 PI. From day 5 PI, there was evidence of a nonsuppurative encephalomyelitis characterized by lymphocytic perivascular cuffing, gliosis, and neuronophagia. Structures resembling apoptotic bodies were apparent in inflamed regions of the olfactory bulb and the brain. Examination of multiple coronal sections revealed a chronological progression of lesions from nasal mucosa to the frontal lobe via the olfactory pathway and eventually to other forebrain, midbrain, and hindbrain regions. In the eye, mononuclear cell and neutrophilic infiltration of the corneoscleral junction and iridocorneal angle, and corneal edema were seen in several infected animals, including those with grossly apparent corneal opacity. However, no alterations were apparent in the optic nerve. Small glial foci were rarely seen in the spinal cord. Microscopic lesions in the piglets infected at 17 days of age were generally similar, but less severe.

Viral Distribution

The distribution of PRuV in the CNS of the piglets infected at 3 days of age closely mirrored the pattern of microscopic lesions. Virus was initially identified in olfactory bulb and subsequently in forebrain (Table 3.9.2). Immunohistochemical labeling of PRuV had a finely granular appearance and was mainly confined to neuronal perikarya and processes. Viral antigen was closely associated with the inflammatory lesions. Virus was rarely identified in optic nerve and spinal cord and only then by virus isolation. Although the tissue distribution of PRuV in piglets infected at 17 days of age was closely related to the olfactory areas and adjacent frontal lobes as in the animals inoculated at 3 days of age, immunolabeling was generally more widespread but less intense in these animals.

Table 3.9.1. Immunohistochemical method for labeling porcine rubulavirus in paraffin sections

1.	Treat deparaffinized sections with 0.5% solution of hydrogen peroxide for 5 minutes.
2.	Wash in tap water followed by TBS.
3.	Treat with 0.1% protease for 5 minutes.
4.	Wash in tap water followed by TBS.
5.	Treat slides with normal goat serum.
6.	Incubate overnight in diluted (1:200) primary rabbit anti-porcine rubulavirus polyclonal antibody.
7.	Wash twice in TBS for 20 minutes.
8.	Incubate in biotinylated goat anti-rabbit immunoglobulin G solution for 30 minutes.
9.	Wash in TBS for 20 minutes.
10.	Incubate with avidin-biotin-peroxidase complex reagent for 30 minutes.
11.	Wash in TBS for 30 minutes.
12.	Incubate with 3,3' diaminobenzidine tetrahydrochloride/hydrogen peroxide solution for 5 to 7 minutes.
13.	Wash in TBS for 30 minutes followed by tap water for 10 minutes.
14.	Lightly counterstain with hematoxylin for light-microscopic examination.

TBS, Tris-bufferend saline.

Table 3.9.2. Distribution of virus in central nervous system tissues of pigs post infection (PI) with porcine rubulavirus (PRuV) at 3 days of age

Tissue	Day 1 PI VI ^a	Day 2 PI IPX ^b	Day 3 PI VI	Day 4 PI IPX	Day 5 PI VI	Day 7 PI IPX	Day 8 PI VI	IPX	VI	IPX	VI	IPX	VI	IPX
Olfactory bulb	– ^c	–	–	–	–	–	–	+	+	+	+	+	+	+
Forebrain	–	–	–	–	–	–	–	+	+	+	+	+	+	+
Midbrain	–	–	–	–	–	–	–	+	+	+	+	+	+	+
Hindbrain	–	–	–	–	–	–	–	+	+	+	+	+	+	+
Optic Nerve	–	–	–	–	–	–	–	+	+	+	+	+	+	+
Spinal cord	–	–	–	–	–	–	–	–	–	–	–	–	–	–

IPX, immunoberoxidase; VI, virus isolation.
^aVI, PRuV isolated from tissue.
^bIPX, PRuV demonstrated in tissue by immunohistochemical labeling
^c+, Virus/viral antigen detected; –, no virus/viral antigen detected

DISCUSSION

The results of this study indicated that 3-day-old piglets were more susceptible to PRuV infection than 17-day-old animals and, therefore, agree with reports of a decreasing age susceptibility in naturally infected pigs (Moreno-López et al. 1986; Stephano et al. 1988). Clinical signs developed at 5 to 6 days PI and were similar to those reported in naturally infected pigs. However, they developed slightly later than in previous experimental investigations. For example, Stephano et al. (1988) reported the onset of clinical signs at 75 and 96 hours PI, respectively, in two piglets intranasally inoculated with PRuV, whereas one pig inoculated by the same route developed clinical signs 3 days PI (Ramírez-Herrera et al. 1997). This variation in incubation period could be due to factors such as differences in viral strains, passage level, or host susceptibility. Not unexpectedly, intracerebral inoculation of 1-day-old piglets with PRuV resulted in rapid development of clinical signs (18 hours PI) (Stephano et al. 1988). Unlike in a previous study (Stephano et al. 1988), corneal opacity developed in several pigs in the current investigation.

A chronological progression of PRuV from the nasal mucosa to the brain via the olfactory bulb and nerve was demonstrated in both age groups. In contrast, virus was not demonstrated in optic nerve of any piglet by immunohistochemical labeling and was identified by the more sensitive virus isolation technique in optic nerve of only one animal (Table 3.9.1). These results, in the absence of detectable viremia (Allan et al. 1996), provide strong evidence that, following combined intranasal and intraconjunctival inoculation, PRuV gains access to the brain via the olfactory pathway; spread from the conjunctival sac to the brain via the optic nerve was not detected. This hypothesis is supported by the absence of apparent choroid plexus or subependymal involvement. These results agree with the finding by Ramírez-Herrera et al. (1997) of viral antigen in olfactory nerve and bulb

and in cerebral cortex 5 days after experimental intranasal infection of a pig with PRuV. As suggested by these authors and by Galina et al. (1989), under natural conditions, anterograde transport of PRuV following intranasal infection is likely to be an important method of viral spread to the CNS. This mechanism could explain the rapid development of clinical signs in spontaneous outbreaks of PRuV disease in piglets.

The nonsuppurative encephalitis that developed in PRuV-infected piglets in this study was typical of a viral encephalitis and similar to that previously reported in natural and experimental cases of PRuV infection (Ramírez-Herrera et al. 1997; Stephano et al. 1988). The presence of structures that resembled apoptotic bodies, and were positive by the in situ end-labeling technique for fragmented DNA, suggests that some PRuV-infected neurons undergo apoptosis rather than necrosis. However, since this technique is not specific for apoptosis, this result must be confirmed by alternative methods. In some pigs, the brain lesions were highly focal and not always associated with detectable PRuV antigen. These findings suggest that histopathologic diagnosis of PRuV-induced encephalitis should be based on examination of a suitably large number of brain sections. Because lesions were usually present in olfactory bulb, this tissue should be included in any histological examination. Although only a few piglets developed corneal opacity, many had microscopic inflammatory lesions in cornea and adjacent ocular tissue. Microscopic examination of the eye may therefore be useful in the diagnosis of PRuV disease.

Ramírez-Herrera et al. (1997) infected piglets with a PRuV isolate by intramuscular or intradermal inoculation at 3 to 8 days of age. Immunohistochemical labeling revealed PRuV within and on the outside of axons of the sciatic nerve 11 and 40 days after inoculation of the medial gastrocnemius muscle in two pigs. The virus subsequently ascended along sensory and motor nerve fibers, respectively, to reach the dorsal root ganglia and motor

neurons of the ventral horn of the spinal cord. In two piglets infected intradermally within the region innervated by the sural nerve, PR virus was detected in axons of the sural and sciatic nerves and in dorsal horn neurons of the spinal cord 11 days PI. These results demonstrate that, in addition to anterograde spread along the olfactory pathway, PRuV can also gain retrograde access to the CNS via peripheral nerves.

Evidence for the persistence of PRuV in the CNS of pigs that had recovered from acute infection has been presented (Wiman et al. 1998). It is not known whether reactivation of persistent infection can lead to recurrent episodes of clinical disease or to spread of viral infection to other animals.

The hemagglutinin-neuraminidase glycoproteins of PRuV bind to cell receptors containing sialic acid (Reyes-Leyva et al. 1993). A higher concentration of such receptors in the CNS of young pigs than in older animals may explain the marked differences in the incidence of neurological lesions between these two age groups.

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3.10 Are the Lesions in the Epididymis of Boars Infected with Porcine Rubulavirus (LPMV) Similar to Those of Mumps Virus in Humans?

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SUMMARY

Porcine rubulavirus, which is a member of the family Paramyxoviridae, causes encephalitis and death in 1- to 5-day-old piglets, abortion in adult females, and epididymitis and orchitis in sexually mature boars. La Piedad Michoacán virus (LPMV) strain is closely related to the human mumps virus, sharing 41% to 47% amino acid sequence homology. Tissue tropism (brain, testicles, and epididymis) is also similar for the two viruses. In three experiments, twelve 9-month-old York-Landrace boars were inoculated with either the PAC-3 or the prototype LPMV strain of the porcine rubulavirus, killed between 5 and 80 days after inoculation, and reproductive tissues examined for lesions resulting from the infection. Gross lesions included swelling and mild-to-severe fibrosis of the head of the epididymis. A marked reduction in motility and concentration of spermatozoa was detected in one boar. Histological alterations included formation of spermatic granulomas and vacuolar degeneration of ductular epithelium. Nodules were seen in the head of the epididymis in seven boars, and the testes of two pigs were atrophic. Viral antigen was detected by immunofluorescence and immunohistochemical assays in the head of the epididymis. A few focally localized inclusion bodies in the tubular walls and epithelial cells, and budding of virus-like particles in intracytoplasmic vesicles and the cytoplasmic membrane, were observed by electron microscopy. Based on these results, it was concluded that the lesions in the epididymis and testes of LPMV-infected boars may resemble those of mumps virus infection in humans.

INTRODUCTION

The porcine rubulavirus LPMV (La Piedad Michoacán virus) was isolated from the brain of a piglet in Mexico

(Moreno-López et al. 1986). Infected piglets primarily exhibit clinical signs of central nervous system involvement followed by death within 48 hours. In older pigs, clinical signs are less severe. Reduced fertility due to epididymitis and orchitis develops in 14% to 40% of cases (Stephano 1999). In previous studies, sequence analysis of genes encoding major viral proteins showed that LPMV was closely related to mumps virus, simian virus 5 (SV5), and other rubulaviruses (Berg et al. 1991, 1992; Linné et al. 1992; Sundqvist et al. 1990, 1992; Svenda 1997, 2001).

During natural outbreaks, LPMV-infected boars may develop orchitis, epididymitis, and unilateral or bilateral testicular atrophy. Abscesses in the head of the epididymis, histologically corresponding to spermatic granulomas, have also been observed. Studies of 79 sexually mature boars with lesions of the reproductive tract showed epididymitis in 24 cases (30%). Sections of the epididymis of the affected boars showed focal lymphoid infiltrates, necrosis, and spermatic granulomas within ductular epithelia (Campos and Carbajal 1994; Stephano 1999; Stephano et al. 1990). Unilateral orchitis was present in 19 of 24 cases and bilateral orchitis in two cases. Histological sections of testes with orchitis showed fibrosis, lymphoid cell infiltration of the tubular walls, and germ cell degeneration. Semen quantity and quality were also affected. The total volume of semen diminished in 10 boars, with alterations in sperm motility, the presence of leukocytes and red blood cells in ejaculates, oligospermia, and azoospermia. The animals recovered after 60 to 70 days, but fertility was affected.

Mumps virus infection in humans causes epididymitis, orchitis, parotitis, pancreatitis, and meningoencephalitis (Gnann 1992). A similar organ tropism is a characteristic feature of LPMV infection. A detailed description of the epididymal lesions in mumps is lacking, but, considering the similarities between LPMV and

Table 3.10.1. Main necropsy and histopathological findings in boars experimentally infected with porcine rubulavirus

Findings	Results											
	Experiment 1 with pigs no.			Experiment 2 with pigs no.			Experiment 3 with pigs no.					
	1 (5) ^b	2 (15)	3 (15)	4 (30)	5 (70)	6 (70)	7 (80)	8 (80)	9 (30)	10 (30)	11 (30)	12 (30)
Necropsy findings	490	375	350	400	620	600	480	580	ND	ND	ND	ND
Weight of right testis (g)	500	400	500	350	450	625	810	600	ND	ND	ND	ND
Weight of left testis (g)	-	-	+	+	-	-	-	-	+	+	+	+
Scrotal edema	-	+	+	+	-	-	-	-	-	-	-	-
Nodules in head of epididymis	-	-	+	-	-	-	-	-	-	-	-	-
Testicular atrophy	-	-	+	-	-	-	+	-	-	-	-	-
Histopathological findings												
Head of epididymis	-	++	++	++	-	-	-	-	++	++	++	++
Spermatic granulomas	-	++	++	++	-	-	-	-	++	++	++	++
Ductular degeneration and necrosis	-	++	++	++	+	-	-	-	++	++	++	++
Interstitial mononuclear infiltration	-	+	+	+	++	++	++	++	+	+	+	+
Body and tail of epididymis	-	+	+	+	-	-	-	-	-	-	-	-
Interstitial mononuclear infiltration	-	-	-	-	-	-	-	-	-	-	-	-
Testes	-	-	+	+	-	-	-	-	-	-	-	-
Tubular degeneration	-	-	+	-	-	-	+	-	+	-	-	-
Interstitial mononuclear infiltration	-	-	++	-	-	-	+	-	+	-	-	-
Immunofluorescence	-	+	+	+	+	-	-	-	ND	ND	ND	ND
Antigen in head of epididymis (polyclonal Ab)	-	-	-	-	-	-	-	-	-	-	-	-
MAb against LPMV-NP	ND	ND	ND	ND	ND	ND	ND	ND	++	++	++	++

clear cells, but no ductular changes. Spermatozoa were absent from the ductuli of the head of the epididymis in two pigs. Interstitial orchitis was seen in the right testis in three pigs and was characterized by degeneration of seminiferous tubules, giant cell formation, and intense interstitial lymphocytic infiltration. Many giant cells appeared to be in the lumina of severely degenerate seminiferous tubules, in which germinal epithelium was absent. These lesions were most severe in the subalbugineal region, with hemorrhage, degeneration of seminiferous tubules, and interstitial mononuclear cell infiltration.

In immunofluorescence assays using polyclonal antibodies, focal cytoplasmic immunolabeling was seen in ductular epithelial cells adjacent to spermatogenic granulomas in the head of the epididymis in four pigs. Similar labeling was detected in the ductular walls and lumina, adjacent to blood vessels, and in interstitial mononuclear cells. Intraluminal labeling was frequently associated with spermatozoa. Tissue sections from the epididymis incubated with the monoclonal antibody against the LPMV-NP protein showed small punctuate fluorescent structures at the apical border of the epithelium and on sloughed cells in the tubular lumens. Moderate vacuolation was observed in epithelial cells in sections of the epididymis prepared for immunohistochemistry using the monoclonal antibody; however, foci of staining in the tubular wall, epithelial cells, and in the lumen were focally observed (Table 3.10.1).

In the third experiment, the histopathologic findings in the head of the epididymis in all boars showed severe, focally localized vacuolation of the epithelial cells and mononuclear cell infiltration. Spermatogenic granulomas, with large accumulation of extratubular sperm, macrophages, and multinucleated cells surrounded by fibrous connective tissue, were present in all cases (Table 3.10.1).

Electron-microscopic studies were performed on the epididymal lesions of four pigs in the third experiment (Table 3.10.1). The initial segment of the head of the epididymis showed a distinct cytopathic effect (CPE). In the cytoplasm, ribosome-like structures resembling the RNA heterochromatin of the nuclei and nucleoli were commonly found as large ovoid inclusion bodies. Electron-dense areas of spherical structures were seen budding into the rough endoplasmic reticulum. Inclusion bodies and electron-dense modified surfaces of the cytoplasmic membrane were also present. From the modified parts of the membranes, rounded virus-like particles budding into the cell outer space were observed. Cells showing more severe CPE had irregular, condensed nuclei and vacuolated cytoplasm. Modified membranes with more electron-dense patches were observed at the nucleus, mitochondria, and vacuoles. In the endomembrane system, viral nucleocapsids with typical herringbone structures were found near and surrounding the cytoplasmic organelles from which budding virus-like particles were

also seen. In apical epithelial cells, less condensed inclusion bodies were observed. From the cytoplasmic membranes, budding of elongated tubular structures and pleomorphic virus-like particles, some of them showing surface projections, were seen. Occasionally, bizarre structures similar to nucleocapsid material were present at the extracellular space. Fibroblast cells showing CPE had dilated, rough endoplasmic reticulum containing widespread, low-density, amorphous material.

This study demonstrated the tropism of porcine rubulavirus for the head of the epididymis in sexually mature boars. The earliest change observed in the ductuli of the head of the epididymis was fine vacuolation of epithelial cells. More severe damage included mononuclear cell infiltration and vacuolar degeneration and necrosis of the ductular epithelium. These lesions were associated with the presence of rubulavirus antigen in the ductular epithelium and lumina and in interstitial inflammatory cells. It appeared that virus-induced damage to ductules resulted in leakage of spermatozoa into the epididymal interstitium, with consequent formation of large spermatogenic granulomas.

Based on these observations and the close genetic relationship to mumps virus in human, it is of particular interest to correlate the lesions observed in LPMV-infected boars with those of mumps virus infection in humans. In both cases, the infection is characterized by epididymitis, orchitis, and meningoencephalitis (Gnann 1992; Ramírez-Mendoza et al. 1997; Stephano 1999).

Morphological studies of the epididymis in an acute fatal case of mumps infection disclosed mononuclear cell infiltration within the stroma of the tubules (Bostrom 1968). Orchitis is a lesion of longer duration of the affected testicle, with pain and generalized inflammatory scrotal symptoms in the acute stage of the disease, ending in atrophy and infertility (López-Pacios et al. 1998). Similar morphological changes and clinical symptoms with an inflammatory response and, later, atrophy and infertility were also observed in boars after either natural or experimental LPMV infection. In mumps virus and LPMV infections, epididymitis may be of a short duration followed by a relatively rapid restoration of affected tissues. Moreover, the lesions of the epididymis are focally distributed, making it particularly difficult to localize them for electron-microscopic analysis. The data suggest that LPMV starts replicating in the epithelium of the head of the epididymis and is then transported to the tubular lumen. For this reason, the semen of LPMV-infected boars should be considered a potential source of virus for infecting sows. It is relevant to consider how the virus reaches the epididymis. Previous reports indicated that receptors for LPMV are sialoconjugates (Neu5Ac- α 2,3-Gal) on erythrocytes (Reyes-Leyva et al. 1993). Sialylated structures interact and play an important role in differentiation and maturation during

cell development. Cell receptors for mumps virus have not been studied in detail, but Hosaka et al. (1998) described binding of influenza virus and paramyxoviruses (mumps and Sendai) to sialyl-galactose linkage receptors of group B *Streptococcus*. Similarly, influenza virus A and B bind to sialyl-galactose (Neu5Ac- α 2,3 or/and 2,6 linkages) receptors of enterocytes in ducks (Ito et al. 2000). Moreover, it is known that similar sialoconjugates exist at high concentrations in the epididymis of sexually mature boars, whereas the concentration in piglets is low (Vallejo et al. 2000). As in other animals, sialylation is modulated by the presence of steroid hormones, and individual variations in androgen secretion in the testis should reflect sialic acid concentrations in the epididymis. We assume that the homing of LPMV to cell receptors in the epididymis may depend on the abundance of sialoconjugates. Similar tissue tropism by LPMV and mumps virus would explain why, after an outbreak of mumps virus in humans or LPMV in pigs, 25% and 25% to 40% of cases, respectively, present epididymitis and orchitis (Gnann 1992; Ramírez-Mendoza et al. 1997; Stephano 1999).

For ethical reasons, it is not possible to study infection in human patients, but LPMV may offer insight into the pathogenesis of mumps virus. In view of the genomic relatedness of porcine rubulavirus to mumps virus, it is not surprising that both viruses have tropisms for a similar range of tissues. Based on the limited information describing the pathology of the reproductive tract in men caused by mumps virus, the histological changes of the epididymis and testes seem to be similar in LPMV and mumps virus infections.

CONCLUSIONS

Considering the similarities of the LPMV lesions in the epididymis and the lack of more relevant information about the lesions in humans with mumps infection, LPMV infection in sexually mature boars may be a useful model for mumps virus infection in the reproductive tract of postpubertal human males.

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3.11 Pathogenesis of Porcine Rubulavirus (LPMV) in Pancreatic Rat Islets

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SUMMARY

It has been suggested that pancreas infection by mumps virus could be a cause of diabetes in humans. Since porcine rubulavirus strain La Piedad, Michoacán (LPMV), is similar to mumps virus, it was decided to use it as a model for pancreas infection. The first experiment found that pancreatic islets isolated from rats were susceptible to infection with the LPMV. The virus caused a cytopathic effect characterized by granulation and formation of syncytia both in islets and in fibroblastoid cells. Virus replication in pancreatic islets was demonstrated by hemadsorption, immunofluorescence, and electron microscopy. After 120 passages, fibroblastoid cells showed evidence of persistent infection, as demonstrated by polymerase chain reaction. It was demonstrated by immunofluorescence that LPMV replicated both in insulin-producing beta cells and in non-insulin-producing cells. Infected cultured islets showed a markedly diminished insulin release when stimulated with glucose. The results indicated that this system could be used as a model to investigate the association between rubulavirus infection and diabetes.

INTRODUCTION

The cascade of events leading to diabetes as an autoimmune response to pancreatic beta cells is believed to be associated with genetic susceptibility, a variety of environmental factors (chemicals, diet, and toxins), and possibly with viral infection (Oldstone et al. 1984; Yoon 1997). Many viruses belonging to different taxonomic groups with putative diabetogenic properties have been associated with the destruction of pancreatic beta cells in animals and humans (mumps virus, cytomegalovirus, rubella virus, encephalomyocarditis virus, Coxsackie virus B1–B6, aphthoviruses, retroviruses, and others), and many reports on diabetes as a sequel to mumps infection have been recorded (Roivainen et al. 2000; Szopa et al. 1993; Von Herrath et al. 1998; Yoon 1995).

Porcine rubulavirus LPMV (La Piedad Michoacán virus), like other members of family Paramyxoviridae, has broad cell tropism and grows in a wide variety of cell types (Moreno-López et al. 1986). The affinity of LPMV

for cells of the reproductive and respiratory tract, as well as cells of the central nervous system (CNS), has been reported. In a sequential study of virus distribution in tissues of pigs experimentally infected with LPMV (Allan et al. 1996), virus was detected in a variety of tissues, including midbrain, olfactory bulb, bronchial mucosa, lung, and pancreas. Considering the close relationship of LPMV to mumps virus (Berg et al. 1991, 1997; Ramírez-Mendoza et al. 1997; Sundqvist et al. 1992) and the observation that LPMV also infects pancreatic cells after experimental inoculation of pigs (Allan et al. 1996), it was decided to use the rat as a model for studies of LPMV pathogenesis in pancreatic islets and to investigate the effect of virus replication on insulin response to high glucose.

GROWTH OF PORCINE RUBULAVIRUS IN PANCREATIC ISLETS

Rat pancreatic islets were inoculated with LPMV, and cytopathic effect (CPE) was observed 2 to 3 days post inoculation (PI). Virus replication and CPE, particularly syncytial formation, in pancreatic islets were confirmed by virological, serological, and molecular methods, including hemadsorption, indirect immunofluorescence (IIF), electron microscopy, and polymerase chain reaction (PCR). We also infected pancreatic islets of murine and porcine origin, with results similar to those obtained in rats.

By dual-labeling immunofluorescence, we ascertained that insulin-producing beta cells and other endocrine cell types from pancreatic islets (including fibroblastoid cells derived from the islets) were susceptible to LPMV infection. Our data on insulin response to high glucose indicated that there was also an impairment of insulin production in infected islets at 72 hours PI. Thus, the results in this *in vitro* model indicated that LPMV infected rat, mouse, and porcine pancreatic cells where infection altered beta-cell function. It was found that LPMV infection of pancreatic islets and fibroblastoid cells was partially lytic. Furthermore, fibroblastoid cells were grown for 120 passages and, based on results from IIF and PCR assays, it was found that LPMV established a persistent infection in pancreatic fibroblastoid cells. Preliminary PCR results have indicated that persistent infection also occurs *in vivo*. In naturally

infected pigs, viral RNA was detected in samples from the pancreas, even following recovery from the disease. These results agree with the observation that other paramyxoviruses are also associated with lytic infection and noncytolytic persistent infection in cultured cells (Hjertner et al. 1997; Holland et al. 1980; Moscona 1991; Murphy et al. 1990; Randall and Russell 1991; Wiman et al. 1998).

CONCLUSIONS

Much evidence suggests an etiologic significance for viral infection in the development of diabetes. However, because of the problems of studying the role of viruses in diabetes in vivo in humans, in vitro models have been used to characterize virus replication and damage to pancreatic beta cells [see reviews by Szopa et al. (1993), Yoon (1995), and Von Herrath et al. (1998)]. Our in vitro study revealed that LPMV is capable of establishing productive replication in murine and porcine pancreatic cells. In vivo studies are in progress to identify the presence of viral RNA in tissues up to 13 months after natural LPMV infection. The type of pancreatic cell in which the virus replicates in vivo and whether there are differences in serum glucose concentration in infected and noninfected pigs remain yet to be determined. Nevertheless, LPMV infection may present a useful in vivo model for studying the effects of viral infection on endocrine pancreatic B cells, including the process of beta-cell destruction, the mechanisms of viral persistence in pancreatic cells, and the possible initiation of an autoimmune response.

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3.12 Menangle Virus: A New Cause of Fetal Mummification and Congenital Defects in Pigs

Robert Love and Peter Kirkland

SUMMARY

In 1997, a new paramyxovirus, subsequently named Menangle virus, was isolated from malformed, stillborn piglets that were a component of a severe reproductive failure, characterized principally by fetal death and mummification, that occurred in a large (3000 sow), intensive piggery. Menangle virus also infected two people working with the infected pigs, causing a severe febrile illness and macular rash. Serological investigations demonstrated that the virus had been recently introduced into the pig population, the probable source of Menangle virus being a colony of fruit bats that roosted within 200 meters of the pig-farm buildings. Menangle virus, along with Mexican blue eye disease virus (or La Piedad Michoacán virus, LPMV), is a member of the genus *Rubulavirus* of the family Paramyxoviridae. The infection does not result in a carrier state nor does the virus survive well in the environment. It persisted in the herd by infection of the growing pigs as they lost the protection provided by maternal immunity. Programmed selective depopulation and repopulation of the pig farm led to eradication of the virus from the pig population. However, infection is endemic in the fruit bat population and so the possibility of reinfection of this and other pig populations remains.

INTRODUCTION

In 1997, sows in a 3000-sow, intensive, farrow-to-finish pig farm near Sydney, Australia, experienced a severe reproductive failure characterized by the presence of mummified fetuses and stillborn piglets with malformations. A paramyxovirus isolated from stillborn piglet tissues was named Menangle virus after the locality of the pig farm (Love et al. 2001; Philbey et al. 1998).

Available evidence indicated that the source of the virus was a colony of fruit bats that roosted in trees close to the pig-farm buildings. How Menangle virus crossed the species barrier from fruit bats to pigs is not known. The virus also crossed another species barrier, infecting and causing serious illness in two pig-farm workers who were in contact with the infected pigs.

In a similar fashion and also in Australia, Hendra virus, another paramyxovirus, crossed from fruit bats to horses and on to humans, causing fatalities among both horses and humans (Halpin et al. 1999; Murray et al. 1995). Hendra virus is not closely related to Menangle virus, but is very closely related to Nipah virus, identified in Malaysia, which crossed from fruit bats to pigs and then to humans with fatal consequences (Johara et al. 2001).

Fortunately, the fruit bat/pig/human experience in Australia has been less devastating than in Malaysia, with infection limited to the one large pig farm and two associated grow-out farms and the disease in humans not resulting in fatalities. Programmed partial depopulation of the infected pig farm resulted in eradication of Menangle virus from the pig population. However, it would appear that Menangle virus is endemic in the fruit bat population and has the potential to reinfect the pig population at any time.

ETIOLOGY

The Paramyxoviridae are typically large (150 to 350 nm), pleomorphic RNA viruses with an outer fringe of "spikes" protruding from the envelope. Contained within the enveloped virion is a long, helically symmetrical nucleocapsid that has a herringbone pattern when examined by electron microscopy. The envelope protrusions consist of attachment and fusion proteins that have both neuraminidase and hemagglutinin activities in some viruses or limited activity in others. These viruses may induce the formation of vacuoles and cause fusion of the membranes of host cells, resulting in the formation of large syncytia.

Menangle virus induces pronounced cytopathic effects in cell culture, including vacuolation of cells and the formation of large syncytia. Electron-microscopic examination indicates that this virus has a morphology typical of the Paramyxoviridae. The virus grows in a wide range of cell types from many species, including cells of porcine and human origin, and is nonhemadsorbing and nonhemagglutinating in tests using erythrocytes from several species, including humans and birds. These last features distinguish Menangle virus from La Piedad

Michoacán virus (LPMV), the cause of Mexican blue eye disease (Moreno-Lopez et al. 1986).

The family Paramyxoviridae consists of two subfamilies: the Paramyxovirinae and the Pneumovirinae. Within the subfamily Paramyxovirinae are the three genera *Paramyxovirus*, *Morbillivirus*, and *Rubulavirus*. Viruses such as human and bovine parainfluenza viruses belong to genus *Paramyxovirus*; canine distemper, human measles, and rinderpest viruses to genus *Morbillivirus*; and Newcastle disease and human mumps viruses to genus *Rubulavirus*. The subfamily Pneumovirinae contains a single genus, *Pneumovirus*, and includes the respiratory syncytial viruses and turkey rhinotracheitis virus. Although the characterization of Menangle virus is not yet complete, available findings indicate that the virus is a member of genus *Rubulavirus* (Bowden et al. 2001) together with LPMV.

EPIDEMIOLOGY

The mechanism of spread, including route of transmission, of Menangle virus has not been established although fecal-oral or urinary-oral transmission is suspected. Studies of archival and newly collected sera indicated that, unlike other members of the family, the virus was not highly contagious in this group of pigs. This was suggested by the relatively slow spread of infection in a building housing pens of sows, i.e., it took several weeks for all the sows to become infected. Six months after the estimated time of entry of the virus into the affected pig farm, a high proportion (>90%) of sera collected from pigs of all age categories contained high virus-neutralizing antibody titers. Positive virus-neutralizing antibody titers ranged from 1:16 to 1:4096 and remained high for at least 2 years after infection. In contrast, all samples collected prior to the estimated time of entry of the virus into the pig population tested negative. All serum samples collected at two pig farms that were populated with only 8- to 12-week-old pigs from the affected pig farm were positive (Kirkland et al. 2001).

Testing of 1114 swine sera from other pig farms throughout Australia, including pig farms with reproductive problems, indicated that infection was confined to the affected pig farm and the two associated grow-out facilities.

Following the initial spread of the infection through the herd, the virus was maintained by infection of young pigs as they lost the protection provided by maternally derived antibodies at about 12 weeks of age. In a large pig herd such as this, the constant availability of susceptible animals was sufficient to ensure persistence of the virus in the herd. In smaller pig herds, such persistence would be unlikely. Almost all selected replacement breeding pigs had been exposed to the

virus (and were seropositive) before mating at around 28 to 30 weeks of age. Therefore, once the infection was endemic in the herd, no further reproductive failures occurred.

It appeared that close contact between pigs was required for spread of infection and that the virus did not survive in the environment for any length of time. Susceptible pigs (i.e., sentinels) that were moved into an uncleaned area occupied 3 days previously by pigs known to have been infected did not become infected.

Infection in pigs appeared to be brief (10 to 14 days) and resulted in strong immunity. Susceptible pigs introduced into the breeding herd after fertility had returned to normal did not become infected, thus providing strong circumstantial evidence that persistent infections (virus carriage) in pigs did not occur.

SOURCE OF INFECTION

During the summer-autumn period, when the virus was thought to have entered the pig farm, there was a large breeding colony of gray-headed fruit bats (*Pteropus poliocephalus*), as well as little red fruit bats (*P. scapulatus*), roosting within 200 meters of the affected pig farm. Sera collected from gray-headed fruit bats in this colony showed virus-neutralizing (VN) antibodies to Menangle virus. A more extensive study of sera collected from various species of fruit bats in New South Wales and Queensland found that approximately one-third were seropositive for the virus, with VN antibody titers ranging from 1:16 to 1:256. VN antibody-positive samples were found in gray-headed fruit bats, black fruit bats (*P. alecto*), and spectacled fruit bats (*P. conspicillatus*). None of 15 little red fruit bats sampled was seropositive. This panel of sera included positive samples collected from colonies of gray-headed fruit bats not associated with the affected pig farm. It also included samples collected prior to the disease outbreak in pigs. These findings indicated that Menangle virus was endemic in the fruit bat population and preceded the infection in pigs. A range of other species in the vicinity of the affected pig farm, including rodents ($n = 19$), birds ($n = 13$), cattle ($n = 60$), sheep ($n = 70$), cats ($n = 25$), and a dog, were all seronegative for Menangle virus.

It is of interest that the fruit bat colony has occupied this roost on a seasonal basis every year since the pig farm was established in 1968 without infection spreading to the pig population. Although Menangle virus has not yet been isolated from fruit bat specimens, it would appear to be just a matter of time. A very closely related virus named Tioman virus has been isolated from fruit bats on Tioman Island in Malaysia, but it is not known whether this virus is infectious for pigs (Chua et al. 2001).

PATHOGENESIS AND CLINICAL SIGNS

Over a 5-month period, from mid-April to early September 1997, the sows in four breeding units of the affected, 3000-sow farm experienced weeks when the farrowing rate decreased from an expected 82% to as low as 38%. Many sows returned to estrus after mating-to-return intervals consistent with early death of the whole litter, i.e., some of these sows returned to estrus around 28 days after mating whereas others remained in a state of pseudo-pregnancy until more than 60 days after mating. The disease occurred sequentially in all four breeding units at the pig farm, affecting the progeny of all parity sows. In the weeks of low farrowing rates, up to 45% of sows farrowed litters with reduced numbers of live piglets and an accompanying increase in the proportion of mummified and stillborn piglets, some of which had deformities.

Individual litters characteristically contained mummified fetuses of varying size, ranging upward in gestational age from 30 days, together with stillborn piglets (some with malformations) and a few normal piglets (Figure 3.12.1). This indicated that, as with parvovirus, transplacental infection occurred early in gestation with infection of a few fetuses followed by progressive spread of infection from fetus to fetus within the uterus. As a

consequence, different fetuses were infected and died at different gestational ages.

There were no clinical signs of infection evident in growing pigs of any age, and the only clinical signs in sows were those associated with reproductive failure.

PATHOLOGY

Affected litters usually consisted of a mixture of mummified fetuses, autolyzed, and fresh, stillborn piglets, and a few normal live piglets. Teratogenic defects, including arthrogryposis, brachygnathia, and kyphosis, were frequently seen, with occasional cases of artiodactyla. The cranium of some piglets was slightly domed.

Viruses from the family Paramyxoviridae are often associated with systemic disease and have a tropism for the respiratory tract and brain. Affected stillborn piglets frequently had severe degeneration of the brain and spinal cord, and, in some cases, the brain and spinal cord were almost absent. Despite such severe deficiency, many of these piglets grew and survived in utero and were often larger than normal because of reduced in utero competition. Gross defects were most common in the cerebrum. Occasionally, there were fibrinous body cavity effusions and pulmonary hypoplasia.



3.12.1. A litter of mummified and stillborn piglets with malformations from a sow infected with Menangle virus in early pregnancy. Reproduced with permission of the Australian Veterinary Journal.

Histological changes were most marked in the central nervous system. There was extensive degeneration and necrosis of gray and white matter of the brain and spinal cord, associated with infiltrations of macrophages and other inflammatory cells. Nonsuppurative, multifocal meningitis, myocarditis, and occasionally hepatitis were also present in some cases. Intranuclear and intracytoplasmic inclusion bodies were observed in neurons of the cerebrum and spinal cord. The inclusion bodies were eosinophilic to amphophilic. Electron microscopy showed that the inclusion bodies consisted of aggregates of nucleocapsids.

DIAGNOSIS

As Menangle virus is a newly recognized agent infectious for pigs, pig populations would be expected to be fully susceptible. The occurrence of a marked reduction in the number of normal live piglets, accompanied by the birth of mummified piglets and stillborn piglets with severe defects, would indicate that Menangle virus infection should be considered. In the first instance, the most rapid method to exclude Menangle virus infection would be to test sows for the presence of specific antibody, either by a virus neutralization test or an enzyme-linked immunosorbent assay using whole virus as antigen.

Fetal specimens should be collected for virus isolation and serology. Virus can be isolated from a number of organs from stillborn piglets, especially brain, lung, and myocardium. A wide range of cell cultures support replication of the virus, but baby hamster kidney cells (BHK21) have been used for the isolation of Menangle virus from field specimens. Three to five passages are necessary for cytopathology to be observed. Cytopathic effects include vacuolation of cells, formation of syncytia, and focal cell lysis. As the virus does not hemagglutinate, identification will depend on electron microscopy and neutralization with specific antiserum. VN antibodies may also be detected in body cavity fluids from some stillborn piglets.

DIFFERENTIAL DIAGNOSIS

The birth of litters containing mummified fetuses of varying size together with stillborn piglets is indicative of an in utero viral infection. By far, the most common cause of this syndrome in pigs is porcine parvovirus but a variety of other viral infections such as encephalomyocarditis, classical swine fever, Aujeszky's disease, Japanese encephalitis, porcine reproductive and respiratory syndrome virus, and blue eye paramyxovirus LPMV (Stephano et al. 1988) can cause this syndrome. A feature that distinguishes Menangle virus from all but Japanese encephalitis is the presence of congenital malformations. However, these were evident in only approximately a third of affected litters. Further, many of these other

viral infections cause disease in piglets and adults. The only other porcine paramyxovirus, LPMV, also belongs to the *Rubulavirus* genus, but clearly differs from Menangle virus in that LPMV agglutinates erythrocytes from mammals and birds (Moreno-Lopez et al. 1986).

CONTROL

Fruit bats are considered the primary source of infection for the pig population, so restricting direct and indirect contact with these bats is important in preventing introduction of Menangle virus to the pig population.

Fruit bats do not enter pig-farm buildings, but they defecate and urinate in flight over and around buildings, and occasionally inadvertently drop their young in flight, so roofing over all areas accessed by pigs (e.g., outside walkways) is important in risk minimization.

Flowering trees and fruiting trees should not be grown in the immediate vicinity of pig-farm buildings, as these may attract fruit bat activity.

In an outbreak of reproductive disease, the infection will probably have already spread through the entire pig-farm population by the time the first affected litters are farrowed. In small piggeries, there would be insufficient numbers of susceptible animals available to ensure that the infection was maintained, as there is no carrier state and environmental survival is poor (in contrast to parvovirus). In large piggeries, infection may become endemic, as it did in the pig farm described, with the infection being maintained in groups of pigs as they lose their maternally derived protection. In such a situation, it is important to maximize the opportunity for infection of all selected replacement breeding stock prior to mating.

ERADICATION

Menangle virus can be eradicated from an endemically infected pig population by removing to another site all of the age groups in which infection is active (e.g., pigs between 10 and 24 weeks of age). Restocking with unexposed pigs or pigs known to be immune to infection into a vacated and cleaned environment will break the cycle of endemic infection in the herd.

PUBLIC HEALTH

Although Menangle virus does not appear to be highly infectious for humans, care should be taken when working with potentially infected pigs or suspect reproductive specimens. Serological studies showed that only two of more than 30 people directly exposed to infected pigs became infected with the virus. These people experienced a severe febrile illness associated with a macular rash followed by prolonged debility (Chant et al. 1998). There was no evidence of infection in a large number of other

people, including veterinarians, abattoir workers, and laboratory workers, who had less direct and less protracted contact with potentially infective material.

This low infectivity for humans has been interpreted to mean that transmission requires the contamination of cuts and abrasions with infectious body fluids or tissues or possibly splashing of material onto the conjunctivae.

FUTURE PROSPECTS

In the last decade, a variety of new viral diseases have emerged affecting domestic animals and humans, with various species of fruit bats as the putative source. Such events would suggest a drastic change in the relationship between fruit bats and our domestic species. This may simply be a further reflection of the worldwide destruction of forest habitat, forcing the wild and domestic species into much more intimate associations.

In Australia, the fruit bat population will remain a potential source of Menangle virus infection for pigs and possibly other species, just as Nipah virus poses a continuing threat in Malaysia. We now know of the existence of these viruses and the consequences for both humans and the livestock industries. One can only speculate as to other viruses that may be lurking in fruit bat and other wildlife populations with the potential to cause devastation.

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3.13 Nipah Virus Infection in Swine

Jasbir Singh and Aziz Jamaluddin

SUMMARY

Initially assumed to be caused by Japanese encephalitis virus, outbreaks of respiratory and neurological signs in swine and encephalitis in farmworkers were discovered to be caused by a previously unrecognized paramyxovirus closely related to Hendra virus. The causative virus was named Nipah virus. The original reservoir of Nipah virus has not been definitively established, although preliminary data have shown that fruit bats of the genus *Pteropus* have neutralizing antibodies against the virus. Transmission of the virus within Malaysia was primarily by transport of infected swine. The outbreak was brought under control through a process of *stamping out* in conjunction with serological testing of swine herds.

INTRODUCTION

In late 1998 to early 1999, a disease of swine characterized by pronounced respiratory and neurological signs, sometimes with sudden death of sows and boars, was observed to spread among some pig farms in Malaysia. In the beginning, it was not recognized as a new problem because the morbidity and mortality rates were not high, and the clinical signs were not markedly different from those of a variety of other diseases known to be present in Malaysia. Furthermore, the disease in pigs appeared to be closely associated with an epidemic of viral encephalitis in pig-farm workers that was assumed to be caused by Japanese encephalitis virus. Japanese encephalitis is a mosquito-borne disease caused by a flavivirus prevalent in most countries in Asia (Burke et al. 1985; Johnson et al. 1985). Attention was again focused on the disease in pigs as numerous and stringent measures used to control Japanese encephalitis failed to contain the increasing incidence of viral encephalitis in pig-farm workers (Lim et al. 2000; Parashar et al. 2000).

From October 1998 to May 1999, a total of 265 cases of viral encephalitis with 105 deaths were recorded among people associated with pig farming, and 11 cases of encephalitis or respiratory illness with one death were reported in Singapore (Lee et al. 1999; Lim et al. 1999; Ling 1999; Paton et al. 1999). A viral agent with the morphology of paramyxovirus was isolated and later con-

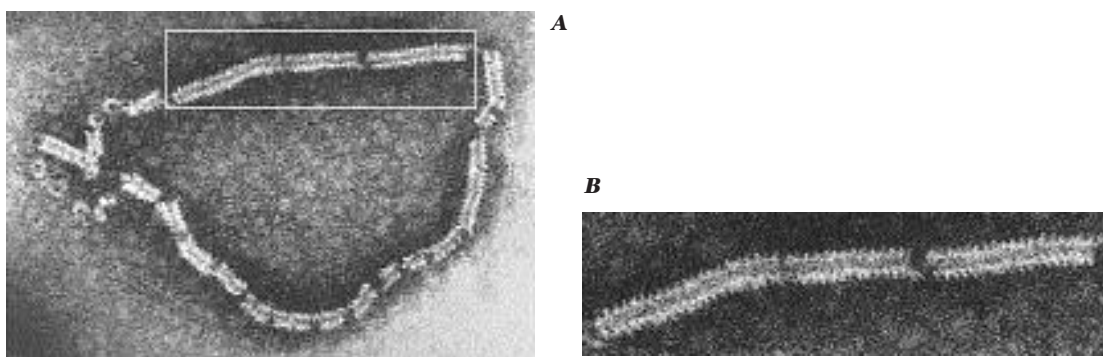
firmed to be the agent responsible for the human and pig diseases (Anonymous 1999a; Chua et al. 2000a,b; Farrar 1999). Late presentation of encephalitis was also seen in patients, even months after being exposed to the virus (Wong et al. 2001).

The name *porcine respiratory and encephalitis syndrome* has been proposed as the technical name for the disease in pigs because of the pronounced respiratory and neurological signs observed. The unusual, loud, barking cough is a characteristic feature of the disease that differs from the other known respiratory diseases of pigs, and thus “barking pig syndrome” has been suggested as a common name for the disease (Nordin et al. 2000).

ETIOLOGY

The agent is named after the village Sungai Nipah in the state of Negeri Sembilan in Malaysia, from which the virus was first isolated in a human case. Electron-microscopic, serological, and genetic studies identified the virus as an RNA virus belonging to the family Paramyxoviridae and most closely related to the recently discovered Hendra virus (Chua et al. 2000b). Hendra virus, formally known as equine morbillivirus, was first isolated during an outbreak of a severe respiratory illness that killed 14 horses and one person in Hendra, Queensland, Australia, in 1994 (Murray et al. 1995).

Molecular characterization of Nipah virus was done at the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia. Phylogenetic analyses of Nipah, Hendra, and other members of the family Paramyxoviridae demonstrated that, although Hendra and Nipah virus are closely related, they are clearly distinct from any of the established paramyxovirus genera and should be considered a new genus (Harcourt et al. 2000; McCormack 2000). Like Hendra virus, Nipah virus is unusual among the paramyxoviruses in its ability to infect and cause potentially fatal disease in a number of host species, including humans. The virus is relatively unstable in vitro and can be readily inactivated by detergents and sunlight. The virus grows well in continuous cell lines such as Vero and baby hamster kidney (BHK). Electron microscopy reveals the herringbone appearance of its nucleocapsid, typical of paramyxoviruses (Figure 3.13.1).

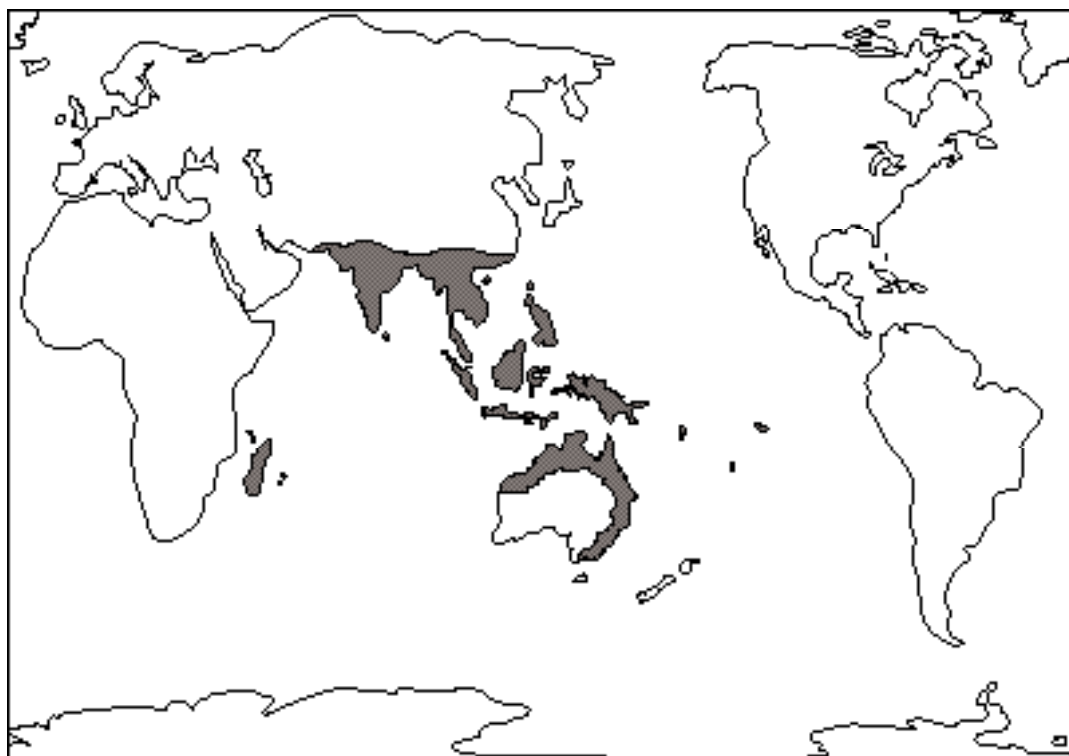


3.13.1. By transmission electron microscopy (**A**) and negative staining (**B**), the helical ribonucleoprotein capsid of Nipah virus has a herringbone appearance.

NATURAL HOSTS

Pigs, horses, dogs, and humans have been infected with the virus in Peninsular Malaysia. Other animals such as cats and goats may be infected only when exposed to infected pigs.

The origin and reservoir of Nipah virus are still not clear, although preliminary wildlife surveillance has shown fruit bats of the genus *Pteropus* to have neutralizing antibodies and identified them as a species meriting further study (Field et al. 2001; Yob et al. 2001). Fruit bats of the genus *Pteropus* are mostly found in Asia (Figure 3.13.2).



3.13.2. Natural habitat of *Pteropus* bats.

TRANSMISSION

It has been established that the mode of transmission of virus between pig farms within and between states of Peninsular Malaysia was by movement of pigs (Figure 3.13.3). Possible modes of transmission between farms within farming communities included contaminated boar semen and perhaps by dogs and cats.

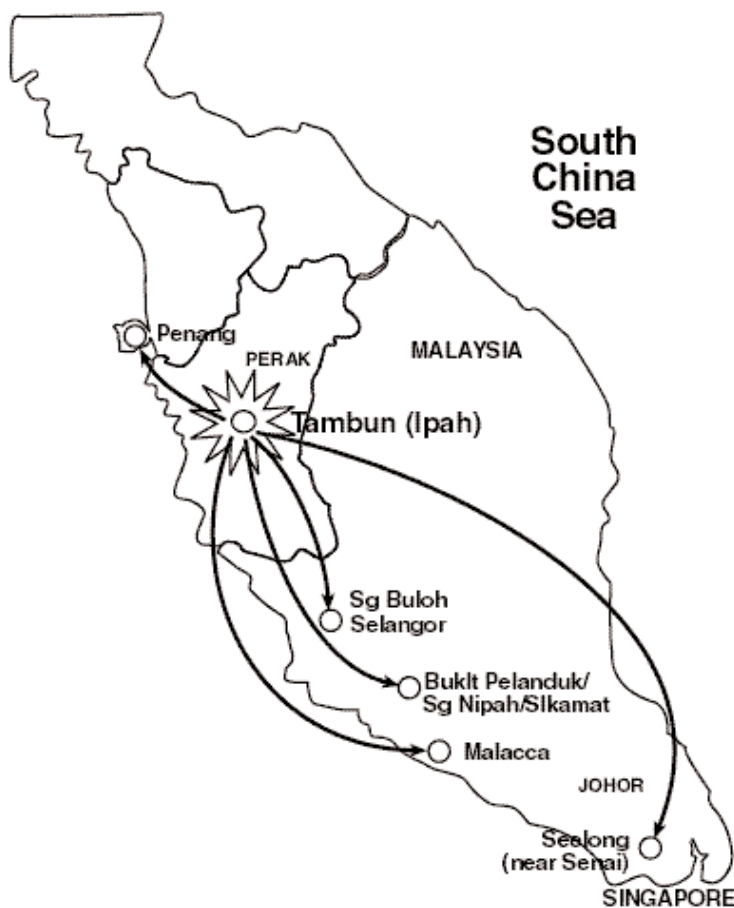
Within infected farms, the disease was observed to spread rapidly among pigs, especially as they were kept in close confinement. Transmission between pigs within a farm probably involved direct contact with virus-contaminated excretory and secretory fluids, such as urine, saliva, and pharyngeal and bronchial secretions. Mechanical transfer by dogs and cats, by the use of contaminated needles or equipment for health intervention and through artificial insemination equipment or virus-contaminated boar semen, was also implicated.

Transmission studies in pigs in the Australian Animal Health Laboratory, Commonwealth Scientific and Industrial Research Organisation (CSIRO), established that

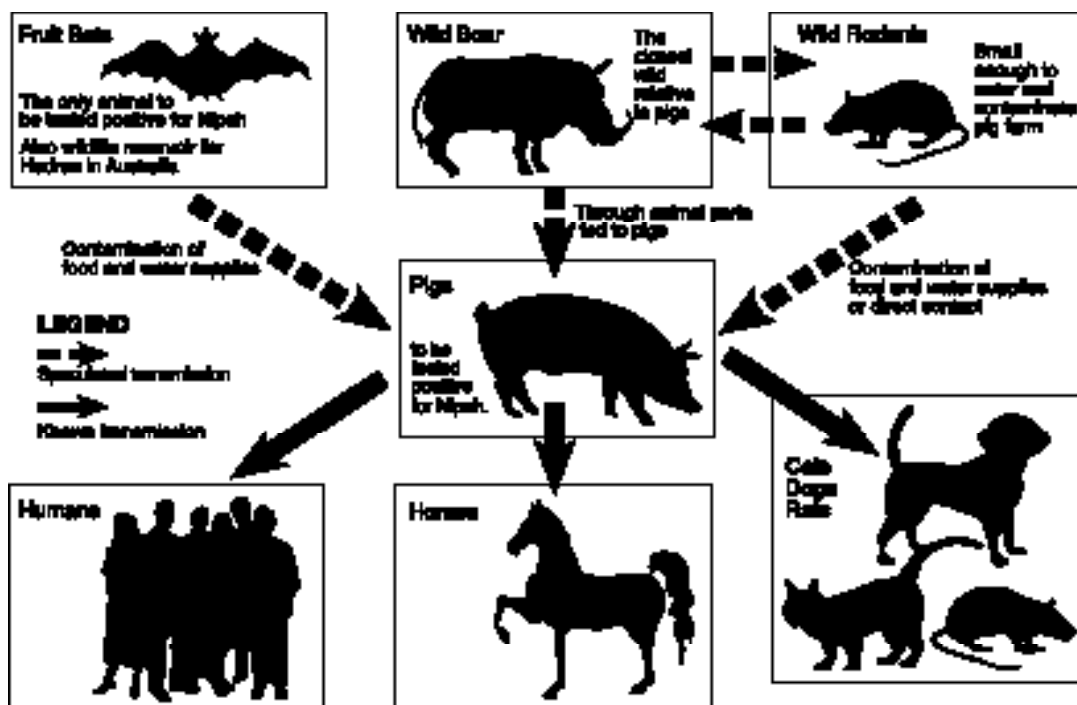
swine could be infected by the oral route (Anonymous 1999b) and parental inoculation. Oronasal excretion of virus was demonstrated, similar to Hendra virus (Hooper et al. 2001), and it was established that infection spread quickly to the in-contact pigs, with detection of neutralizing antibodies by day 14 (Middleton et al. 1999). Figure 3.13.4 illustrates possible modes of transmission of the Nipah virus.

CLINICAL SIGNS

Based on field observations of pigs in Malaysia, the clinical patterns varied with the age of the pigs. Sows presented primarily with the neurological signs, whereas in pigs at 1 to 6 months of age the respiratory syndrome predominated. Clinical disease in pigs, however, could also be very subtle. A large proportion of pigs on farms sometimes appeared to be infected asymptotically, as farmers reported that farmworkers developed disease after the pigs had recovered. The incubation period in swine was estimated to be 7 to 14 days (Middleton et al. 1999).



3.13.3. Transmission of Nipah virus among pig farms between the states of Peninsular Malaysia by the movement of pigs. From Tambun, Nipah virus spread via infected pigs to farms in Penang, Perak, Selangor, Malacca, Negri Sembilan, and Johor. Farmers in Tambun had "fire sales" to get rid of pigs.



3.13.4. Possible modes of transmission of Nipah virus.

Weaners and Porkers

Pigs between 1 and 6 months of age usually showed acute febrile ($>39.9^{\circ}\text{C}$) illness, with respiratory signs ranging from rapid and labored respiration to harsh nonproductive cough (loud barking cough). Severe cases presented with hemoptysis and less severe cases with open-mouth breathing. One or more of the following neurological signs accompanied the respiratory signs: trembles and neurological twitches, muscle spasms and myoclonus, rear leg weakness and varying degrees of spastic paresis or lameness, uncoordinated gait when driven and hurried, and generalized pain, especially in the hind quarters.

The mortality rate was low (1% to 5%), but the infection rate approached 100%. Clinically, the infection ranged from asymptomatic to fulminant. Severe cases were observed to recover, or the severity of signs to moderate, under conditions of lowered stress.

Sows and Boars

Boars and sows are similar in clinical presentation. Infection may be accompanied by sudden death or sometimes with acute febrile ($>39.9^{\circ}\text{C}$) illness with labored breathing, increased salivation (drooling or frothy), nasal discharge (serous or mucopurulent or bloody), and possible early abortion in some sows (first trimester). Some or all of the following neurological signs were also present: agitation and head pressing,

tetanus-like spasm, seizures, nystagmus, champing of mouth, and apparent pharyngeal muscle paralysis, which may explain an inability to swallow, frothy salivation, and dropping of the tongue.

Suckling Pigs (Piglets)

The disease was identified in suckling pigs, with a mortality rate of approximately 40%. However, it was very difficult to confirm whether this was due to the disease in piglets or the sow's inability to nurse the piglets. Apparently healthy, but confirmed seropositive sows were observed to nurse apparently healthy piglets. The majority of the infected piglets showed the following signs: open-mouth breathing, leg weakness with muscle tremors, and neurological twitches.

PATHOLOGY

The majority of the cases showed mild-to-severe lung lesions with varying degrees of consolidation, emphysema, and petechial-to-ecchymotic hemorrhages. The cut surface showed distention of the interlobular septa. The bronchi and trachea were sometimes filled with frothy fluid, with or without blood. Brain tissue sometimes showed generalized congestion and edema. Kidney tissue was congested in some cases, both on the surface and the cortex, but was apparently normal in many cases. Other visceral organs were apparently normal.

Histologically, the main lesion was a moderate-to-severe interstitial pneumonia with widespread hemorrhages and syncytial cell formations in the endothelial cells of the blood vessels of lung. Generalized vasculitis with fibrinoid necrosis, hemorrhages, and infiltration of mononuclear cells, sometimes associated with thrombosis, were observed, most notably in the lung, kidney, and brain tissues. Nonsuppurative meningitis with gliosis was the other significant finding in the brain. Immunohistology showed a high concentration of viral antigens in the endothelium of the blood vessels, particularly in the lung. Evidence of viral antigens in the cellular debris in the lumen of the upper respiratory tract suggested the possibility of virus transmission through expired air (Hooper et al. 2001). The virus induces syncytial cell formation in vascular tissues and is vasotropic and/or neurotropic, resulting in interstitial pneumonia or encephalitis. It is also epitheliotropic in respiratory epithelium and, thus, contagious (Hooper et al. 2001).

LABORATORY TESTS

Serological tests, such as enzyme-linked immunosorbent assay (ELISA) and the virus neutralization test, are currently available in Malaysia. The Nipah virus antigen used in the ELISA is whole virus inactivated by gamma irradiation. The test sera are inactivated by adding sodium dodecyl sulfate and Triton X-100 and by heating at 56°C for 1 hour. Laboratory diagnosis by immunohistochemistry, virus isolation, serum-virus neutralization test, and polymerase chain reaction is recommended to be carried out in a biosafety level 4 laboratory.

TREATMENT

No treatment in pigs is recommended, since the disease is transmissible to humans. In humans, the antiviral drug ribavirin has been found to reduce mortality caused by acute Nipah encephalitis (Chong et al. 2001).

CONTROL AND ERADICATION

With the discovery of the etiology of the pig disease, an immediate stamping-out policy was instituted to cull all pigs in the outbreak areas in the first phase. A total of 901,228 pigs from 896 farms were destroyed in the infected areas between February 28 and April 26, 1999. The culling of pigs in these areas successfully controlled the human epidemic in the states of Negeri Sembilan, Perak, and Selangor. The culling program was stopped when an ELISA was made available to identify infected farms in a national swine-testing and surveillance program.

The cross-reactivity between Nipah and Hendra viruses facilitated the early application of an indirect ELISA for screening. Rapid screening using an ELISA for

Hendra had shown that, on infected farms, most of the adult pig population, particularly the sows, had been exposed to infection. An indirect immunoglobulin G ELISA using Nipah virus antigen was developed in Peninsular Malaysia, and initial studies indicated that screening of sow blood gave the highest confidence of detecting an infected farm. This observation, together with the availability of ELISA for testing Nipah virus infection, formed the basis of the second phase of the national swine-testing and surveillance program that was launched on April 21, 1999.

The second phase of the program stipulated that each farm was to be sampled twice, with a minimum interval of 3 weeks between sampling. A statistically significant number of sows, with a minimum of 15, were tested on each farm. If a farm had sows housed in physically separate barns, each barn had at least six sows sampled and tested. A total of 889 farms were tested nationwide from April 21 to July 20, 1999. Among these, 50 farms were found positive. A total of 172,750 pigs from these farms were destroyed by the end of July 1999. On average, 5.6% of all the pig farms examined in Peninsular Malaysia were found to be positive for Nipah virus.

Currently, a control program is being developed to provide continued monitoring of all pigs entering abattoirs for slaughter. The program uses an ear-notching system to identify pigs and allow them to be traced back to farms of origin, if tests reveal the pigs to be infected. An ongoing educational program for farmers on the danger of the new pig disease has been undertaken. Farmers are educated on the detection of clinical signs and basic on-farm personal safety practices. They are to report immediately to the veterinary department any occurrence of abnormal morbidity or death among pigs or other animals on the farm. They are advised to avoid direct contact with sick or infected pigs or other animals and to wear appropriate protective attire, including boots and gloves, while handling pigs and excreta. They need to exercise good personal hygiene by washing hands with soap or detergent after handling pigs. Disinfectants such as sodium hypochlorite and Lysol are recommended for use in the pig farms of Malaysia.

CONCLUSIONS

The Nipah outbreak had a significant impact on the pig industry in Malaysia, with a significant reduction in the pig population and the total number of farms with swine. As of the end of July 1999, the total standing pig population was reduced from 2.4 million to 1.32 million, and the number of farms was reduced from 1885 to 829.

The episode also resulted in a dramatic change in the direction of the pig-farming industry. In the state of Negeri Sembilan, for example, where pig rearing in previously infected areas has been completely prohibited. Pig farming is only allowed in identified Pig Farming

Areas (PFAs), as drafted by the Ministry of Agriculture, Malaysia. Restocking of farms culled to control the disease is subject to state oversight. Policies and conditions for restocking previously infected farms require further study. As an alternative, farmers are being encouraged to undertake other agriculture and livestock activities.

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3.14 Nipah Virus Diagnosis and Control in Swine Herds

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SUMMARY

Nipah virus (NiV) is a dangerous zoonotic agent. Successful control depends on early diagnosis, ideally before human cases occur. This chapter outlines approaches to working safely with NiV and infected animals, the observations that may lead to a diagnosis of suspected NiV infection on farms, and the sampling and laboratory testing strategies to confirm a diagnosis. NiV control cannot be achieved by government action or legislation alone, but depends on an active partnership between the veterinary services and the farming industry. The aim is to identify and support farming practices that will reduce the risk of farms becoming infected and maximize the chance of infection being detected in a timely manner.

INTRODUCTION

The emergence of Nipah virus (NiV) encephalitis of humans marks an escalation in the severity of emerging zoonotic diseases. At least 115 people died as a result of the one outbreak. Of some 265 cases diagnosed in Malaysia before the infection was controlled, 105 were fatal (Chua et al. 2000). Outside Malaysia, two farm-workers who returned home to Indonesia (Mohamad Taha Arif, personal communication) and one abattoir worker in Singapore (Paton et al. 1999) died. Seven more people infected during the outbreak in Malaysia have died subsequently, and 20 or more surviving encephalitis cases suffer nervous sequelae (Mohd. Nordin Mohd. Nor, personal communication). Most of the human cases were infected as a result of exposure to infected pigs. The infected people were employed mainly in the pig industry and had close contact with pigs (Parashar et al. 2000). No other zoonotic infection has caused such a fatal human disease, with the risk of exposure being the normal tasks of husbandry of a livestock species on farms. The steps taken in the NiV outbreak investigation have been outlined (Daniels 2000; Daniels et al. 1999, 2000; Nor and Ong 2000).

The causative infectious agent, NiV, is a paramyxovirus new to science, first isolated from human cases of encephalitis during the outbreak (Chua et al. 1999). NiV

has subsequently been isolated from pigs and from dogs on infected pig farms (Chua et al. 2000), and experimental infections of pigs have confirmed their susceptibility to both infection and disease (Middleton et al. 2000). The virus appears to be highly contagious among pigs. They excrete virus via the oronasal routes, and asymptomatic infections among them are common. They may be considered an amplifying host for NiV.

Fruit bats of the genus *Pteropus* have been identified as the natural reservoir host for NiV. Serological studies of a range of bat species as well as other animals were conducted during the investigation of the outbreak. A significant proportion of the wild-caught pteropid bats that were sampled had specific neutralizing antibodies to NiV (Johara et al. 2001). Subsequently, NiV was isolated from the urine of a free-living colony of *Pteropus hyomelanus* in Malaysia (Chua Kaw Bing, personal communication). Experimental infections of an Australian species, *Pteropus poliocephalus*, showed that the bats supported a permissive cycle of infection with a human isolate of NiV (D. Middleton and P. Daniels, unpublished).

NiV has been analyzed molecularly and shown to be a paramyxovirus in the subfamily Paramyxovirinae and more closely related to Hendra virus (Murray et al. 1995; Wang et al. 2000) than to viruses in the recognized genera in the subfamily (Chua et al. 2000). Hendra virus is also a severe zoonotic agent causing disease in humans, horses, and cats and having pteropid bats as a natural reservoir host (Williamson et al. 1998). The level of danger to people associated with Hendra virus infections has led to its being classified as a Biological Safety Level 4 (BSL4) agent (Abraham et al. 2001). Because of the relatedness of Nipah and Hendra viruses, the similarities in their biological characteristics, and the obvious seriousness of NiV infections in people, NiV has also been designated a BSL4 agent.

WORKING SAFELY AND EFFECTIVELY DURING NIPAH VIRUS INVESTIGATIONS

Since NiV is classified internationally as a BSL4 agent, propagation of the virus is best conducted under Physical Containment Level 4 (PC4) conditions. The principles of working safely during investigations of dangerous zoonotic

agents have been reviewed (Abraham et al. 2001). A number of specific questions must be considered: What precautions are necessary during investigations on farms where NiV infection may be suspected? How may diagnostic specimens be handled in the laboratory where NiV infections are suspected but not confirmed? How should sera from suspected outbreaks be handled? Is a PC4 facility needed to initiate virus isolation in the diagnostic phase of an investigation?

On Farms

Obviously, investigations of other respiratory diseases of pigs must be conducted on a continuing basis, even in countries where NiV infections may have occurred or been suspected previously. Where there is no history of NiV infections and a low probability of their presence, routine veterinary investigations should continue in the normal way, but the veterinary profession in all countries should show leadership in practicing basic precautions while dealing with sick animals.

Just as the medical profession has developed basic universal precautions to prevent nosocomial infection of medical staff, so veterinarians have a need for a recognized approach to the handling of animals and of the samples submitted to laboratories that will minimize the risk of zoonotic disease. Development of such broadly based recommendations by national veterinary services is recommended. A basic requirement is the prevention of exposure of the skin and mucous membranes to the body fluids of sick or potentially infected animals. Hence, internal examinations and necropsies should not be conducted without gloves and other protective measures such as appropriate clothing and footwear. Depending on the circumstances, personal judgment should be exercised regarding the need for protection of the mucous membranes of the face and the need for respiratory protection.

During the initial phase of the NiV outbreak in 1997–1998, private veterinarians serving infected pig farms reported that pig-farm workers developed febrile illness after assisting sows with difficult birthing. This observation may indicate the potential role of reproductive tract fluids in disease transmission, hence requiring the need for greater awareness of potential risks in handling diseased pigs (Aziz et al. 1999b).

During the NiV outbreak in Malaysia, two types of respiratory protection were used (Daniels et al. 2000). In situations where risk of infection was highest, investigators wore a positive air-pressure respirator that pumped air through a HEPA filter and into a hood enclosing the head and face. Where the risk was considered to be less, respiratory protection was achieved using a disposable face mask that incorporated a HEPA filter. Face masks that can be adjusted to fit snugly to the skin to ensure that all inspired air is drawn through the filter are preferred.

The precautions needed for working safely on farms extend beyond issues of apparel. Thought must be given to a range of measures to ensure that the activities of the investigation do not spread the infection or increase the risk of exposure of others (Daniels et al. 2000). The approach is to define the infected and noninfected areas of the farm or district, and practice disinfection where people, equipment, samples, and rubbish or used clothing are moved across the boundary of the designated infected area. Where necropsies are conducted, appropriate arrangements must be made for disposal of carcasses.

In the Laboratory

Banning attempts at virus isolation at less than PC4 from cases where NiV is just one of the differential diagnoses would not be practical. Laboratories should consider carefully what can be done safely in their facilities and develop written standard operating procedures (SOPs) that are approved by senior management and in which staff are regularly trained and retrained. In the case of Malaysia, the health and veterinary authorities have commonly agreed on the development of at least a PC3 facility to handle animal disease diagnostic cases suspected of NiV (Shamshad et al. 2000).

Primary virus isolation from new cases of NiV infection has been conducted in some laboratories under PC3 conditions. At the University of Malaya, a PC3 laboratory was developed for virus isolation from new Nipah encephalitis cases. Where a cytopathic effect with the formation of large syncytia develops in such cell cultures, the local SOPs must be followed to ensure operator safety. At the Australian Animal Health Laboratory, these cultures are transferred to a PC4 laboratory. Where PC4 facilities are not available, it is recommended that the culture be forwarded to an international reference laboratory.

Another point at which safety measures should be applied is in the handling of porcine sera for NiV investigations. Irradiation is an option. Alternatively, sera may be treated by heat inactivation at 56°C for 30 minutes and diluted 1:5 in phosphate-buffered saline containing 0.5% Tween-20 and 0.5% Triton X-100 prior to testing (Daniels et al. 2001).

In general, diagnostic tests that do not require growth of the virus are preferred where a PC4 laboratory is not available. Hence, enzyme-linked immunosorbent assays (ELISAs) are preferred over serum neutralization tests for serology, and immunohistochemistry (IHC) is preferred as an initial test for the presence of viral antigen, rather than routine propagation of virus by virus isolation.

Preparedness based on planning and establishment of lines of communication with reference laboratories is an ideal method of approach. Hence, SOPs can be developed that use a stepwise diagnostic approach, with more dangerous procedures being undertaken only if the results of

less dangerous screening tests indicate a need. Built into the SOP should be the sampling strategy to be adopted in suspect outbreaks or during surveillance, to ensure that an adequate number of sera are collected, an adequate number of animals necropsied, and an appropriate range of tissues collected. Recommendations for some SOPs can be found in Nor and Ong (2000) and Daniels et al. (2000), and a more complete discussion of the diagnostic tests can be found in Daniels et al. (2001) and below.

THE DIAGNOSTIC APPROACH ON-FARM

Clinical Signs in Swine

Although many NiV infections in pigs were asymptomatic, many farmers reported a recognizable clinical syndrome in their herds immediately prior to the occurrence of human disease. These observations, supported to a limited extent by laboratory confirmation of diagnosis and by experimental infections, have been distilled into a clinical case description to aid in the early recognition of NiV infections in swine herds (Aziz et al. 1999a; Nor et al. 2000). It is currently believed that NiV will infect pigs of all ages and induce clinical signs, depending on the husbandry situation. Respiratory disease with marked coughing was seen in weaners and growers able to move around in their pens, whereas death without such premonitory signs occurred in a small proportion of individually stalled animals (Shahirudin et al. 1999). Abortions were reported in sows. Where clinical signs were observed prior to death, there were manifestations of central nervous system disease. There was a small but significant increase in mortalities of both breeding stock and younger animals in infected herds.

Involvement of Other Species

Dogs were reported by farmers to be affected by a fatal disease at the same time as the pigs during the Malaysian outbreak. Two such animals were examined and found to be showing a distemper-like syndrome. NiV was isolated from the tissues of one and NiV antigen demonstrated in both by IHC on tissue sections (Chua et al. 2000). Serological studies showed that up to 50% of dogs in the infected areas had seroconverted to NiV. Similarly, farmers reported cats to be affected. The susceptibility of cats was confirmed by experimental infections (Deborah Middleton, personal communication).

A limited survey of small mammals that included rats (*Rattus* species), house shrews, and *Tupaia glis* trapped around an infected pig farm indicated that these animals were not infected by NiV. Hence, their involvement in the spread of NiV to pigs is unlikely (Asiah et al. 2001).

In countries or situations where NiV infections may be suspected, the occurrence of disease in pigs consistent with the case description accompanied by unexplained sickness and the death of dogs and cats should be regard-

ed with concern. Similarly, the occurrence of human disease characterized by the early signs of encephalitis (Chua et al. 1999) on a farm with a suspect clinical syndrome in pigs should be viewed as a potential disease emergency.

Epidemiological Aspects

NiV in Malaysia was spread from farm to farm by the movement of infected pigs. A check for any violation of farm biosecurity should be conducted.

Since it is believed that NiV infections have been eradicated from the Malaysian pig herd, it is most probable that any new outbreak of NiV infection will be as a result of a new jump of the virus from the pteropid wildlife reservoir. It should be a part of any future investigation to establish whether contact could have occurred between affected pigs and fruit bats. Factors to consider would be the system of housing the pigs, and the presence of fruit or flowering trees that could attract foraging bats to the vicinity of the farm.

Sampling During the Farm Investigation

Necropsies should be conducted of recently dead and acutely diseased pigs. Animals chosen should be representative of the affected ages and types, and should include a number of animals to increase the sensitivity of the sampling procedure. The postmortem changes of NiV infections in pigs are relatively nonspecific. There may be consolidation of lungs and frothy or blood-tinged exudate in the airways. Tissues should be taken unfixed for possible virus isolation from lung, spleen, kidney, and central nervous system. Inclusion of the tonsil in this series may be useful. Formalin-fixed tissues for histopathology and IHC should be taken from a range of organs, including several levels of the lungs and major airways.

Serology is a useful aid to diagnosis in NiV investigations, as the finding of specific antibodies is always significant. In determining a sampling strategy, it should be remembered that NiV is highly contagious on a pig farm. In experimental studies, pigs have shown disease approximately 7 days post inoculation (PI) and seroconverted 10 to 14 days PI (Middleton et al. 2000). By the time a farm is suspected of being infected, it is likely that a proportion of pigs will have antibody. As an approximate guide, if it is expected that more than 20% of the pigs could have seroconverted, collection of serum samples from 15 of each of the adult, grower, and weaner age groups should give a 95% probability of detecting seropositive animals (Cannon and Roe 1982).

LABORATORY DIAGNOSTIC TESTS

Serology: Serum Neutralization Tests

The serum-virus neutralization test (SVN) is accepted as the reference serological test, but is performed only in

PC4 laboratories. In surveillance programs and in the development of a diagnostic capability, developing a partnership with an international reference laboratory is recommended. Establishment of an ELISA capability will require standardization of the ELISA in each participating laboratory, which involves testing of panels of sera from each country in parallel in both tests.

Serology: ELISAs

To manage the emerging paramyxoviruses, like NiV, in epidemiological studies and for surveillance, there is a need for serological tests that can be conducted safely and quickly without access to PC4 facilities. ELISA meets these requirements (Daniels et al. 2001).

Problems with nonspecific reactions have been encountered in the ELISA, and the test is undergoing refinement to improve the specificity relative to the SVN. In the initial work in Malaysia, a simple indirect test configuration was used, with a specificity of 98.4% during the later phases of the National Swine Surveillance Program (Ong et al. 2000). After active foci of NiV transmission had been identified and removed, the need was for a test with even greater specificity so that reactors in the test would not cause alarm in public health forums. Again, issues of sensitivity of the testing procedure can be addressed through careful epidemiological design of the sampling strategy (Nor and Ong 2000; Ong et al. 2000).

In the ELISA configuration currently being used, control antigen from uninfected Vero cells is prepared in a manner identical to that used for virus-infected cell lysates. This is used both in a blocking or preabsorption step and as a mock antigen in parallel with viral antigen on the test plates. In this way, any high levels of nonspecific binding are removed or identified (Muniandy 2001). Recombinant NiV G and M protein antigens, generated using baculovirus expression systems, have been used experimentally, but have not yet been adopted routinely.

Where laboratories are establishing an ELISA capability, it is recommended that, as well as standardizing against the positive and negative controls, a reference panel of at least 500 sera be established that is representative of the type of sera to be routinely tested in that country. This reference panel can be used in refining test performance in the new laboratory. A random selection of the 500 can be tested by SVN to give assurance that there is a low probability of sera with NiV antibodies in the panel, and the panel of 500 presumed NiV-negative sera used while test parameters are fine tuned. When the test is considered ready for application, an estimate of test specificity relative to the SVN can be calculated from the results achievable with the panel.

As previously indicated, the ELISA can be a most useful aid in the diagnosis of infected farms. The test is also a useful surveillance tool. The surveillance program should be designed carefully, based on epidemiological

principles, and in the knowledge that ELISA screening does not have 100% specificity. There will be false positives. The response to such ELISA reactors must be planned with the relevant veterinary and public health authorities in advance. For pig producers, false positives in the ELISA create much anxiety, whereas, for the public health care providers, the possibility of false negatives is more worrying (Mokhtar et al. 2000).

Histopathology, Electron Microscopy, and Immunohistochemistry

The pathogenesis of NiV infection involves primarily vascular endothelium in all species and, particularly in the pig, the respiratory epithelium (Hooper et al. 2001). However, the histopathologic changes are not pathognomonic. Although syncytia formation is a feature of NiV histopathology, these structures are not identifiable in all cases. Syncytia may be observed in lymphoid tissue (Hooper et al. 2001). Electron microscopy (EM) of tissues from affected pigs has been useful in confirming NiV infections by demonstrating the presence of a viral agent with a morphology typical of paramyxovirus (Hyatt et al. 2001).

Immunohistochemistry is highly recommended for initial NiV diagnosis. It is one of the safest of tests because it is performed on formalin-fixed tissues. Since the primary pathology occurs in the vascular endothelium, viral antigen can be detected in a wide range of tissues. Importantly, the diagnostic submission should include a range of tissues, not just lung. Viral antigen has been detected in porcine meninges (but not brain tissue), lung, trachea, and kidney (Daniels et al. 2001). In pregnant animals, the uterus, placenta, and fetal tissues should be included.

It should be remembered that IHC, in common with most laboratory tests, will suffer from not being perfectly sensitive or specific. Issues of sensitivity can be addressed by submitting an adequate number of animals to necropsy, perhaps at an interval of a few days if disease is progressing on the farm. In addition, an adequate range of tissues should be sampled from each animal. Laboratories planning to use IHC diagnostically should practice the test, keeping records of their observations. There will be apparent reactions that are difficult to interpret, and the specificity of the test in any laboratory will be greatly improved if the operators maintain a familiarity with the range of conditions and artifacts that are normal in their region. Consultation and sharing of specimens with colleagues internationally are recommended as a means of mutual self-help and is one of the key points in development of a laboratory quality-assurance system.

Virus Isolation

In confirming any new NiV outbreak, virus isolation will be necessary at some point. Each country must decide

whether it will undertake that task or send specimens to international reference laboratories with PC4 facilities. Proof that wildlife species act as natural hosts of the viruses also requires virus isolation from captured animals.

NiV grows well in Vero cells and develops characteristic syncytia with the nuclei arranged around the periphery of the multinucleated cell. This arrangement differs from most syncytia seen in cell cultures with the closely related Hendra virus (Bryan Eaton, personal communication). Brain, lung, kidney, and spleen should be cultured. Cytopathic effect usually develops within 3 days, but two 5-day passages are recommended before discontinuing the attempt.

Identification of virus isolates may be attempted by immunostaining of fixed, infected cells, neutralization with specific antisera, polymerase chain reaction of culture supernatants (a procedure that requires stringent quality control to avoid spurious results), and EM. Suspected new isolates should be sent to an international reference laboratory for molecular characterization. Teamwork among the international community is recommended in the handling of these emerging infections.

MANAGING PIG INDUSTRIES FOR FREEDOM FROM NIPAH VIRUS INFECTION

An Industry-Government Alliance

Experience in Malaysia has shown that successful management of the pig industry for freedom from NiV requires a partnership approach among government agencies, the industry representatives, and the individual farmers. The expectations that each group has of the others can be defined through consultation and communication. Ultimately, the government must legislate and the industry must adopt methods of operation that will protect individual farms from infection and prevent any new infection from spreading from farm to farm.

Biosecurity of Farms

Farm-gate biosecurity is obviously an important first principle. Where it is necessary to purchase new breeding stock, the methods for the introduction of these animals to the herd must be clearly defined. This step may include serological testing, depending on the agreed protocol between government and industry.

Herd Health Monitoring

Early detection of newly infected farms is an important prerequisite for public health, and herd health monitoring has an important role to play. Early recognition of syndromes consistent with the clinical case description (Nor et al. 2000) followed by laboratory testing will be the most efficient means of containing any future potential outbreak. Full implementation of this approach im-

plies a strong involvement of veterinarians, probably employed as consultants to the farms, who have skills in epidemiology for the management and analysis of such farm records.

Serosurveillance

The aforementioned activities are necessary to maintain freedom, but in addition the general public and trading partners may also require active demonstration of freedom. For some period after an outbreak, this will involve serological surveillance, as has been applied successfully in Malaysia (Muniandy 2001; Ong et al. 2000). The design and management of such programs will again require veterinarians trained and proficient in epidemiological procedures. Cost-effective sampling strategies applicable to local circumstances have to be designed and acceptance of the suitability of the program negotiated with the client bodies—public health agencies, trading partners, or Office International des Epizooties (OIE), as the case may be. A laboratory testing capability has to be established and maintained, as previously discussed. Test results have to be interpreted by using epidemiological principles and knowledge of test performance. Ideally, a partnership with an international reference laboratory will enable follow-up testing on any samples giving results of concern.

CONCLUSIONS

Among the emerging viruses in Asia and the western Pacific, it is the viruses that have been identified in pteropid bats that are of most concern and, among these, NiV has been the most dangerous.

There are good indications that NiV has been eradicated from the Malaysian pig herd, but ongoing surveillance is necessary to increase confidence that this is the case. NiV preparedness is warranted in those countries with pteropid bats. Laboratory tests that do not require propagation of this BSL4 virus are best for preliminary screening. All tests will be subject to false-positive and false-negative reactions, and programs applying these tests should be designed according to epidemiological principles to take account of this. International partnerships where networks of labs assist one another in the quality control of laboratory tests, especially external proficiency testing programs, have an important role to play.

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4 AFRICAN SWINE FEVER VIRUS

4.1

African Swine Fever

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SUMMARY

African swine fever (ASF) is caused by an icosahedral, complex DNA virus classified as the only member of the family Asfarviridae and the genus *Asfivirus* (Murphy et al. 1999). ASF is a very complex viral disease that affects only porcine species (both wild and domestic), producing clinical signs and lesions ranging from acute to a subacute, chronic, and/or inapparent. Montgomery first described the disease in Kenya in 1921. Clinically, acute and subacute forms of ASF may resemble a variety of other swine hemorrhagic diseases, and, especially, it can be easily confused with classical swine fever (hog cholera) and erysipelas. Laboratory tests are required to establish a correct diagnosis. The disease is endemic in many sub-Saharan African countries. In Europe, it is still endemic on the island of Sardinia (Italy), and a new outbreak was identified in the Alentejo (Portugal) in November 1999. No treatment or effective vaccine is available against ASF, and disease control is based on rapid laboratory diagnosis and the enforcement of strict sanitary measures.

INTRODUCTION

African swine fever is classified as a List A disease by the Office International des Epizooties (OIE) because of the potential for serious and rapid spread and resultant socioeconomic consequences of great magnitude on the international trade of animal and swine products.

ETIOLOGY

The ASF virus (ASFV) is a complex icosahedral DNA virus that exhibits features common to both the iridovirus and poxvirus families. It is the only member of the family Asfarviridae and the genus *Asfivirus* (Murphy et al. 1999).

Size and Structure

By electronic microscopy, viral particles show an average diameter of 200 nm (Breese and De Boer 1966). The virion is formed by several concentric structures with an external hexagonal envelope (Carrascosa et al. 1984). The virus has a double-stranded linear DNA composed of between 170 and 190 kilobases (kb), depending on the

virus strain (Blasco et al. 1989), with terminal inverted repeats (Sogo et al. 1984), a conserved central region of about 125 kb, and variable ends (Blasco et al. 1989). The complete analysis of the sequence of ASFV strain BA71 has been reported (Yañez et al. 1995). The ASF viral DNA is composed of 170,101 nucleotides, contains 151 open reading frames, and encodes five multigene families.

The ASFV has a very complex structure. More than 100 infectious proteins have been identified in infected porcine macrophages, and at least 50 of them react with sera from infected or recovered pigs (Alcaraz et al. 1992). Some of these proteins are very antigenic, i.e., p73, p54, p30, and p12. Even though protection is not induced by these proteins, they are very good reagents as antigens for ASF serodiagnosis (Sánchez-Vizcaíno 1999).

Virus Replication and Persistence

The main target cells for ASFV replication are monocytes and macrophage cells (Malmquist and Hay 1960; Minguez et al. 1988). Virus replication has also been observed in endothelial cells (Wilkinson and Wardley 1978), hepatocytes (Sierra et al. 1987), renal tubular epithelial cells (Gomez-Villamandos et al. 1995), and neutrophils (Carrasco et al. 1996). No infection has been described in T- or B-lymphocytes (Gomez-Villamandos et al. 1995; Minguez et al. 1988).

The ASFV has also been adapted to grow in several stable cell lines, including VERO, MS, and CV (Hess et al. 1965). The virus also replicates in nature, principally in some soft ticks: *Ornithodoros moubata* (Plowright et al. 1970) and *Ornithodoros erraticus* (Sánchez Botija 1963).

The ASFV is very resistant to inactivation in the environment, particularly temperature and acid pH. The virus can be isolated from sera or blood stored at room temperature for 18 months. However, it is inactivated by heat treatment at 60°C for 30 minutes (Plowright and Parker 1967) and by many lipid solvents and commercial disinfectants. In meat products, ASFV may persist for several weeks or months in frozen or uncooked meat. In cured or processed products, such as Parma ham, infectious virus was not demonstrated after 300 days of processing and curing (McKercher et al. 1987). Spanish cured pig-meat products, such as Serrano hams and Iberian hams and shoulders, were free of viable ASFV by day

140 and Iberian loins by day 112 (Mebus et al. 1993). In cooked or canned hams, no infectious ASFV has been found when these products were heated to 70°C.

EPIDEMIOLOGY

Montgomery first described ASF in Kenya in 1921 when the virus spread from infected warthogs (*Phacochoerus aethiopicus*) to domestic pigs (*Sus scrofa*), causing a disease with a 100% mortality rate. Since then, it has been reported in a large number of African countries, including Angola, Zimbabwe, Sudan, the Republic of South Africa, Mozambique, Santo Tome, and Principe, where ASF has been shown to be endemic. In 1957, ASF was detected for the first time outside the African continent. It appeared in Lisbon as a peracute disease with a mortality rate of almost 100% (Manso Ribeiro et al. 1958). In 1960, the disease reappeared near Lisbon, apparently as a new outbreak, and spread through the rest of Portugal, reaching Spain the same year (Polo Jover and Sánchez Botija 1961). Portugal and Spain remained endemically infected until 1995, when, after a strict and successful eradication program, they were declared ASF free. In 1978, ASF again was detected outside of Africa, reaching Malta, Sardinia (Italy), Brazil, and the Dominican Republic. It appeared in Haiti in 1979, and in Cuba in 1980.

The ASF is currently present in Africa, mainly in countries located south of the Sahara, and, in most, the disease is endemic. In Europe, ASF is still endemic in Sardinia (Italy), and a new outbreak was reported in Portugal in November 1999. Elsewhere, the disease has been successfully eradicated.

Reservoirs and Susceptible Animals

Pigs are the only domestic animal species that is naturally infected by ASFV. European wild boars are also susceptible to ASFV infection, with clinical signs and mortality similar to those observed in domestic pigs in Spain and Portugal (Sánchez Botija 1982) and in Sardinia (Italy) (Contini et al. 1982), as well as in experimentally infected feral pigs (McVicar et al. 1981). In contrast, in the African wild boar population, ASFV usually induces an inapparent infection in three wild boar species: Warthogs (*Phacochoerus aethiopicus*), Giant forest hogs (*Hylochoerus meinertzhageni*), and bushpigs (*Potamochoerus porcus*).

The ASFV also replicates in two different species of soft ticks, which are considered ASFV reservoirs and vectors: *Ornithodoros moubata* in Africa (Plowright et al. 1969) and *O. erraticus* in the Iberian Peninsula (Sánchez Botija 1963). Other tick species widely distributed in North and South America have also been identified as capable of harboring and transmitting ASFV (Groocock et al. 1980), and a new species of soft tick present in Africa, *Ornithodoros savignyi*, can experimentally transmit ASFV to domestic pigs (Mellor and Wilkinson 1985).

Epidemiological differences exist in ASFV transmission between Africa and Europe. In Africa, ASFV usually induces an inapparent infection in wild boar species with low levels of virus in tissues and low or undetectable levels of viremia. Virus levels are sufficient for transmission to domestic pigs through a biological vector, *O. moubata*, but usually not by direct contact between animals. Thus, ASFV is maintained in Africa by a cycle of infection between the wild boars and soft ticks. Only when domestic pigs are present are clinical signs and mortality observed. In contrast, in Europe, wild pigs are susceptible to ASFV infection, but, in this case, the animals show clinical signs and mortality similar to those observed in domestic animals. The epidemiological dynamic observed between the wild boars and domestic pigs in Europe is very similar. Therefore, direct transmission by contact between sick and healthy animals is the most common route. Indirect transmission by biological vectors, like *O. erraticus*, has also been described in the Iberian Peninsula, especially in outdoor pig productions. Another important difference between Africa and Europe is related to ASFV replication in soft ticks. In Africa, *O. moubata* transmits ASFV by both transovarial and transtadial routes (Plowright et al. 1970), whereas, in Europe, only transtadial transmission has been observed in *O. erraticus*.

IMMUNOLOGY

The mechanisms involved in immunity to ASF are not well understood, and many questions remain unresolved. The fact that great variability is observed among the different ASFV isolates and that the main target cells for the ASFV replication are cells of the immune system (monocytes and macrophages cells) could be related to the persistence and the evasion mechanisms. On the other hand, although some studies have shown that cell-mediated immunity to ASFV infection may be effective (Martins and Leitaó 1994; Sánchez-Vizcaíno et al. 1981), the precise role of cellular immunity is still uncertain.

High levels of specific antibodies are produced during ASFV infection. Immunoglobulin M (IgM) can be detected by 4 days post infection (PI) and IgG by 6 to 8 days PI (Sánchez-Vizcaíno 1999). Antibodies are detectable for a long period following the initial exposure. Several studies have shown that virus-specific antibodies delay the onset of clinical signs and reduce the level of viremia, thereby protecting the pigs from lethal infections (Onisk et al. 1994). However, antibodies do not possess the capacity to fully neutralize the virus (Gomez-Puertas et al. 1996).

PATHOGENESIS

The entrance of ASFV into pigs is normally through oral or nasal routes. Other routes, such as cutaneous

scarification, intramuscular, subcutaneous, intraperitoneal, or intravenous injections and tick bites, have also been described (Colgrove et al. 1969; McVicar 1984). The incubation period varies widely (4 to 19 days), depending on the ASFV isolate and the route of exposure. The primary sites of replication are the monocytes and macrophages of lymph nodes nearest to the virus entrance. The monocytes and macrophages of tonsils and mandibular lymph nodes are the first affected when the exposure is oral. From these sites, the virus spreads through the blood associated with erythrocyte cell membranes and/or lymphatic drainage. Viremia usually begins 4 to 8 days PI and, due to the absence of neutralizing antibodies, persists for a long period, even months. As the virus reaches different organs, i.e., lymph nodes, bone marrow, spleen, kidney, lung, and liver, a second replication takes place.

CLINICAL SIGNS AND LESIONS

Clinical signs associated with ASFV infection are highly variable, depending on the virulence of the virus isolate and the breed of pig. African ASFV isolates usually induce peracute and acute disease, whereas the European ASFV isolates range from an acute to subacute, chronic, and/or inapparent disease. European domestic pigs and wild boars are very susceptible and exhibit a wide range of clinical disease, from acute and subacute to chronic. African wild boars are very resistant to infection and normally do not have lesions (Oura et al. 1998).

The acute form of the disease is usually characterized by fever (40°C to 42°C), loss of appetite, leukopenia, and thrombocytopenia. In white pigs, reddening of the skin at the tips of ears, chest, and abdominal areas is frequently observed. Vomiting and hemorrhagic diarrhea may occur. Anorexia, cyanosis, and incoordination may be present 1 or 2 days before death. Abortion in pregnant sows is also frequently described. Acute ASF results in mortality rates of 90% to 100% (Mebus et al. 1983; Moulton and Coggins 1968).

The lesions observed in the acute and peracute forms primarily consist of extensive hemorrhages in lymph nodes (mandibular, renal, and gastrohepatic), spleen, kidney, and sometimes in the heart. Lymph nodes are hemorrhagic, edematous, and friable. The spleen is enlarged, infarcted, and friable. Petechial hemorrhages are observed in the renal cortex, medulla, and renal pelvis of the kidney. Hydropericardium with petechiae in epicardium and endocardium is usually described. Other lesions are petechiae in the mucous membrane of the urinary bladder, larynx, and pleura. Congestion of the liver, edematous lungs, and fluid in the abdominal and thoracic cavities are frequently reported (Gomez-Villamandos et al. 1996; Sánchez Botija 1982).

Milder lesions than those described for the acute form characterize the subacute form and include extensive hemorrhages in lymph nodes and kidney, enlarged and hemorrhagic spleen, and pulmonary congestion, edema and, in some cases, interstitial pneumonia (Arias et al. 1986).

The chronic form is characterized by a variety of clinical signs and mortality rates from 2% to 10%. Clinical signs include respiratory signs, abortions, arthritis, and chronic skin ulcers or necrosis. Overall, the clinical signs associated with the chronic form do not resemble the typical clinical picture of ASFV infections. The lesions in the chronic forms may be minimal or absent (Gomez-Villamandos et al. 1995; Mebus et al. 1983). Histopathologic findings are characterized by enlarged lymph nodes and spleen, pleuritis and fibrous pericarditis, and infiltrated pneumonitis. Focal caseous necrosis and mineralization of the lung have also been described (Mebus et al. 1983).

DIAGNOSIS

The clinical signs and lesions caused by ASFV infection are very similar to lesions associated with other hemorrhagic diseases of pigs. Therefore, laboratory confirmation is essential to establishing the diagnosis of ASF (Sánchez-Vizcaíno 1999).

Laboratory Diagnosis

A wide variety of laboratory techniques are available for the detection of ASFV and/or specific antibody. Most of these assays have been successfully used in control and eradication programs. The assays most commonly used for virus detection are direct immunofluorescence (Bool et al. 1969), hemadsorption (Malmquist and Hay 1960), and the molecular detection of ASFV by polymerase chain reaction (PCR) techniques (Wilkinson 1996).

Direct immunofluorescence (DIF) is based on the demonstration of viral antigen in impression smears or frozen tissue sections with an immunoglobulin conjugated against ASFV. It is a fast (1 hour), inexpensive test with high sensitivity for acute ASF. For subacute or chronic forms, the DIF test exhibits a diagnostic sensitivity of only 40%. This decrease in sensitivity seems to be related to the formation of antigen-antibody complexes that do not allow the reaction with the ASF conjugate (Sánchez-Vizcaíno 1986). The use of DIF together with an indirect immunofluorescence makes it possible to detect 85% to 95% of all ASF cases (acute, subacute, and chronic) in less than 3 hours (Sánchez-Vizcaíno 1986).

The hemadsorption test (HAD) is a universal technique that is used for ASFV identification due to its sensitivity and specificity. The HAD is based on the hemadsorption characteristics that most ASFV isolates induce in infected pig macrophages in the presence of the erythrocytes; that is, a characteristic rosette around the

infected macrophages develops before a cytopathic effect appears. It is important to point out that a small number of field strains produce a cytopathic effect without producing the hemadsorption phenomenon (Sánchez Botija 1982). These strains are identified by using the direct immunofluorescence test on the sediments of these cell cultures. The HAD is relatively inexpensive, but ASF-free pigs and sterilized facilities are needed.

ASF-DNA Detection

The detection of the ASFV genome by PCR has been developed with the use of primers from a highly conserved region of the viral DNA. PCR detects a wide range of ASFV isolates belonging to all the known virus genotypes, including both nonhemadsorbing isolates and low-virulence strains (Wilkinson 1996). This test is particularly useful for detection of viral DNA in poorly conserved or putrefied tissues or samples where the virus may have been inactivated. It is an excellent and relatively rapid diagnostic technique, but is expensive, and the necessary equipment is still not available in all laboratories.

Antibody Detection

Since effective vaccines are not available, the presence of ASFV-specific IgG is diagnostically significant. ASFV-specific serum antibody is detectable in blood from 6 to 10 days after inoculation for long periods, even years. Thus, serum antibodies are useful for studying subacute and chronic forms of ASF, as well as for ASF eradication programs (Arias and Sánchez-Vizcaíno 1992). Several techniques have been adapted to ASF antibody detection, but the most common, practical, and inexpensive tests are the indirect fluorescent antibody (IFA) test (Bool et al. 1969; Sánchez Botija et al. 1970), enzyme-linked immunosorbent assay (ELISA) (Sánchez-Vizcaíno et al. 1982), and immunoblotting (IB) assay (Arias et al. 1993; Pastor et al. 1989).

The IFA test is a fast technique with high sensitivity and specificity for the detection of ASF antibodies from either sera or tissue exudates. It is based on the detection of ASFV-specific antibodies that bind to a monolayer of cells infected with an adapted ASFV. The antibody-antigen reaction is detected by adding a protein A fluorescein-labeled conjugate and examining under an ultraviolet-light microscope.

The ELISA is the most useful assay for large-scale serological studies. It detects anti-ASFV antibodies that react with the viral proteins that are attached to a solid phase. The reaction is visualized by the addition of protein A conjugated to an enzyme. A visible color reaction occurs when it reacts with the appropriate substrate (Sánchez-Vizcaíno et al. 1982).

The IB assay is a highly specific, sensitive, and easy-to-interpret test that has been successfully used as an alternative to the IFA for low or doubtful ELISA sera confirmation (Arias and Sánchez-Vizcaíno 1992).

The samples that should be collected for ASF laboratory diagnosis are lymph nodes, kidney, spleen, lung, blood, and serum. Tissues are used for virus isolation (HAD test), antigen detection (DIF test), and detection of viral DNA (PCR), while blood is used for virus isolation and viral DNA detection. Serum is used for antibody detection by IFA, ELISA, or IB assay. Tissue exudates can be used for viral detection by PCR and for antibody detection by aforementioned serological tests.

PREVENTION

Due to the significant economic losses caused by ASF and the great cost of eradication (the last 5 years of the ASF Spanish eradication program budget was about \$92 million US), preventive measures have to be established in order to avoid the disease gaining entry into free areas. Epidemiological studies have demonstrated that the key points to ASF entry in free areas are primarily related to feeding pigs with contaminated garbage from international airports and seaports. Thus, all food leftovers from planes and ships should be incinerated or efficiently sterilized.

In endemic areas outside of Africa, like Sardinia, where chronic or inapparent forms are present, the prevention of ASFV dissemination should be based on the control of animals and the detection of carrier pigs. In endemic areas of Africa, the most important factor is the control of the natural reservoirs (*O. moubata* and wild pigs) in order to prevent contact with domestic pigs.

In ASF outbreaks, all animals on affected farms should be rapidly and cleanly slaughtered. The proper disposal of cadavers is critical. Other additional important points include cleaning and disinfection of affected farms, designation of the infected area, and imposition of control over animal movement. A serological survey of the area surrounding the infected zone is necessary. It is important to remember that low-virulence ASFV strains do not produce extensive lesions.

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4.2 Eradication of African Swine Fever in Cuba (1971 and 1980)

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SUMMARY

In Cuba, African swine fever (ASF) virus was detected for the first time in 1971 in the western region and then, in 1980, in the eastern region. The ASF eradication campaign was carried out through a program coordinating national, provincial, and municipal administrative structures throughout the country. An educational campaign was developed through the press, radio, and television. With the integration and cooperation of these activities, ASF was eradicated.

BACKGROUND

The Republic of Cuba, with a territory of 114,524 km² and 3500 km of coastline, consists of the Isle of Cuba and more than 1600 keys and islands. The country, which is divided into 14 provinces and 169 municipalities, is situated at the entrance of the Gulf of Mexico and surrounded by the Caribbean Sea and, on its northern coast, the Atlantic Ocean.

In Cuban agricultural history, the breeding and fattening of pigs on large farms was practically unknown, and farmers raised swine on small pig-producing farms. Production was for self-use, with the surplus being sold to market. As part of the changes produced in our economic and social structure beginning in 1959, a solid foundation was established in breed improvement, nutrition, and facilities, as well as in the training of professionals and the introduction of modern animal production technologies (Anonymous 1975). By 1971, we were seeing the evidence of a new, well-structured industry with an important concentration of pork production in Havana Province and the western region of the country. During this period, classical swine fever (CSF) was endemic, and a national program against it was begun. This included compulsory vaccination of the entire swine population with the Chinese lapinized vaccine that had begun to be produced in Cuba at that time.

THE FIRST OCCURRENCE OF ASF IN CUBA, 1971

On May 6, 1971, ASF was first diagnosed in Cuba. Its presence was confirmed in a finishing site, in the province of Havana, that held 11,425 pigs. This site received most animals from specialized porcine farms, plus some private owners. After this outbreak, three other focus areas were detected involving a total of 9000 pigs. There were links among these farms in terms of animals, transportation, and personnel. Contacts between the fattening center with the other farms, plus a delayed diagnosis of ASF, enabled the infection to spread throughout the province. In total, 33 focus areas were detected, and 32,524 animals were involved. The affected area was limited to the region that today comprises two provinces: Havana City with 15 and Havana with 18 focus areas. Of the 32,524 animals within the area, 12,173 died and 20,351 were destroyed. The entire swine population of the two provinces, totaling 463,332 animals, was eliminated. Private owners were allowed to slaughter from three to five pigs for self-consumption and were required to sell the remaining animals to the state.

A set of control measures was put into practice, among which were (1) movement of the swine population of the country was restricted, (2) a census of the swine population in the affected provinces was conducted, (3) the foci of ASF were eliminated by means of hygienic sacrifice, (4) affected pigs were incinerated and buried, (5) privately held pigs were slaughtered sanitarily, and (6) ingress and egress were controlled, with registration and disinfection at the boundaries. In addition, vaccination against CSF and erysipelas was carried out, and the epidemiological surveillance of the whole country was organized.

In parallel, other measures that were followed in order to avoid the dissemination of ASF to other countries included prohibiting the supply of ships or aircraft leaving the country with pork or by-products, disinfection of shoes and luggage of passengers traveling abroad, and not allowing travelers leaving Cuba to carry pork or derivatives.

In the 1971 outbreak, no pathognomonic signs were observed, and clinical examinations of affected animals were insufficient to provide a definitive diagnosis of the disease. The incubation period ranged from 5 to 12 days, but was most frequently 7 days. Clinically, subacute, acute, and peracute forms were seen. Grossly, pathological examination primarily showed the effects of the virus on the vascular endothelium and lymphoid tissues. In addition, lesions were seen in the liver, spleen, and kidneys, where there was congestion, hypertrophy, and hemorrhage. The initial diagnosis was carried out in the research laboratories of our country, with the support and collaboration of Soviet, French, and Canadian specialists. These efforts resulted in the confirmation of ASF on June 17. The diagnosis was based on virus isolation in leukocyte cultures from bone marrow and identification of viral antigen in tissue impressions and infected cell cultures by direct immunofluorescence with specific conjugate. These results were combined with other features, such as clinical, pathological, and epidemiological data, to complete the picture.

When the diagnosis was determined and the presence of ASF virus (ASFV) confirmed, a commission was created with the responsibility of guiding and performing all measures for the eradication of the disease in the national territory and to avoid its spread outside of Cuba. Thus, emergency measures were immediately adopted. A crucial role for the success of this campaign was played by the civil society in these zones. The Defense Committee of the Revolution (Comités de Defensa de la Revolución, CDR) and the National Association of Small Farmers (Asociación Nacional de Agricultores Pequeños, ANAP) actively participated in carrying out disinfection and control measures, thereby contributing to the successful culmination of the work (Anonymous 1971). Once the epidemic was controlled, a recovery plan was carried out with the objective of repopulating the affected areas, after first monitoring with sentinel pigs and validating the completion of the sanitary control measures.

THE PERIOD BETWEEN OUTBREAKS

After the 1971 experience, efforts to protect the country from a new introduction of ASF were increased. Compliance with the measures put into place was sought in ports and airports, including

- Prohibition of any kind of animals living around ports and airports. In addition, food waste extraction and incineration from ships and planes coming from abroad were conducted under strict control.
- Freezers of ships coming from ports of countries with dangerous diseases for cattle were sealed.

- Luggage of passengers coming from affected countries was searched for potentially dangerous devices or materials.
- In addition, steps were taken to assure that pig-producing farms, either state owned or private, fulfilled the sanitary measures mandated by the national order.

The experience acquired in controlling ASF was extremely useful for all workers associated with swine production. Training plans were kept at the Veterinary Medicine Institute, providing the technical staff basic information regarding the characteristics of this disease and the means of prevention and/or response. In addition, the National Center of Animal Health (Centro Nacional de Sanidad Agropecuaria, CENSA) actively participated in the campaign against ASF in 1971 and has subsequently maintained a group of specialists of several disciplines dealing with this illness. This group has closely followed the course of this disease in different countries.

In 1978, when Brazil, Haiti, and the Dominican Republic faced ASF outbreaks, a national sanitary alert was declared by the government of the Republic of Cuba due to the risk of introduction of ASF into our country. In response, the National Headquarters (Poder Popular or Popular Power) and the Provincial Headquarters were created (Anonymous 1978). In July 1978, several measures were implemented in which the following aspects were considered: (1) Retroanalysis of the port and airport zones, as well as the international mail. (2) Intensification of internal protection barriers in ports and airports. (3) Updating and distribution of vaccines against CSF and erysipelas for the entire swine population. (4) Sanitary surveillance of 1 km around the state-owned swine units. (5) Increase in epidemiological surveillance. The health status of the country's swine population was surveyed, and serological testing determined that ASF was not present.

BACKGROUND OF THE SECOND EPIDEMIC

The highest elevations are in the eastern region of Cuba. In addition, heavy rivers cross Guantánamo, the easternmost province, making access to some areas difficult. The Maisí region (La Punta de Maisí) is located on the extreme eastern end of the island, only 77 km from the Republic of Haiti and surrounded on the northeast, east, and south by coastline. At this last point is a US military base, established in 1903, that occupies 118 km². On the southern and eastern coasts, seacraft often arrive from Haiti manned by people guiding themselves to other countries looking for better economic and social conditions. The people aboard these craft travel with food for the journey and even with live animals. The policy of the

Cuban government has been to assist them by supplying fuel, clothing, and food. Medical assistance is given whenever required, as well as repairs to their craft, so that they can continue traveling in safety. In addition, measures have been implemented to attempt to prevent the introduction of human or animal pathogens. These steps have primarily consisted of isolation of persons, incineration of animals and animal by-products, and disinfection of the landing zones. However, strict implementation of these control measures is difficult due to lack of control over the time and place of the arrival of the craft. In 1979, Guantánamo Province received craft carrying 2801 persons.

The economic foundation of Guantánamo Province lies primarily in agricultural and animal production. Among these resources are two swine production facilities, one in the municipality of Baracoa on the northeastern side and the other at the municipality of Guantánamo, in the southwestern part of the province. Swine were held in these provincial facilities until they reached the finishing stage. Finishing and slaughter were carried out in the province of Santiago de Cuba, which lies to the west of Guantánamo. The majority of the 90,000 pigs in the province were raised on the land of peasant families. These animals were raised in humble conditions and fed with fruit and domestic food waste. Although there is no evidence of the existence of wild pigs, feral (or rustic) pigs are frequently found roaming freely in the hills. Some enterprises and farmer cooperatives had pig herds for the purpose of supporting the workers' dining halls, but often with less than ideal hygienic conditions in their facilities, although these conditions were better than those of private ones.

Due to the geographical characteristics of the zone, and taking into account the ruggedness of the area and its proximity to the Republic of Haiti, it received the greatest attention with regard to epidemiological surveillance.

REINTRODUCTION, 1980

The index case was identified on January 26, 1980, in the municipality of Baracoa. On January 27, necropsies were undertaken on affected animals, and samples were collected that would enable definitive diagnosis within 4 days. Once the presence of ASF was confirmed, a state of emergency was declared for the eastern provinces (Guantánamo, Santiago de Cuba, Holguín, and Granma), and the rest of the country remained on sanitary alert—previously declared since the appearance of ASF in the Caribbean. The main goals were considered to be (Anonymous 1980d)

- Eradication of the disease
- Protection of the swine population in the unaffected areas

- Application of sanitary control measures to prevent the transmission of the disease to other countries

Initially, actions were taken to confirm the diagnosis at Baracoa, and a specialized laboratory was established capable of meeting the containment requirements to prevent the circulation of biological material potentially contaminated with ASFV to uninfected areas. The staff from the Institute of Veterinary Medicine and CENSA was quickly moved. The province and municipal administrative structures, created in July 1978, were activated, and emergency measures were implemented (Anonymous 1980d):

- An up-to-date province swine population census was performed.
- The “backyard herds” of private peasants and state farms were visited to carry out clinical examinations and sample collection. Initially, samples were sent to the Baracoa laboratory and, later, to the Guantánamo laboratory.
- Animals originating from the affected area were incinerated and buried in trenches. First the foci of infection and surrounding areas, and finally the entire province, were depopulated of swine.

A total of 37 foci of infection was confirmed in 9 of the 10 municipalities of the province. The municipality of Baracoa with 12 focus areas and the municipality of Guantánamo with 8 recorded the highest incidence. To eliminate the affected swine population, 57 sanitary slaughterhouses were organized and conditions established to recover meat for consumption, without risk of spreading the infection. Waste in these sanitary slaughterhouses was collected in septic tanks, then treated with sodium hydroxide, and again covered. When necessary, bones or whole animals were cremated in trenches, then covered with a layer of lime and a layer of soil, and finally fenced off.

The depopulation started on February 7, 1980, and ended on February 18. A total of 59,211 pigs were gathered up. Within the focus area, 2704 pigs were cremated. From the pigs used, 40 tons of de-boned, canned meat were sent to workers' dining halls, hospitals, and schools. The swine depopulation demanded the effort of all of the people, both state bodies and others. The state bodies required the formation of brigades of volunteers from the urban areas, and, once more, CDR and ANAP worked day and night in gathering, moving, slaughtering, cremating, and meat processing (Anonymous 1980c).

At the same time, a strict quarantine was declared in the province. Control and disinfection points at the focal area bordering the neighboring provinces were created

for pedestrians and vehicles. Also, train, seaport, and airport terminals were checked. Hunting brigades were organized to recheck the affected zones and to exterminate wild pigs, killing 216 feral swine. Furthermore, rustic facilities used by small farmers and nonspecialized pig-raising enterprises were burned, as well as the feed and medicines remaining on the state farms that were part of the original focus area. The owners of animals and facilities destroyed in the eradication effort were indemnified for their losses.

Within the first days, an effort was made to limit the disease to Guantánamo Province, but before it could be confined, ASF had spread to the provinces of Santiago de Cuba and Holguín at Guantánamo's western border.

ASF in Santiago de Cuba was confirmed on February 18, 1980. Thanks to the measures already put into effect, the disease was limited to the municipalities of Santiago de Cuba, with nine outbreak foci, La Maya with four, and San Luis and Julio Antonio Mella with one each. Overall, four of nine municipalities were affected, with a total of 15 foci: four in state enterprises and 11 in backyard herds belonging to small farmers and other nonspecialized pig-raising enterprises.

As was done at Guantánamo, pigs were slaughtered and incinerated, and measures were taken to control the outbreak area. Once the animals were destroyed, cremation, cleaning, and disinfection were performed to eliminate the virus within the focal area. In addition, a program to eliminate rodent populations was conducted by the Ministry of Public Health.

In Holguín Province, a dead pig was reported with clinical signs compatible with ASF. Samples were sent to Guantánamo for diagnosis, and the result was positive for ASF on February 24, 1980. On February 26, three more cases were reported: two from the municipality of Urbano Noris and one at the municipality of Báguano. All three outbreaks involved small backyard herds behind the farmers' houses. In total, there were four foci of infection in three of the 14 municipalities located in the province. Control measures were immediately taken: slaughter, cremation, cleaning, disinfection, and control of virus spread at points of movement within the municipalities.

OUTBREAK EPIDEMIOLOGY

In the 1980 epidemic, it is believed that the first backyard herd affected was located several kilometers to the southwest of the city of Guantánamo. The owner of this herd had sent animals to the abattoir in Guantánamo, as well as the abattoir in Baracoa. Producers around these abattoirs fed offal to their pigs, which would explain the concentration of affected herds around both cities. The state-owned herd at Guantánamo also sent pigs to Santiago de Cuba to be finished, so animal movement contributed to the spread of the virus within the province. In

the three farms at Holguín Province that were affected, the (lesser) movement of animals and people explains the limited propagation of the disease and the fact that control measures were effective in eliminating the outbreak and protecting other zones of the country.

Initial analysis indicated that the disease entered Cuba by means of food products brought by Haitian immigrants arriving in an uncontrolled way on our coasts. However, later studies pointed out that the illness could have been introduced intentionally, as was confirmed later in the case of the first outbreak (Franklin 1997).

DIAGNOSIS

An investigation of the first diagnosed focus area at the municipality of Baracoa made evident the presence of two clinical forms: acute and peracute. The acute form, which was most common, started as anorexia, followed by a fever of 41° to 42°C (105.8° to 107.6°F), ataxia primarily affecting the rear limbs, constipation and in some cases bloody diarrhea, mucous secretions of the eyes and nose, and prostration. Pregnant sows aborted. In a large percentage of affected animals, subcutaneous hemorrhage was observed, particularly of the ventral region. Death occurred from 5 to 10 days following the onset of the first signs. Clinical data obtained by experimental inoculation did not differ in general from field observations. Lesions found in animals infected either in the field or under experimental conditions were classic for those described for ASFV. The diagnosis was determined using the same methods as described for the first epidemic.

MONITOR AND CONTROL PROGRAM

In July 1978, a program was developed based on a structure consisting of a National Board, provincial boards, and municipal boards. The President of the Chief Government (Poder Popular), in each instance, led the boards, along with representatives of state bodies and civil societies. Information flowed from the level of the municipality to the nation, whereas the operation flow was vice versa, from the national level to the municipality.

To organize epidemiological surveillance, each municipality was divided into zones that were created based on geographical and political characteristics and the density of pork production. Among a total of 134 municipalities, 939 zones were created. They were plotted on maps, and foci and focal areas were marked, as well as geographical features and other information relevant to the work. Assigned to each zone was a veterinarian or technician familiar with the number of pigs, the sanitary situation, the transportation of pigs, and the hygienic status of animals and by-products. Zones were subordinate to the municipal board. Daily information flew with

extraordinary speed from the zone, no matter how far it was to the National Board.

By means of this structure, pig owners were visited and the health status of the swine population was controlled. In total, 3,478,308 pigs were vaccinated against CSF and 917,601 against erysipelas. A total of 899,122 visits were carried out to private owners. On the farms, rectal temperatures of animals were taken and, in cases of suspicious signs, the *diagnosis group* was alerted to collect samples for submission to specialized laboratories.

In all provinces, diagnostic groups were created, composed of clinicians, pathologists, and epidemiologists, plus support personnel responsible for conducting post-mortems, collecting samples, and sending samples to the laboratories. Once notified of a suspect case, the group was in charge of guiding the investigation, collecting samples, and overseeing the implementation of the quarantine measures necessary to prevent dissemination of the disease. To carry out this work, the diagnostic groups were provided with suitable clothing and shoes, and all precautions were taken for necessary disinfection. These groups, which used the laboratories of the province as a base, visited sites on the instructions of the Provincial Board, evaluated the situation and, in suspect cases, collected samples in order to arrive at a definitive diagnosis.

In provinces with high pig density, a provincial diagnostic group would sometimes create separate teams within itself to work either in state swine herds or in private herds. Since private herds were considered at higher risk of infection, this was an effort to protect state herds from infection.

Samples were not only collected from sick or dead pigs. As part of the effort to track the disease, samples were collected from clinically unaffected pigs at the boundary or periphery of affected areas or, in municipalities or provinces with outbreaks or places of high pig density, near areas where pigs had been killed as part of the control effort, abattoirs, packing plants, and canned pork factories.

All samples collected in this effort were strictly controlled. In total, the laboratories received 9587 cases, of which 89 were positive for ASFV.

With the goal of reducing the risk of transmission, a nationwide freeze was declared on the movement of all samples of porcine origin for diagnostic purposes. All work of this type was left to the diagnostic groups.

Once the diagnosis of ASF was confirmed, the quarantine and cleanup procedures were implemented in the affected zone, according to a system of classification (Anonymous 1980a).

In areas diagnosed with ASF, all pigs were collected and killed. Hunting brigades were established with the support of the army that searched the zones for feral pigs. Within the three provinces, 137,287 pigs were killed, of which 123,250 were cremated, and all offal was

incinerated. On the state farms, all swine transport was stopped. In a general sense, prevention and control measures were strengthened on state farms and in the private sector.

Control and disinfection points were established, taking into account the means of transmission of the agent and the existing transportation routes. In general, these points were placed at the access points of the cities in question, although, according to the real risk of transmission, these could be located in other places, such as at the entrance and exit of towns. In these places, vehicles and pedestrians were inspected carefully. Bags were examined in the presence of the owner. If the bags contained shoes, they were disinfected. All products of porcine origin were confiscated and then burned in trenches created for this purpose at the point where the confiscation occurred. Similar points were placed at bus and rail stations and the same procedure followed. Once the inspection was finished, the luggage was sealed and stamped, assuring that the contents remain unaltered until the end of the journey and that the check would be done only once per trip.

The 229 control points that were created functioned 24 hours a day and were manned by personnel from the Civil Defense and the National Policemen of the Revolution. The Municipality Commission supervised the technical aspects of the disinfection activity. For disinfecting vehicles and shoes at the control points, disinfecting pools with a 1200-gallon capacity, 2000-liter sprayers, and backpack sprayers were used. In general, formalin was used as the disinfectant, with the concentration of the solution dependent on its use, except in ports and airports, where 2% sodium hypochloride was applied to stairs.

Once depopulated of pigs, a program was carried out to clean the affected areas. This was done in four stages, with the primary measure consisting of the collection and burning of all objects that had been in contact with pigs. Subsequent measures included breaking up the soil, applying calcium oxide, and removing weeds. The program also included four applications of disinfectant at 10- to 15-day intervals, and the eradication of rats. This sanitization program was also conducted at the sites where pigs were killed, once this task was definitively completed, in addition to the routine disinfection that was in place.

MASS BROADCAST

The campaign against ASF was widely publicized, primarily through the written press and radio. Journals from the provinces informed the public of the work performed to eliminate the disease in affected areas and the efforts to protect unaffected areas. Educational information was disseminated by these means about the characteristics of the disease and the actions to be

taken at the appearance of signs compatible with ASF in pigs.

FINAL PHASE OF THE CAMPAIGN

The program of eradication was carried out in conjunction with intense surveillance, which enabled the level of propagation of ASFV in the population to be determined. In turn, this made it possible to coordinate the technical information and epidemiological data in order to determine when the disease was controlled.

The intensive phase ended on May 30, 1980, progressing to quarantine and recovery phases. During this second phase, the organizational structure was kept intact and prepared to continue the campaign against ASF. Efforts were modified in the controlled zones. In privately owned holdings, however, efforts continued at the same level that was in force during the intensive phase of the campaign because of their greater risk of infection. The ASF diagnosis laboratories at Santiago de Cuba continued their work and conducted a program designed to train the technical staff.

Affected areas were kept free of vegetation to allow the sunlight to act. In areas difficult to clear, trees were pruned and bushes removed. The prohibition against movement of pigs, meats, or by-products from the three affected provinces to other regions remained in force. At the same time, measures taken in seaports and airports to avoid possible transport of the disease to other countries, including a ban on the export of live pigs and semen, remained in place.

The quarantine and recovery phases began on May 30 and lasted until September 1, 1980. At the end of August, the situation and the measures taken during this phase were evaluated (Anonymous 1980b). The results of the evaluation were satisfactory, so on September 25 the initiation of the test phase was authorized.

Thereafter, 1500 sentinel pigs were placed within the outbreak focus areas and elsewhere. This was considered necessary to be certain that no ASFV remained. At the end of the observation period, the pigs were killed under rigorous measures of protection and control. More than 20% were sampled and the samples assayed for virus in the specialized ASF laboratories. Results of the clinical, pathological, and biological studies found no evidence of the virus. Once this phase was completed, the specialized swine farms were repopulated in a minimum amount of time, but with epidemiological preventive measures against ASF in place.

ECONOMIC IMPACT

In estimating the economic losses due to ASF and the costs of the eradication campaign, the following factors were taken into account:

1. Animal losses (dead or killed). The estimated value of cremated animals was \$936,994. This figure represents animals lost with no salvage value. The value of the animals collected during the eradication from which meat was salvaged was \$1,041,781.
2. Payments to owners for confiscated animals or removal of facilities. Private producers were indemnified for confiscated pigs by payment in cash according to the animal weight and price in force for pork. These owners were also paid for recently born pigs, although the meat had no value. The cost in this category was \$4,218,074.
3. The current expenses of the campaign. The total costs that were possible to count amounted to \$3,972,962, with wages constituting 30% of the total.
4. Export losses. Losses accrued as a result of products or animals not being sold included exports to Canada valued at 477,221 Canadian dollars and to other export markets for a value of 1,820,303 rubles. Additional losses to the national economy reached \$896,349 due to the necessity of placing products on the domestic market at a price lower than would have been received on the export market.

Total losses amounted to \$9,359,414, but to have a more complete idea of the effort that it represented for the economy of the country, it should be recognized that the campaign required the mobilization of 42,312 persons who worked for more than 2 million hours at specialized technical tasks, such as the killing and cremation of animals, checking of luggage at the control points, inspection, and other auxiliary activities. For the movement of personnel, animals, animal by-products or meat, materials, etc., 936 vehicles were used for a total of 130,468 hours.

Expenses and financial losses that were incurred by the various institutions as a consequence of ASF were reimbursed by the state through a contingency fund. It is considered, however, that the financial and human resources that the state placed in the hands of the authorities responsible for conducting the campaign against ASF avoided the even greater economic losses and repercussions that would have been felt if ASF had spread throughout the entire territory (Anonymous 1980e).

CONCLUSIONS

The structure that was created involving the participation of agencies at different levels—national, provincial, and municipal—as well as the information system used on this occasion, enabled rapid, coordinated action dur-

ing the intensive phase of the campaign. The ability of the population of the affected zones to understand the necessity to accomplish the required measures, together with the massive organized participation in the diverse activities of the campaign, especially in the collection, cremation, or killing of pigs, were crucial in the successful ASF eradication campaign in Cuba.

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4.3 African Swine Fever Eradication: The Spanish Model

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SUMMARY

This chapter reviews the Spanish African swine fever (ASF) eradication program (1985 to 1995). Since no vaccine was available, the program was primarily based on the detection of ASF-positive and carrier animals by laboratory diagnosis and the enforcement of strict sanitary measures. The key components of the program included (1) a network of mobile veterinary field teams responsible for the control and diagnosis of the disease, (2) serological testing of animals, (3) improvements in sanitary infrastructure, (4) elimination of all ASF outbreaks and identification and slaughter of carrier animals, and (5) veterinary control of all swine movement, with individual identification of every animal moved for fattening or breeding purposes. In 1995, Spain was declared free of ASF. At present, Spain is the second largest swine-producing country in the European Union with a total population of 22,435,000 pigs and 2,478,000 breeding sows.

INTRODUCTION

African swine fever (ASF) is a highly contagious viral disease of swine and of such concern that it is included among the List A diseases by the Office International des Epizooties (OIE). The disease causes significant economic losses in affected countries due to the high mortality rates associated with the acute and peracute forms and the potential for extensive and rapid spread at international levels. Such an occurrence would have important socioeconomic consequences in the international trade of animals and swine products.

Domestic pigs and European wild boars are very susceptible, and infection produces a wide range of clinical forms: acute, subacute, chronic, and subclinical (Arias et al. 1986; Sánchez-Vizcaíno 1999). African wild boars, bushpigs (*Potamochoerus porcus*), and warthogs (*Phacochoerus aethiopicus*) are very resistant to the disease, present inapparent infections, and serve as reservoir hosts in Africa (Heuschele and Coggins 1965). Soft ticks, especially *Ornithodoros erraticus* and *Ornithodoros moubata*, act as reservoirs and transmission vectors of

ASF virus (ASFV) (Plowright et al. 1970; Sánchez-Botija 1963).

African swine fever was confined to Africa until the end of the 1950s, when it appeared in Portugal in 1957 from Angola. It subsequently spread to other European countries: Spain in 1960, France in 1964, Italy in 1967, 1969, and 1993, Belgium in 1985, and the Netherlands in 1986. Between 1978 and 1980, ASF appeared in several American countries: Brazil, Cuba, the Dominican Republic, and Haiti. In the latter two countries, ASF was successfully eradicated by *stamping out* all the pigs on the island of Hispaniola. In Europe, the disease remained endemic in Portugal and Spain for decades until 1994 and 1995, when both countries were declared ASF free. In November 1999, ASF appeared again in Alentejo, Portugal, but was successfully eradicated (OIE information).

Currently, ASF is primarily present in sub-Saharan countries of Africa. It has acquired a greater importance since 1997 because of increased outbreaks in Angola, Benin, Botswana, Cameroon, Cape Verde, Ghana, Madagascar, Malawi, Mozambique, Namibia, Nigeria, Senegal, Togo, Uganda, and Zambia. In these countries, ASF is maintained by a cycle of infection between wild boars (reservoir) and soft ticks (vector) with transmission to domestic pigs. This vector-borne cycle of transmission makes eradication difficult. Outside of Africa, ASF is present in only Sardinia (Italy), in which carrier wild boars and domestic pigs recovered from ASFV infection are believed to play a role in maintaining the disease.

ASF ERADICATION PROGRAM IN SPAIN

Since there is no vaccine against ASF, the Spanish eradication program (1985 to 1995) was based on the detection of ASFV-infected animals by laboratory diagnosis and the enforcement of strict sanitary measures. In the final stages of the program, a new Coordinated Program was prepared jointly with Portugal to intensify the fight against ASF in the southwestern portion of the Iberian Peninsula, the last remaining infected area. In 1995, Spain was declared free of the disease.

HISTORY AND EPIDEMIOLOGICAL SITUATION

In 1960, when ASF appeared in Spain, it spread within what was essentially an undeveloped sector. During the 1960s, as the Spanish economy began to take off, a marked change took place in pig production. In a few years, Spain went from family-type holdings to an industrial organization characterized by intensive swine production systems, the incorporation of European breeds, and the use of new handling techniques. Pig production, which until then had been located mainly in the south and southwest and operated as outdoor production systems, began to be carried out in industrial farms that were found mainly in six regions. Galicia, Castilla, León, and Murcia specialized in the production of piglets, and Aragón, Cataluña, and Segovia in large feeding farms. This situation resulted in the extensive movement of livestock within the Spanish territory.

In 1960, the virus spread widely in certain areas of the country. Initially, the usual picture of ASF was acute clinical signs and high mortality. This changed through the years to an endemic disease characterized by mild-to-subclinical forms and a mortality rate below 5% in infected herds. Thereafter, confirmation of the disease by laboratory diagnosis was required. In addition, the virus also could be transmitted by the soft-tick vector, *Ornithodoros erraticus*, found in certain southwestern areas where the disease was endemic and where the outdoor production of Iberian pigs was located.

In spite of ASF, the pork-producing sector experienced strong development, and swine production increased from 6 million head in 1960 to 16.7 million in 1989 (1.9 million breeding animals), representing an increase of 178.3% from 1960 to 1989 (Bech-Nielsen et al. 1995). At this time, the sector employed 200,000 people, taking into account secondary industries, and produced nearly 1.2 million tons of meat. Of this, 50% was consumed as manufactured products, of which cured products constituted 45%—a very high percentage compared with the European countries. On the whole, the contribution of the pig industry to the total annual agricultural production was 240,000 million pesetas (1.4 million Euros). Only export restrictions restrained significant development of the Spanish pig sector. The ASF imposed economic hardships on pig producers and created barriers to intracommunity trade in live pigs, fresh pig meat, and certain pork products. Furthermore, ASF control measures produced great economic costs for the Spanish administration. An analysis of the effort to control ASF in Spain in the year 1983 estimated costs at 1900 million pesetas (11.4 million Euros).

The Spanish administration, aware of the situation, established a Coordinated Program to eradicate ASF in

Spain (Royal Decree 425/1985) in March 1985. It was approved in its totality and provided an initial financial support of 7200 million pesetas (more than 43 million Euros) by the European community (Council decision 86/650/EEC).

KEY ACTIONS OF THE ERADICATION PROGRAM

The new regulations introduced the following:

1. A network for the control and diagnosis of the disease implemented by mobile veterinary field teams (127) exclusively dedicated to the program. The field teams worked in the sanitary control of holdings, animal identification, epidemiological surveys, sample collection for the serological surveillance of breeders, serological control at slaughterhouses, epidemiological investigations, and also promoted and encouraged pig producers to create Sanitary Associations.
2. Serological surveillance of 100% of the pig farms. To achieve this goal, it was necessary to put into place a simple, fast, accurate, and specific diagnostic test and a reference laboratory to harmonize the techniques to be used in regional and province laboratories. The indirect enzyme-linked immunosorbent assay (ELISA) was selected as the best assay for obtaining a rapid and reliable diagnosis of the disease (Sánchez-Vizcaíno et al. 1982). The Department of Animal Health of the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) became the National Reference Laboratory. At present, this is known as CISA-INIA, the ASF reference laboratory for the OIE and the European Union.

At the beginning of the program, the regional laboratories used the indirect ELISA to screen samples and an indirect fluorescent antibody (IFA) assay to confirm results. In the final stages, the National Reference Laboratory of INIA developed an improved ELISA based on a new soluble antigen that contained all the ASFV proteins (Sánchez-Vizcaíno 1986). This enabled better recognition of carrier animals. In addition, an immunoblotting assay was developed as a serological confirmatory test in place of the IFA (Arias and Sánchez-Vizcaíno 1992; Pastor et al. 1989). This assay enabled easier and more objective interpretation of results and a better recognition of weak-positive samples. The virological studies for the detection of new outbreaks were carried out at the National Reference Center.

A total of 13 regional laboratories was involved in serological surveillance during the eradication program (actually there are 39 laboratories located in the 17 Autonomous Communities). To assure the quality and

reliability of results, these laboratories received the reagents required to perform the serological assays from the National Reference Center.

3. Improvements in animal holding facilities. The primary objective was to improve sanitary barriers to prevent the spread of the disease. This involved adoption of basic measures of hygiene, including fences, sanitary enclosures, and safe disposal of manure. For this purpose, it was possible to obtain funds to cover part of the investment or loans at low interest rates. More than 2175 holdings were improved between 1985 and 1990.
4. Elimination of all ASF outbreaks, killing of ASFV carriers, depopulation of infected herds. As soon as the National Reference Laboratory confirmed an outbreak of ASF, all pigs in the infected holdings had to be killed immediately. Sample collection for virological and epidemiological investigations was also performed and reported. The responsible authorities arranged immediate and adequate compensation to be paid to the pig producers of the infected holdings, in compliance with the relevant legislation.

Once the outbreak was identified, depopulation by killing all the pigs in the affected herd was initiated, and additional measures were adopted. The buildings underwent complete cleaning and disinfection about 1 month after depopulation. This included insect and rodent extermination and the removal and destruction of all animal feed, animal products, and thorough cleansing of manure pits by using 2% sodium hydroxide, as well as the incineration of straw bedding at the pig holding facility. A sanitary zone of a few hundred meters radius was designated and movement of animals, animal products, animal feed, and waste into or out of the sanitary zone and the movement of people to and from the area were restricted. The restrictions in the sanitary zone were gradually eased although some specific biosafety measures were maintained for at least 3 months. After complete cleaning and disinfection, ASF-seronegative sentinel animals were introduced into the holding and taken through all the facilities. The number of sentinel pigs was usually equivalent to 10% to 20% of the population at the time when restocking was completed. If after a month they did not become sick, they were retested serologically. If the results were negative, the authorities allowed the repopulation of the farm, with identified animals from a health status-controlled farm. Restocking procedures were usually completed within 3 months after the entry of sentinel pigs.

On holdings known to be infested with *Ornithodoros erraticus*, no restocking could take place unless special arrangements were made after consultation with the Central Veterinary Administration.

Biosafety and sanitary measures developed to avoid transmission of the virus between herds played an important role in the eradication of the disease. Reports on sources of ASF outbreaks collected through epidemiological investigations indicated that the 84% and the 93% outbreaks in 1989 and 1990, respectively, listed neighbor contact as the most likely source of the outbreak (Bech-Nielsen et al. 1995).

RESTRICTED AREAS

A protection zone and a surveillance zone were established upon confirmation of an outbreak. The radius of the zones depended on the initial findings of the epidemiological investigation, but the protection zone had a radius of at least 3 km and the surveillance zone a radius of 10 km. Pigs kept on all holdings situated within the protection zone were serologically screened immediately after an outbreak was confirmed. Further screenings in the 3-km and 10-km zones were done no sooner than 30 days after the preliminary cleaning and disinfection of the infected holding were completed. Movement of live pigs within the zones was prohibited for 30 days, but this limitation could be lifted after completion of serological tests proved that the area was negative. Live pigs could not be moved out of the zones. The traffic police and other competent authorities carried out the control of movement of animals.

LIVESTOCK MOVEMENT AND ANIMAL IDENTIFICATION

Livestock movement was strictly controlled. Vehicles were required to be properly washed and disinfected. Animals in transit, which had been previously identified, required an official veterinary certificate stating their origin and sanitary situation. At all times, program administrators retained authority over transportation of animals and their destination (abattoir or breeding farm). When pigs arrived at an abattoir, the official veterinarian checked the sanitary certificate prior to slaughter. In addition, live animals were inspected antemortem and tissues postmortem. The abattoir retained the sanitary certificate for at least 1 year following slaughter. Throughout the process, manufacturers retained identification of the origin of the meat from the moment of the arrival of the animal to the final manufactured product.

All of these direct measures worked together with others, such as an improved producer registry, a census of pig holdings, a list of infected farms, and an annual report of the program development. However, all of these measures would have been useless without the direct involvement and the active participation of the farmers. Thanks to a wide publicity campaign in the mass media, including radio, primarily directed to the rural environment, the pig producers became aware of the need to

fight against the ASF. The pig producers created associations for sanitary defense (Health Protection Group) and took a leadership role in the eradication program. The associations were originally started by a group of farmers who voluntarily adopted, at the municipal level, a common approach against ASF. These farmers maintained serological surveillance of breeders and the correct sanitary infrastructures. They maintained sanitary programs suitable for ASF and other pig diseases and received important aid from the administration. In 1990, more than 979 associations had been created in Spain that included over 41,321 farmers and 922,996 breeding animals. A registry was created with the aim of classifying holdings in accordance with the health status of the pig herd and the facilities on the holding, and the different types of farms: holdings with confirmed health status, holdings with special health protection, and holdings free of African swine fever located in the free area.

THE ERADICATION PROGRAM EVOLVES INTO REGIONALIZATION

As a result of the progress of the eradication program, in December 1989 the European Community Council (ECC) adopted a new rule. This decision (89/21/EEC) divided Spain into two regions: an *ASF-free region* and an *ASF-infected region*. Regionalization lifted the prohibitions on trade in certain areas in Spain and allowed the trade in live pigs, fresh pig meat, and certain meat products from the free areas to other European countries. The free region, in which the last outbreak was recorded in 1987, covered the largest part of the country and included about 70% of the pig population of Spain. The infected region covered complete or partial areas of the provinces of Salamanca, Cáceres, Badajoz, Huelva, Córdoba, Sevilla, Cádiz, and Málaga (Figure 4.3.1, Decision 89/21/EEC).

Since 1990, no outbreaks of clinical disease were recorded in certain parts of the infected region, although serological evidence of ASFV was observed in certain herds (in a very few animals). Progressively, new divisions of the Spanish territory were established: a *free area*; a *surveillance area* adjacent to the Portuguese border that included parts of the provinces of Salamanca, Cáceres, Badajoz, Cádiz, and Málaga; and an *infected area* that covered a small part of the southwest of the country and included the province of Huelva and parts of Córdoba and Sevilla. Outbreaks continued in the infected area until 1993 (Figure 4.3.1, Decisions 91/112/ECC and 93/443/ECC). In the infected area, virus persistence was primarily due to the following factors: production facilities lacking basic sanitation and biosafety features; the presence of soft ticks (*Ornithodoros erraticus*) that acted as ASFV vectors; and the presence of an uncontrolled wild boar population.

At that juncture, new measures were put into place that included a specific serological screening program as follows:

In the free area, serological sampling of 5% of the national sow herd and wild boar population was required each year. Samples were collected mainly from areas adjacent to surveillance and infected areas and from herds in the vicinity of abattoirs authorized to kill pigs originating from the surveillance area. Wild boars killed during hunting in areas at risk were also screened.

In the surveillance area: (1) in each breeding herd, 30% of the breeding animals were tested each year; (2) in farms using *open* or *mixed* production systems, 50% of the breeding animals in all herds were tested each year; and (3) pigs greater than 40 kg in weight had to be tested once. The sampling could be increased by a factor of 2 in the case of herds where carrier animals had been identified during the previous 6 months, or in areas where evidence of ASF in the wild boar population had been found.

In the infected area: (1) 30% of breeding animals from breeding farms had to be serologically screened twice each year; (2) in farms using open or mixed production systems, 50% of the breeding animals in all herds were tested every 6 months with sampling performed in such a way that every breeding animal was tested at least once per year; (3) pigs greater than 40 kg in weight had to be tested once each year; and (4) wild boars killed by hunting had to be examined for ASFV by virological and serological techniques.

In addition, special measures were implemented in the surveillance and infected areas. These included the destruction of the unsanitary animal production facilities, serological surveillance by ELISA of pigs at risk of being bitten by ticks (Canals et al. 1990), and construction of metal fences of about 100-meter radius around the animal facilities with historic value or good sanitary conditions to avoid the contact with feral animals.

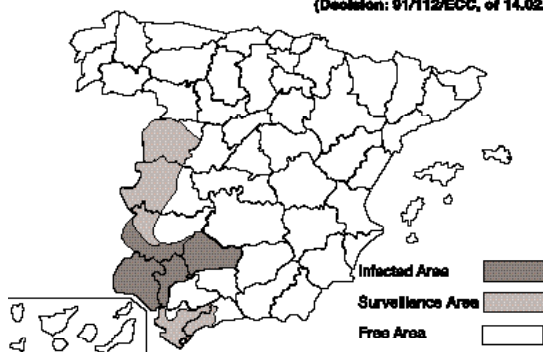
FINALE: THE COORDINATED PROGRAM ON THE IBERIAN PENINSULA

In 1994, the epidemiological situation presented the need to join efforts with Portuguese authorities to eradicate ASF from the remaining infected areas located on the Spanish side of the border between the two countries. The last outbreaks in Portugal, in 1993, had been located in the Alentejo area (Portuguese border) in which mixed production systems were common. In June 1994, a coordinated program to eradicate ASF from the remaining infected areas of the Iberian Peninsula was arranged with the approval of the European Community (EC). Between July 1994 and December 1996, the EC funded 50% of the cost of serological testing, slaughter, and destruction of pigs, cleaning and disinfection, and

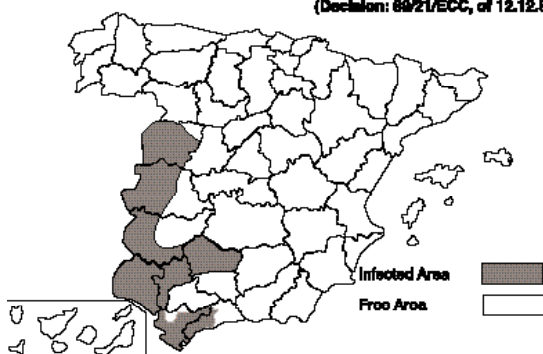
ASF Situation In 1985



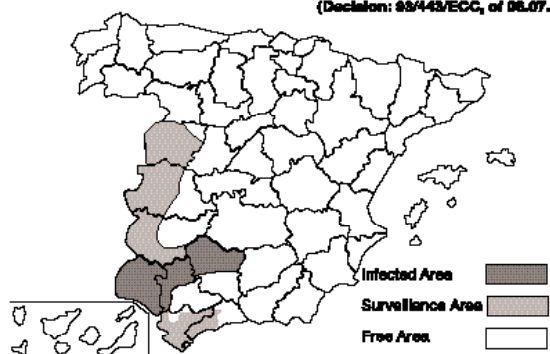
ASF Situation In 1991 (Decision: 91/112/ECC, of 14.02.91)



ASF Situation In 1989 (Decision: 89/21/ECC, of 12.12.89)



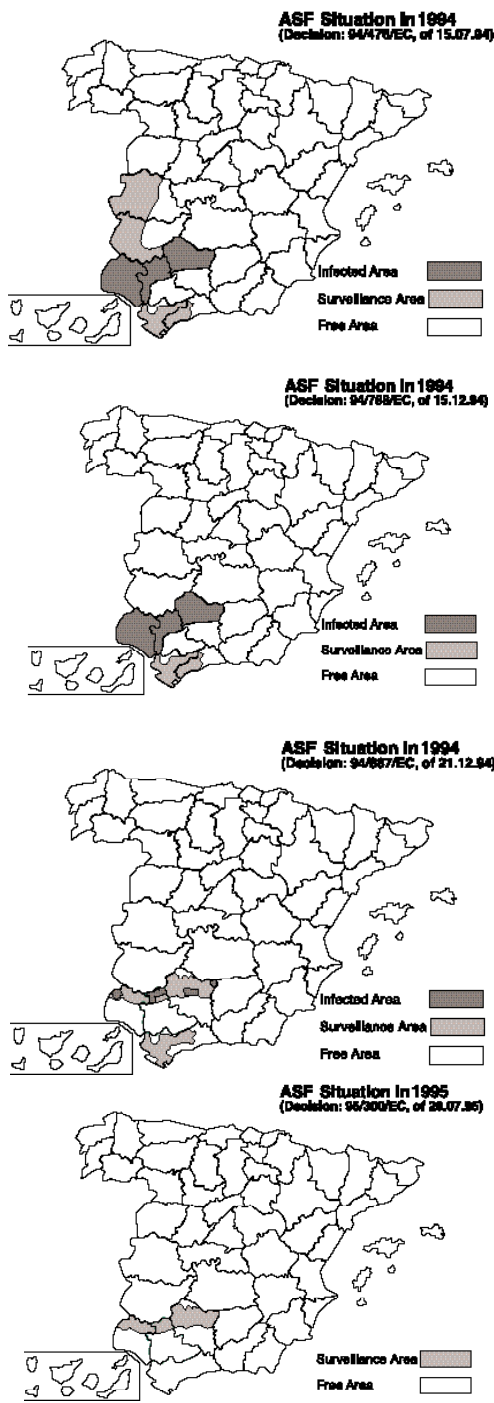
ASF Situation In 1993 (Decision: 93/443/ECC, of 06.07.93)



4.3.1. African swine fever in Spain: 1985 to 1993.

epidemiological investigations of ticks and wild boars, up to a maximum of 7,210,000 Euros. This program involved the member states of the EC and the Commission, and pig producers, the pig-meat industry, and the authorities of Spain and Portugal. Furthermore, the program established different National Committees and Monitoring Centers, as well as an Advisory Board that

was responsible for supervising the eradication program. The program consisted of a number of health status areas: free area, surveillance area, and an infected area in Spain and a monitoring area in Portugal. The evolution of this coordinated eradication program is shown in Figure 4.3.2 (Decisions 94/476/EC, 94/788/EC, 94/887/EC, and 95/300/EC).



4.3.2. African swine fever in Spain: final stages of the eradication program.

ASF ERADICATION: FINAL REMARKS

The methods used to eradicate ASF in Spain involved a great effort on the part of the Agriculture Ministry, primarily due to the need to create the necessary infrastructure and because of the need for highly qualified personnel. Coordination with the Spanish Regional Communities was also an important factor in the successful outcome of the program. Since 1987, no new outbreaks of ASF appeared in the areas that used confined production systems (about 96% of Spanish territory). In these regions, ASF eradication was easier and faster because of the absence of vectors or wild boars, compared with those using open swine production systems, in which ASF eradication was particularly difficult. Beginning in 1987, ASF outbreaks appeared in only specific southwestern areas of the country, i.e., in the regions of Salamanca, Sevilla, Córdoba, Huelva, Cádiz, and Málaga. As a consequence of the increased control and intensification of the effort in the provinces that were still affected, together with the Spanish-Portuguese Coordinated Program, since 1994 no new outbreaks have appeared anywhere in Spain. It should be noted that vaccine was not essential for ASF eradication, even in endemic areas.

In December 1995, Spain was declared officially free of the disease. At present, Spain is ranked as the second largest pig-producing country in the European Union, with a total population of 22,435,000 pigs and 2,478,000 breeding sows.

ACKNOWLEDGMENT

We thank the Dirección General de Ganadería from the Ministerio de Agricultura, Pesca y Alimentación of Spain, for its help and support in preparing this review.

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5 CLASSICAL SWINE FEVER VIRUS

5.1

Reemergence of Classical Swine Fever in Cuba, 1993 to 1997

María Teresa Frías-Lepoureau

SUMMARY

After an absence of more than 20 years, classical swine fever (CSF) reemerged in Cuba in the 1990s due to a breakdown in biosecurity and the presence of a highly susceptible population that had developed because of a worsening economic situation on the island. This favored the reemergence of strains that had previously circulated in the population. The 1993 outbreak was detected early through an active surveillance system, but the outbreak expanded and 13 of 14 provinces and almost all of the country's genetic centers were infected by 1996. This brought about the declaration of a national emergency. This chapter reviews Cuba's experience with the reemergence of CSF.

INTRODUCTION

Classical swine fever virus (CSFV), also known as hog cholera virus, is the causative agent of a highly contagious disease of swine that continues to cause significant economic losses in the pig industry on a worldwide basis. For this reason, CSF is included among the List A diseases of the Office International des Epizooties (OIE) Animal Health Code.

Classical swine fever was first reported in 1833 in the United States. Currently, it is present throughout the world and is found in 54 countries. Eradication programs were established many years ago in the United States (with annual losses estimated in the millions of dollars), Canada, Australia, and the countries of the European Union. However, Western Europe—a great producer of pigs—still has significant outbreaks, such as the epidemics that have occurred in recent times in Germany, Belgium, Italy, the Netherlands, and Spain, resulting in great economic losses. The disease is endemic in Asia, South America, Central America, and Mexico, with recent outbreaks in Argentina, Costa Rica, the Dominican Republic, and Haiti.

CLASSICAL SWINE FEVER IN CUBA

In Cuba, CSF was reported for the first time in the 1940s, probably originating from pigs imported from the Unit-

ed States. Initially, a crystal violet-inactivated vaccine was used against the disease. Then, in 1962, a Chinese lapinized strain of CSFV was introduced, and local production of vaccine was begun (LABIOFAM). This development facilitated the organization of a National Control Program. Thereafter, a progressive decrease in the occurrence of cases was reported until 1975, after which time no more cases were reported (Frías 2000a,b). After a long period free of CSF, new outbreaks began in 1993, but the clinical-epidemiological presentation of disease had changed. The affected area expanded as the occurrence of outbreaks in the eastern provinces rose to epidemic levels. At the same time, an outbreak of rabbit viral hemorrhagic disease in Havana caused a decrease in the rabbit population destined for vaccine production. The economic losses that resulted, although not yet quantified, were high. Losses included 455 outbreaks that resulted in 4926 deaths, 58,755 animals sent to sanitary slaughter, and more than 1600 tons of meat wasted. This alone represented losses estimated at more than 5 million dollars (US), a severe shortage in the supply of animal protein, and an increase in the price of pork products. In 1996, it became necessary to declare a national emergency and to activate the Civil Defense System and its respective provincial offices.

Bearing in mind that CSF was clinically silent under a vaccination program for more than 20 years, the reemergence of the disease and the appearance of atypical clinical manifestations led us to consider the possibility of the external introduction of viral strains. However, it should also be taken into consideration that at that time the economic situation of the country had greatly changed the zoosanitary and zootechnical conditions of industrial pig farming and that 69% of the pig population of the country was raised under this type of farming (Frías 2000a,b).

These developments made clear the need to develop new tools for the diagnosis and molecular characterization of the virus. In recent years, the development of molecular tools for specific amplification, sequencing, and phylogenetic analysis has made it possible to analyze CSF epidemiological information at the molecular level (Frías 2000b).

It was necessary to perform epidemiological studies to have a clear idea of the origin of the epidemic and to

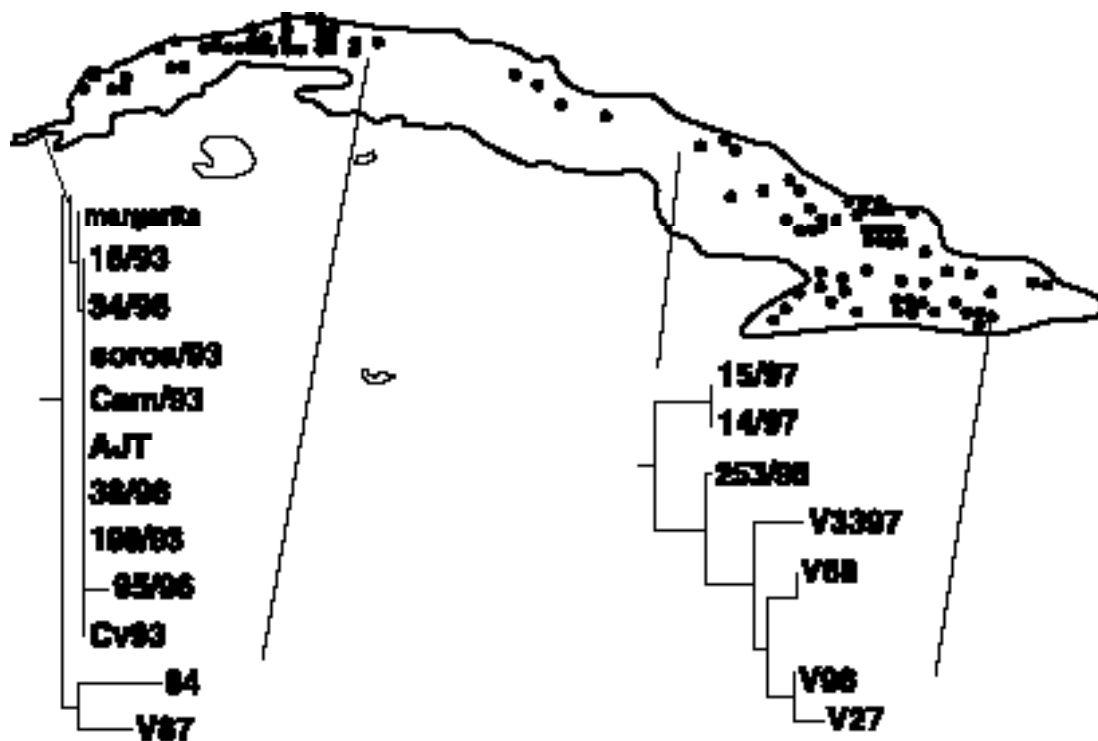
improve prevention and control efforts. Several methods have been used for molecular characterization and comparison of virus isolates, among them reactivity patterns of monoclonal antibodies, restriction endonuclease analyses, and sequencing of the viral genome. In particular, the latter methods provide a great deal of information.

The CSFV genome consists of a positive-sense RNA molecule of about 12.3 kilobases encoding a single polyprotein that contains both structural glycoproteins and nonstructural proteins. Among the structural proteins, E2 glycoprotein is highly immunogenic and induces neutralizing antibodies. Phylogenetic analyses based on nucleotide sequences spanning the highly variable N terminus of the E2 protein have provided the most sensitive discrimination among field isolates. Lowings et al. (1994, 1996) compared 112 isolates from all over the world, based on the sequences of 190 base pairs (bp) of the N' region of E2. The existence of two large groups was described: group 1, consisting of all vaccine strains, the American and ancient European isolates; and group 2, comprising the recent European and Asian isolates.

Sequence data from vaccine and challenge strains and Cuban isolates from the entire country were repre-

sented in our study, including the "Margarita" strain that was isolated in Havana in 1958 and later used as a challenge strain to test the potency of the Cuban vaccine. The field strains were selected from various production categories with different kinds of zootechnical management and with clinical signs ranging from those typical of the disease to inapparent forms. The alignment of sequences was done using Clustal V software for the multiple alignment of sequences (Higgins et al. 1992).

Lowings's primers detected all the strains and isolates evaluated. The alignment of the nucleotide sequences of the 228-bp fragment showed both highly conserved genomic regions and regions that were more variable. Among the 18 field isolates and the strain "Margarita," the nucleotide substitutions detected in the 228 nucleotides affected 12 positions (81.6% transitions and 18.4% transversions), with 65% of changes being silent substitutions. The Cuban isolates were located in group 1 subgroup 1.2 and were very closely related to each other (Díaz de Arce et al. 1999). The most pronounced genetic distances were observed between the seven isolates from the eastern zone of the country and those from the western zone (Figure 5.1.1). The strain "C" used in our country for vaccination was located in the same phylogenetic subgroup as these isolates.

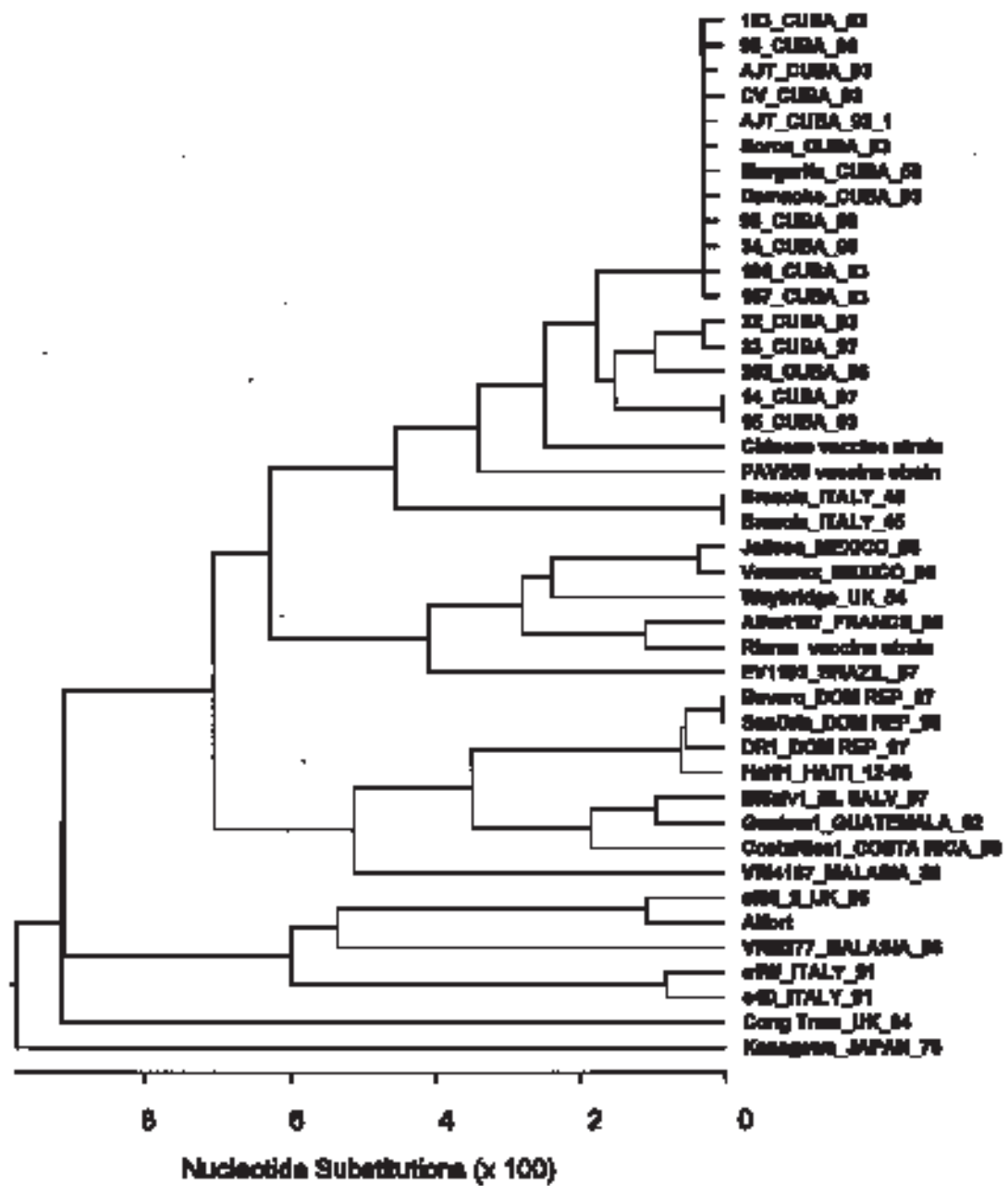


5.1.1. Map of Cuba indicating the spatial distribution of outbreaks (•) and the areas from which specific classical swine fever virus isolates were recovered.

As Figure 5.1.2 shows, the genetic distances (Dopazo 1997) calculated among the western isolates were equal to zero. This suggested the possible reintroduction of the challenge strain used in the Cuban vaccine potency trials as the cause of the epidemic. In the eastern region, in contrast, the isolates formed an identifiable group

independent of the isolates from the western region but with heterogeneity within the group.

Assuming an epidemiological link between the 1993 western outbreaks and the eastern isolates, the estimated mutation fixation rate used to derive the phylogenetic tree ranged from 4.6 to 6.7×10^{-3} substitutions per site



5.1.2. Phylogenetic tree (Clustal V software) based on 190 nucleotide sequences of the E2 region of representative classical swine fever virus strains (Lowings et al. 1996) and isolates recovered between 1993 and 1998 in Cuba, Haiti, the Dominican Republic, Honduras, El Salvador, and Mexico. Brescia_ITALY_45 was sequenced twice as a measure of quality control.

per year. A higher rate was obtained among 1996 western than eastern isolates, i.e., 2.6×10^{-2} substitutions per site per year. These values are higher than estimates previously reported for viruses associated with European outbreaks (Lowings et al. 1996). In Europe, the fixation rate mutation of CSFV was estimated taking into account the variability of nucleotide sequences and comparing viruses of a common source but with different isolation dates. For this estimate, isolates from a specific geographical area (central Italy) were considered over a 6-year period. Estimated values ranged from zero to 2.7×10^{-3} substitutions per site per year in the E2 region. A similar value was obtained when the mean of the fixation rate mutation was calculated, taking into account all the probable progenitor-progeny relations (3.3×10^{-3}) of the E2 tree (Lowings et al. 1996).

Overall, the results from our studies in Cuba suggested that the eastern isolates were independent of the western; that is, there were no epidemiological links between the two groups and, phylogenetically, they represented different virus clusters. Possibly, the eastern isolates represented the emergence of viruses that had previously circulated in the population but had gone unnoticed, or they may have been introduced from the surrounding countries. This last assumption was rejected by Ward and Lubroth (2000). Although the Cuban isolates were within group 1, by comparing the Cuban isolate sequences to those of viruses from Haiti and the Dominican Republic, they found that these latter isolates formed a subgroup within group 1 distinct from the Cuban strains (Figure 5.1.2).

The methods used to characterize the genomes of the various strains and Cuban isolates allowed for the analysis of their phylogenetic relationship, as well, as an assessment of their mutation fixation rate. At the nucleotide level, most of the variations in the isolates did not result in amino acid substitutions. Some isolates possessed identical sequences in the regions of the genome that were examined. In every case, these viruses were closely related in their date of isolation and geographical origin. This suggests that they could be the same viral variants.

The phylogenetic grouping of the strains was in no way related to their virulence, which was reported as varying from clinical signs and lesions compatible with chronic or atypical infections to acute forms of the disease. These data were consistent with the fact that the sequenced region has not been reported to contain virulence markers (Lowings et al. 1996). However, it is necessary to emphasize that virulence is based not only on the intrinsic characteristics of the viral strains, but also involves characteristics of the host that, up to the moment, have not been taken sufficiently into account (Depner et al. 1996).

The most likely origin for the western outbreaks was the reemergence of the disease from the strain "Margarita" used in the vaccine potency trials. However, the out-

breaks that occurred in the eastern part of the island may have been caused by the spread of the disease from the west or may have had an independent origin. This origin seems to be related to the reemergence of the co-circulating strain before the epidemic. There does not seem to be a unique origin or a relation with the strains circulating in the Caribbean.

The unexpected appearance of outbreaks of the disease has been explained, in some cases, by the existence of persistently infected (Depner et al. 1996) and chronically affected animals that have not been detected because of their atypical symptoms (Westergaard 1996). These animals constitute a reservoir for the virus. In addition, as it is well known, viral vaccines generally do not produce clinical signs even though the virus is replicating in the animal. This means that even though there is a good program of vaccination, subclinically infected animals may be present that help spread the disease. In Cuba, the disease reemerged and spread coinciding with worsening economic conditions.

CONCLUSIONS

We can conclude that the 1993 to 1997 CSF outbreak in Cuba was not due to the introduction of new virus variants, but to the presence of previously circulating strains that emerged because of the economic crisis that had affected the country since 1990. This crisis was brought about by (1) the weakening of biosecurity measures (reduced availability of food, deterioration of disease control measures, and the decline in zoosanitary management of herds), (2) an uncontrolled increase in the number of backyard pigs (poor availability of animal protein and fat), and (3) insufficient vaccine production as a result of the emergence of rabbit hemorrhagic viral disease in 1993. At present, family pig rearing has increased to 65% of the total pig population in the country. This has hindered the implementation of a national program of control and eradication. However, Cuba has a well-organized national veterinary service; an effective epidemiological surveillance system; well-structured legislation; field, and laboratory facilities; and highly skilled veterinarians, scientists, and technicians. Nevertheless, the lack of monetary resource has been a major impediment to a major eradication project in Cuba.

In March 2000, a Food and Agriculture Organization (FAO) technical cooperation project for CSF prevention, control, and eradication was approved. This will provide the technical and organizational support needed to protect swine production in Cuba and will assure food for the population by applying state-of-the-art technology in the field of animal health with the establishment of effective CSF epidemiological surveillance and control. The project includes coordination strategies for CSF control and eradication in Cuba, as well as in other affected countries of the Americas.

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5.2 Reemergence of Classical Swine Fever Virus in Mexico

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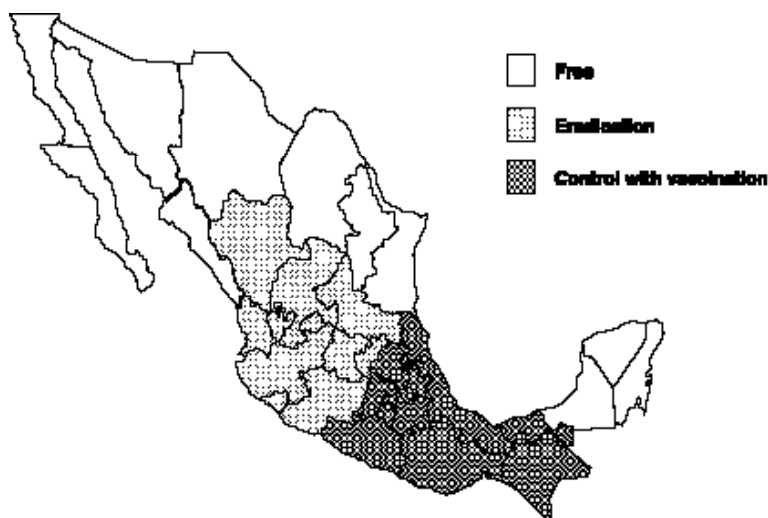
SUMMARY

In 1996, after an intensive classical swine fever (CSF) vaccination campaign, Mexico was divided in three zones: the *free area*, the *eradication area* where CSF had been eliminated and vaccination was prohibited, and the *control area* where CSF was endemic and vaccination was mandatory. In 1997, however, the number of outbreaks in the control area increased, and, by 1998, the disease had again reached the eradication area. An epidemiological analysis done during 1997 found that the increase in virus circulation in the control area was primarily in the “backyard” pig population, i.e., small private holdings. The increase in virus circulation resulted from the entry of susceptible (unvaccinated) backyard pigs from the eradication area, the intensive trade in backyard pigs at animal markets, and the constant mixing among the local backyard pig populations. Herd immunity in these small private holdings was less than 50%, in spite of official vaccination campaigns. When backyard pig owners suspected CSF, they sold their pigs or sent them to the abattoir, which then became a source of contamination for trucks and personnel. In addition, virus-contaminated

meat entered the human food chain and from there to the swine population by garbage feeding. The growing levels of CSF virus (CSFV) circulating in the backyard pig population during 1997 eventually reached commercial farms in the control area and the eradication area. It was concluded that the backyard pig population maintained an endemic cycle of CSFV infection and was the source of new outbreaks in the eradication area.

INTRODUCTION

In Mexico, CSF had previously been endemic in most of the country. Animal health authorities established a control and eradication campaign based on certification of the potency of all the vaccine lots, intensive vaccination, quarantine of affected premises, killing of animals, and control of animal movement. By 1996, the country was divided into three areas: (1) an area that had been internationally certified as CSF free and that consisted of the states of Sonora and Yucatán, (2) an eradication area where the disease disappeared and vaccination was stopped, and (3) a control area where CSF was endemic and vaccination was continued (Figure 5.2.1).



5.2.1. In July 1998, Mexico was divided in three classical swine fever areas. By the end of 1998, the disease had again appeared in the eradication area.

The incidence of the disease declined to its lowest point during 1996. Then, in January 1997, there were 29 outbreaks. This increased to 162 in December. By 1998, CSF reappeared in the eradication area, and vaccination was allowed in order to avoid further spread. By the year 2000, any difference in the CSF status between the eradication and control areas had disappeared because the disease was present and vaccination enforced in both areas (Solis 2000). By 2001, the official CSF situation was that the states in the northern part of the country and the states on the Yucatán Peninsula (Yucatán, Quintana Roo, and Campeche) were free of CSF. The disease was endemic in the remaining states in the central part of the country.

Several factors led to an increase in virus circulation in the control area during 1997 and to the subsequent reemergence of CSF in the eradication area in 1998. An epidemiological analysis of the outbreaks found that 87% of the outbreaks occurred in backyard pigs and 13% on commercial farms, indicating that viral circulation occurred primarily within the backyard pig population. In Mexico, approximately 34% of swine production involved backyard pigs (Rosales et al. 1997, 2000).

To determine some of the epidemiological factors involved in the reemergence of CSF, several surveys were done in the control area during 1997 (Estrada et al. 1998, 2001), and the results are described in the following sections.

Backyard Pig Production Units

Backyard producers finished very few animals and only occasionally produced their own pigs, generally maintaining one boar and up to five sows (Suárez and Barkin 1990). However, because a large number of people living in rural and suburban areas were involved in this type of production throughout the central part of the country, the backyard pig population reached a high density in some areas. In most cases, backyard herds surrounded commercial farms.

In 94% of backyard herds, facilities were rustic, located near the house, and used no sanitary preventive measures. Pigs were attended to by family members as side work. In some cases, pigs were allowed to roam and seek their own food. Alternatively, commercial food was provided and, in around 10% of the herds, animals were fed swill. Up to 20% mortality was considered normal.

Trade in live animals was done by buying a few pigs from other backyard pig premises, from commercial farms, or at animal markets. Animals were fattened and sold to other pig producers, animal markets, or the abattoir, or were slaughtered for private consumption. On 30% of the premises, the herd “turned over” every 3 months.

It was found that 31% of the outbreaks were associated with the introduction of animals that had been purchased at animal markets. When owners noticed that the

animals were sick, they killed them, sold their meat, and tried to sell the rest of the pigs in the herd.

Official personnel from the animal health authorities vaccinated backyard pigs. In 14 municipalities of the control area that were surveyed, animal health authority officials reported vaccination of an average of 25% (range, 9% to 100%) of the pigs. A serological survey found that 51% (range, 21% to 86%) of the pigs in the 14 municipalities surveyed had serum antibodies against CSF virus (CSFV), as determined by an enzyme-linked immunosorbent assay E2 test. In only a few cases were pig deaths reported to the animal health authorities.

Backyard Pig Trade

The backyard pig trade was carried out primarily at animal markets or *tianguis*. The results of the surveys showed that an average 40% of the pigs came from the CSF eradication and free areas and, therefore, were susceptible to infection. The remaining 60% came from the control area where CSF was endemic. Pigs were taken from one animal market to another until sold. This marketing process allowed pigs to be exposed to many other pigs in the same conditions for several days, thereby increasing the chance of becoming infected. An average of 37% (range, 25% to 67%) of susceptible pigs were vaccinated against CSF when bought by the dealer.

Commercial Farms

Commercial farms typically provided production units for breeding, farrowing, weaning, growing, and fattening. Pigs generally were sold to slaughter or as weaners or breeders to other farms. Boars, gilts, or semen were introduced into the herd on an ongoing basis to improve the genetics of the herd. Balanced nutrition and vaccination programs were used.

CSF vaccine was used in only 87% of the farms where CSF vaccine and few biosecurity measures were in place. Only 83% had fence facilities. In 83% of the farms, the truck-loading area was inside the farm, and trucks were cleaned but not disinfected.

In most herds, farmworkers changed their clothing, but only 6% of the farms provided shower-in/shower-out facilities. The majority (82%) of workers owned backyard pigs, and some of them gave veterinary care to sick pigs in the community. In addition, 87% of the workers brought their own food to work.

When CSF appeared, the owner commonly attempted to sell the sick pigs and vaccinate the remainder. Very few reported the disease to the animal health authorities.

Abattoirs

Pigs delivered for slaughter came from all areas: free, eradication, and control. The sanitary conditions of the loading and unloading facilities at abattoirs were poor, and trucks were not cleaned or disinfected after delivering pigs. Workers in the facilities generally did not have

the appropriate clothing and sufficient training or education in sanitation and hygiene.

Animal remains were taken by abattoir laborers, placed in heaps beside the abattoir, or removed to municipal landfills. In 1997, officials reported that pigs with CSF lesions were being condemned at slaughter.

Movement of Live Animals or Meat Products

Animal health authorities prohibited the movement of swine or meat products from the control area into the eradication and free areas. However, when market prices for pigs were low in the control area, traders sometimes bought and smuggled live animals into the eradication area, sometimes probably carrying CSFV with them.

Surveys Done in the Eradication Area

A survey done in the state of Guanajuato in central Mexico found that 13% of the processed meat products, such as ham and sausage, sold in some grocery markets, came from the control area and could potentially be contaminated with CSFV. Extensive serological surveys carried out in the backyard pig population in the state of Guanajuato, a part of the eradication area, showed that animals did not have antibodies against CSFV during 1997 to 1998, demonstrating that CSFV was not present.

DISCUSSION

Based on the results of these surveys, the primary means by which CSFV spread in the control area were established. A number of factors responsible for the 1997 to 1998 increase in the circulation of CSFV directly related to the backyard pig population. Historically, pigs were freely introduced from the eradication area into the control area. In 1995 and the first half of 1996, the vaccination of backyard pigs in the eradication zone was enforced. Therefore, the introduction of these animals into the control area reinforced herd immunity and reduced the number of outbreaks (Morilla et al. 2000). When vaccination was stopped in the eradication area in the middle of 1996, a susceptible backyard pig population appeared. These pigs were introduced into the control area through animal markets and mixed with other pigs of diverse origins, some of which were possibly infected with CSFV strains of low virulence. Pigs not sold at one animal market were taken to the next market and the next in turn until sold. This process increased the chance of becoming infected with CSFV.

It has previously been reported that endemic CSFV can produce vague clinical signs, inapparent infections, and a variable degree of mortality in backyard pig populations (Carbrey et al. 1977; Koenen et al. 1996; McCauley 1993). Thus, CSFV survived easily in the backyard pig trade chain because of the subacute and chronic forms of presentation of the disease, the low level of vaccination, and the intensive trade in animal markets. Although the

mortality rate was 10% to 20% of the pigs, it was rarely reported to veterinary officials (Barreto 1995; Beal et al. 1970; Zepeda 2000). For this reason, the eradication of CSF in the Rivas area in Nicaragua led to an increase of 30% in the number of pigs in the region (J. Rooijakkers, personal communication).

Some commercial farms in the control area became infected with CSFV. In part, this was because vaccination gave a false sense of security to the owners. The perception was that vaccinated herds would not become infected with CSFV, and, therefore, biosecurity measures were lax or not enforced. A variety of people who had previously had contact with pigs easily entered these farms, i.e., service, sales, and repair persons, veterinarians, buyers of live or dead pigs, friends of the owner, family members, and others. They were not required to change clothing or shower before or after entering the farm. Dogs and cats, which commonly travel among farms and may play a role in the mechanical transport of virus, were also not controlled. In addition, 82% of the workers at the commercial farms also owned backyard pigs or gave medical assistance to pigs in the community during their free hours. These people, therefore, could serve to transport the virus into the farm on clothes, shoes, bicycles, etc. Furthermore, 87% of the workers brought food onto the farm, including foodstuffs that might contain CSFV, such as pork products.

In commercial farms, the virus may remain endemic in spite of vaccination. Upon entry, CSFV infects some susceptible animals by contact, but the common practice of injecting groups of animals with one needle promotes the spread of virus to other susceptible pigs and results in the contamination of bottles of vaccine, antibiotic, iron, etc., thereby maintaining the virus endemically on the farm (Hamdy 2000; Terpstra 1992, 1996).

When CSFV entered into a backyard or commercial herd, it took between 1 week and up to 2 months before the owner suspected the disease and, if the outbreak was reported to animal health authorities, it took on average an additional 25 days for depopulation. Therefore, during that period, viremic animals were sold to the abattoirs or other farms, and vehicles and workers of the farm became contaminated, inadvertently spreading the virus.

Abattoirs were important sources of CSFV. Clinically healthy, but viremic, animals contaminated the premises. Consequently, vehicles and workers from commercial farms delivered pigs, became contaminated with virus, and carried the virus back to their herds. Recall that, in 83% of the farms, the loading area was on the premises, and the trucks were not disinfected. In addition, contaminated pig meat entered the human food chain and, from there, the pig food chain, reaching any part of the country where garbage feeding of pigs was practiced (Helwing and Keast 1966; Mengeling and Packer 1969). A study done at the abattoirs during the eradication cam-

paign in the United States found that 1% to 2% of healthy pigs harbored CSFV in their tissues (Anonymous 1981).

CONCLUSIONS

In conclusion, to control CSF, a campaign will need to be developed to eradicate the virus from the backyard pig population—the main reservoir of the virus in Mexico.

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5.3

The Reappearance of Classical Swine Fever in England in 2000

David Paton

SUMMARY

After a 14-year absence, classical swine fever virus infected 16 English pig farms between June and November 2000. As on previous occasions, the likely source for the introduction of the virus was pig-meat products, rather than live pigs. However, on this occasion, swill feeding was thought not to have been a factor. The causal virus was closely related to others that have been periodically introduced into Europe over the last 10 years. There is a lack of knowledge about the nature of pig-meat movements around the world and the risks that they pose to animal health. Secondary cases were attributed either to pig movements or to local viral spread, possibly involving wildlife vectors. Outdoor pig keeping may increase the risk of exposure to infected meat products and to neighboring infection. Although multisite pig production increases pig movements, the integration of many local farms into a single pig-rearing enterprise may help to ensure that movement of infected pigs does not result in distant viral spread. Restricting pig movements, slaughtering affected herds, and investigating dangerous and neighbor contacts controlled the outbreaks. Concurrent cases of porcine dermatitis and nephropathy syndrome complicated the clinical diagnosis. Testing pooled blood samples by reverse transcriptase-polymerase chain reaction was found to be a sensitive, rapid, and reliable method for detecting infected herds.

INTRODUCTION

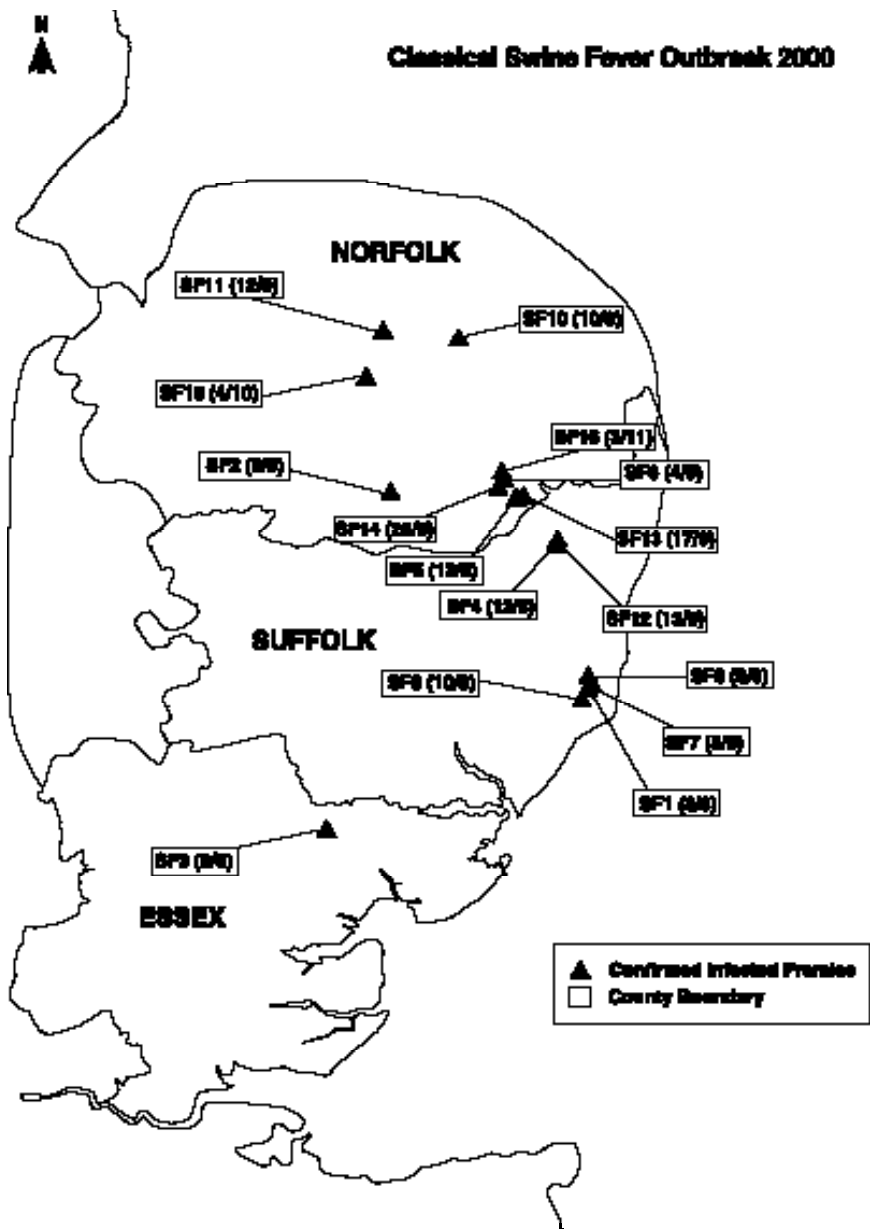
Classical swine fever (CSF) was eradicated from the United Kingdom (UK) in 1966 by means of a compulsory slaughter policy introduced in 1963. This policy was used to control outbreaks involving three herds in 1971 and ten herds in 1986. Both of these series of outbreaks were attributed to feeding unprocessed waste food containing imported pig-meat products (Williams and Matthews 1988). In August 2000, a new outbreak was detected, and subsequently disease was found at 15 other pig farms. This chapter provides a brief description of the epidemiology and control of this latest incursion.

The British pig population is quite small in European and global terms, at around 6.5 million. Changes in recent years have seen more farms under the ownership of

fewer companies, with increasing integration between feed production, multisite pig keeping, and abattoirs. In response to welfare demands, there has also been a move to keeping more pigs outdoors, with some 25% to 30% of sows now kept at pasture. The two main areas of pig production are in the east of the country, in coastal Yorkshire and farther south in the counties of Norfolk and Suffolk within East Anglia (Figure 5.3.1).

Control of CSF is subject to legislation, which is harmonized for all European Union (EU) member states. EU legislation is enshrined in directive 2001/89/EC (Anonymous 2001; Edwards et al. 2000; Moennig 2000). It is aimed at eradication, based on rapid detection, notification, and slaughter of all infected herds. Member states detecting CSF in their national herd are required to gain the approval of the European Commission for their control measures. Importation of pigs and pig products into the EU is illegal, except from other countries with an approved CSF health status. Feeding pigs with waste food (i.e., swill) of porcine origin is a major risk factor for the spread of CSF virus. Restrictions on the use of imported materials in pig food and a requirement for heat treatment of swill help to reduce this threat. The UK has had strict regulations controlling the feeding of swill since 1973. In Great Britain, responsibility for control of CSF is vested in the Ministry of Agriculture, Fisheries and Food (MAFF), and enactment of policy falls to the Chief Veterinary Officer's group based in London. They control a veterinary field service with regional bases throughout the country. Scientific consultancy, surveillance, and diagnostic services are provided by the Veterinary Laboratories Agency (VLA), which is largely government sponsored. An EU Reference Laboratory in Hannover, Germany, supports national swine fever laboratories in Europe, such as the one at the VLA.

The global CSF situation has been reviewed recently (Edwards et al. 2000), and features of the disastrous 1997-1998 epizootic affecting domestic pigs in the Netherlands have been described (Elbers et al. 1999; Pluimers et al. 1999; Stegeman et al. 2000). Although some countries have eradicated the disease, it is still present in parts of Europe, Asia, and South America. This poses a continuous threat of reintroduction to cleared areas, especially through trade in pigs and pig products. In Western Europe, CSF is not endemically present in



5.3.1. Map of East Anglia showing the location of infected premises and dates when disease was confirmed.

domestic pigs, but has a reservoir in free-living wild boar (Laddomada 2000). The virus is reintroduced to domestic pig herds on a sporadic basis, mainly through direct or indirect contact with wild boar or through importation of infected pig products.

CHRONOLOGY OF THE CASES

The presumed index case in England was the second herd identified (SF2) and was a mainly outdoor breeding

unit with approximately 550 sows. (Note that each case identified was given a unique sequential reference number prefixed by the letters "SF.") Extensive neighborhood and backward tracings from this farm and from subsequent cases failed to identify a specific source of infection. No artificial insemination had been used, and all replacement breeding stock came from a single source that remained CSF negative. There were no nearby pig-keeping enterprises or feral pigs. The two most likely sources of infection were considered to be adjoining footpaths

open to members of the public and an abattoir approximately 1.5 km away. The abattoir, which received pigs only from within the UK, appeared to have no means of direct contact with the affected unit. Serological tests indicated that the disease had probably been present at SF2 since early to mid-June, although few of the infected sows had become ill. The disease became much more obvious when it affected housed young stock in early August.

From the index case herd, weaned pigs were sold under contract to a large pig-producing business that owned feed mills and abattoirs, and controlled more than 140 farms, mainly in East Anglia. Four grow-out units belonging to this company were supplied with CSF virus (CSFV)-infected pigs between the end of June and the beginning of August (SF1, SF3, SF4, and SF5). Each was located in a different part of East Anglia, and all were outside the 10-km surveillance zone around SF2. Other grow-out herds supplied prior to this period remained uninfected. One of the affected grow-out units was actually the first CSF case to be confirmed (SF1) in early August. This farm operated an all-in/all-out policy, receiving batches of recently weaned pigs from a variety of company-owned or contracted breeding herds. The pigs were kept for approximately 6 weeks before dispatch to finishing units and thence on to slaughter. There had been significant disease in one of the supply herds for several months, ascribed by the experienced company veterinarian to porcine dermatitis and nephropathy syndrome (PDNS). When CSFV-infected pigs arrived from the index CSF case (SF2), a diagnosis of PDNS was initially made, and CSF was not suspected for a further 2 weeks; that is, after losses had accelerated steeply. By the time of the official visit, about a third of the 3600 pigs were sick, and some 200 had died.

Approximately 3 weeks later, a further cluster of cases was identified in farms adjoining case SF1 (cases SF7, SF8, and SF9). All of these units kept pigs outdoors, and local spread was suspected, possibly from dead pigs that had been scavenged by wild animals from an open yard at SF1, prior to its recognition as a CSF virus-infected premises. Two further outbreaks resulted from pigs being supplied by SF7 to two distant units in North Norfolk, which became case SF10 and case SF11, respectively. SF15 had received pigs from SF11.

The origins of infection for cases SF6, SF12, SF13, SF14, and SF16 are less certain. SF12 was very close to SF4, while the others were all within a 10-km radius of one another close to SF5. A variety of indirect links could explain the spread of infection among these cases (Sharpe et al. 2001). The patterns of disease and the serological findings indicated that these herds had become infected after the earlier cases. The last outbreak (SF16) was confirmed CSF positive at the beginning of November, approximately 3 months after the first case.

CONTROL MEASURES

Foremost among these was the imposition of movement restrictions within protection and surveillance zones of approximately 3 and 10 km, respectively, around infected herds. A total of 850 premises were placed under movement restrictions during the investigations, 264 of which resulted from the presence of suspicious signs of disease. At the start of the epidemic, movement restrictions within the protection and surveillance zones were enforced only once CSF had been confirmed. Later, however, restrictions were put in place as soon as disease was suspected in order to prevent preemptive pig movements. Infected herds were slaughtered, and thereafter cleansing and disinfection was carried out. From these herds, approximately 41,500 pigs were destroyed and rendered. Cleansing and disinfection procedures on large outdoor pig units were very thorough and therefore protracted, and in some cases took over a month to complete. Forward and backward tracings and epidemiological enquiries were used to identify possible routes of virus spread. Restricted herds were subject to regular health checks and laboratory tests. As the epidemic progressed, there was an increased use of preemptive slaughtering, whereby dangerous contacts and herds especially close to confirmed cases were killed without waiting for laboratory confirmation of the CSF health status. Forty such herds with approximately 31,900 pigs were slaughtered out, but were not subsequently shown to be CSF infected.

MAFF headquarters in London retained overall responsibility for coordinating and regulating the control effort. Meanwhile, a CSF control center was established at the local animal health office, and the resident veterinary team was augmented by a large number of seconded veterinarians and administrative support staff from other parts of Britain. Assistance was also received from overseas, including veterinarians with experience in CSF outbreak management and others using their participation as a training opportunity. The control center organized local epidemiological investigations and all farm visits for clinical inspections, data gathering, and sample collections. Prioritizing activities under these circumstances was difficult. Farms with suspect disease tended to always attract the highest priority, followed by hot tracings from affected premises. More difficult judgments were required as to whether routine surveillance testing took precedence over low-level tracings. Where several nearby farms became infected at different times, the surrounding herds could end up being visited on numerous occasions for different reasons. Integrating the findings from all such visits was a monumental challenge.

Where pigs were compulsorily slaughtered because of CSF, owners were compensated by the government, receiving full market value for healthy animals and 50%

for sick animals affected by CSF. There was no government compensation for consequential loss as a result of swine fever. More problematic was the difficulty of dealing with premises in protection and surveillance zones that were under restrictions for a protracted period. Many farmers kept their pigs for as long as possible, in the hope that restrictions would be lifted. This led to welfare problems as they ran out of space. The government introduced a unique scheme, the Pig Welfare Disposal Scheme, jointly funded by government and the industry. Under this scheme, following a clinical examination of the herd, animals were killed and rendered. The owners then received some compensation for them.

LABORATORY DIAGNOSIS

The testing strategy was based on the recommendations of the diagnostic manual produced by an EU working group (Anonymous 2000). From herds with clinical signs suspicious of CSF, blood and organ samples were submitted for both CSF and African swine fever (ASF) diagnosis. For the purposes of this narrative, only CSF testing is covered. Blood samples in ethylenediaminetetraacetic acid (EDTA) were taken from living sick pigs. Tonsils and other organ samples were taken from dead or euthanized sick animals. Clotted blood for serology was collected from in-contact animals. In the laboratory, pools of up to 10 EDTA blood samples were tested by a single-tube reverse transcriptase-nested polymerase chain reaction (RT-nPCR) employing a CSFV-specific fluorescent probe (McGoldrick et al. 1999). Meanwhile, organs were tested by a fluorescent antibody test (FAT) on frozen sections and by virus isolation in PK15 cell cultures. Isolates were confirmed as CSFV with a monoclonal antibody that did not recognize other pestiviruses. At least one sample from each case was further characterized by partial genetic sequencing (Paton et al. 2000). Sera were tested for antibodies to CSFV by a screening enzyme-linked immunosorbent assay (ELISA) (Colijn et al. 1997) with positive results confirmed by comparative neutralization tests employing CSFV, bovine viral diarrhoea virus, and Border disease virus.

In herds with an epidemiological link to known case herds, but where no clinical disease was evident, testing was for CSFV but not ASF virus. Clotted blood samples for serological testing were collected from a proportion of the pigs. The sampling strategy depended on the link involved and whether high-risk groups could be identified. Latterly, an ELISA (Chekit-CSF-Virus-III; Bommeli Ag, Liebefeld, Switzerland) was used to screen some sera for the presence of CSFV antigens.

At depopulation, blood samples were also collected from a proportion of pigs for serology and viral detection. There were two reasons for doing this: firstly to establish how long virus had been present in herds where disease had already been confirmed, and secondly to

check whether virus was circulating in herds that had been preventively culled as dangerous contacts. Viral detection in these cases employed either RT-nPCR on pooled bloods (as above) or was by means of microwell virus isolation (De Smit et al. 1994).

Having some redundancy in the testing regimen was very valuable in confirming the results. This was particularly important for the first case where there was the greatest concern that the results might have been nonspecific. FAT staining did not always give conclusive results, compared with RT-PCR and virus isolation. RT-nPCR was very reliable and rapid, and false-positive results were not a problem. More emphasis might be given to the use of this method in future. Using the method on pooled samples enabled a four-person team to test over 8000 blood samples in 3 months. Partial genetic sequencing showed that all 15 virus isolates recovered in the epidemic were identical to one another (SF15 was confirmed by serology alone). Since the sequence obtained was different from any of those in our database, we were confident that the isolates were not adventitious laboratory contaminants. The new UK CSFV was in genetic subtype 2.1, a group that is endemic in Asia but not Europe (Paton et al. 2000).

DISCUSSION POINTS

The most likely source of the index case for these outbreaks was an imported pig product. This is consistent with the genetic typing of the virus, which suggested an introduction from a part of the world from which live pigs or semen are most unlikely to have been imported. Furthermore, evidence for other plausible means of virus introduction are lacking. This introduction into Europe may be considered as the latest in a series involving CSF viruses of genetic type 2.1 (Sandvik et al. 2000). The incident highlights the need for authorities to have effective, tight controls on imports of pig products whether they are commercially traded or personal imports. The low probability of contaminated products ever being fed to pigs leads one to suppose that the actual incidence of such imports must be a great deal higher. The keeping of pigs outdoors may increase the risk of their being exposed to contaminated pig products, either fed to them by members of the public or scavenged from landfill sites by wild animals.

A lack of pathognomonic signs, particularly in older pigs, has been a common problem in CSF diagnosis in recent years (Koenen et al. 1996; Williams and Matthews 1988). The presence of PDNS greatly exacerbated this difficulty. PDNS became a serious problem in Britain about a year previously (Gresham et al. 2000a,b; Sandvik and Gresham 2000). Most cases had occurred in East Anglia, and the particular breeding company most affected by the CSF outbreaks had also been seriously affected by PDNS. The similarity of the clinical signs between CSF

and PDNS made on-farm diagnosis of CSF exceedingly difficult. There is a serious danger that consideration of CSF is overlooked when PDNS is prevalent. Where CSF and PDNS occur concurrently, there is also a risk that even when CSF investigations are instigated, random sampling of small numbers of sick pigs may target PDNS-affected animals and fail to detect CSF.

When designing sampling strategies, there is a very difficult balancing act between being sufficiently prescriptive and sufficiently flexible. Ultimately there is no very satisfactory substitute for trained field staff with a proper understanding of epidemiological considerations.

Placing restriction zones around newly discovered outbreaks reduces the probability of local disease spread from neighboring herds that may have infected, or been infected by, the already detected outbreak. In this epidemic, local spread was confined within the 3-km protection zones set up around the outbreaks and did not extend into the 7 km of surrounding surveillance zones. On the other hand, spread by pig movements was frequently to areas completely outside the surveillance zones. An argument has been made previously for reducing the size of restriction zones from 10 km to 6 km in order to target effort where it will be most effective (Roberts 1995). The difficulty of detecting local spread of relatively low-virulence virus, until after several weeks had elapsed, was the main reason for increasing the use of preemptive slaughter in the protection zones. A trend to increased use of preemptive slaughter during an epidemic was also reported from the Netherlands (Pluimers et al. 1999). Nine of the 16 outbreaks, and all of the first seven, involved farms that were part of a single business enterprise. It could be argued either that the integrated pig flow contained the outbreaks from the wider pig population or that it helped to spread it within the particular company.

On the diagnostic front, the major problem continues to be early detection before widespread disease is apparent. This is particularly difficult in very large units with many thousands of pigs. Large-scale temperature testing was used, although current methods requiring individual application of rectal thermometers are arduous and unpopular with field staff when dealing with outdoor units. Random virological testing of large numbers of pigs was only attempted latterly and using antigen ELISA, by which time CSF had already disappeared. Due to its higher sensitivity, RT-nPCR has a much higher likelihood of achieving a preclinical diagnosis. We managed to examine large numbers of samples by a manual RT-nPCR method. However, without robotic sample handling, it was not possible to scale up to the extent needed to carry out random testing of a significant proportion of apparently healthy animals within suspect herds. Serological and, to a lesser extent, virological testing of a cross-section of the pigs slaughtered in confirmed CSF-

affected herds was useful to determine how long the units had been affected (Laevens et al. 1998; Stegeman et al. 1999), as well as to show the pattern of spread within the unit. It was observed that spread within outdoor herds could be quite slow and could be considerably hampered by electric fences between animals in adjoining paddocks.

All such episodes of disease emphasize the need for thorough and integrated contingency plans and an ability to switch on large-scale field and laboratory resources at short notice (Pluimers et al. 1999). As elsewhere in Europe, the killing and disposal of many thousands of pigs and dealing with welfare and financial issues in relation to long standstill periods were very challenging. It is interesting to contrast these long standstill periods with the much shorter ones used when CSF was first eradicated from Britain (Beynon 1969). The drawbacks for such a precautionary approach have to be balanced against the advantage of international acceptance and regionalization of trading restrictions. Public relations is, in fact, of increasing importance, whether it be convincing a growing international audience of the provenance of one's control measures in order to avoid punitive trade sanctions or in maintaining goodwill and cooperation from hard-pressed local farming communities.

ACKNOWLEDGMENTS

I am grateful to colleagues at the Ministry of Agriculture, Fisheries and Food (MAFF) and the Veterinary Laboratories Agency (VLA) for their helpful advice in preparing the manuscript of this chapter. My own input was supported by MAFF Grant SV3000.

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5.4 Experiences with Classical Swine Fever Vaccination in Mexico

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SUMMARY

Vaccination with attenuated, live strains of classical swine fever (CSF) virus is currently used for prevention, control, and eradication of CSF in Mexico. However, at the end of the 1980s, it was found that only 64% of vaccinated herds were fully protected. Possible causes of vaccine failure in the remaining 36% of vaccinated herds were analyzed. It was concluded that the most likely causes of vaccine failure were a lack of efficacy in some lots of vaccine and maternal antibody interference. Advantages and disadvantages of controlling CSF through vaccination are discussed, as well as new developments in vaccine technology, such as subunit vaccines.

INTRODUCTION

Vaccination is used to prevent and reduce the number of outbreaks of classical swine fever (CSF) and, together with other control measures, was an important factor in the eradication of CSF in Holland in 1985 (Terpstra 1992) and the Rivas region in Nicaragua (Zepeda 2000). Historically, CSF vaccine has been based on attenuated strains, i.e., lapinized Chinese and tissue culture-adapted strains. Recently, subunit vaccines have been developed. These have the advantage of providing the ability to distinguish vaccinated versus field virus-infected animals by means of differential serological assays (Moor-mann et al. 2000).

In most countries where CSF is endemic, vaccination is the method of choice for controlling the disease because unskilled workers are readily trained to administer vaccine, the vaccine is relatively inexpensive, and vaccination reduces the cost of clinical disease. However, vaccination also has disadvantages. In particular, it may allow the virus to remain endemic in herds. For this reason, as prevalence declines in a CSF eradication program, it is necessary to stop vaccination and begin the depopulation of infected herds. In this chapter, we describe the experiences we have had in Mexico with vaccines and vaccination in the control of CSF.

EFFICACY OF ATTENUATED VIRUS VACCINES

In the 1980s, the vaccine strains used in Mexico were PAV-1, GPE, PAV-250, and Chinese. During that time, a study was done to determine the efficacy of the vaccines at the herd level. The experimental design involved vaccinating pigs under farm conditions and then challenging groups of five pigs with virulent CSF virus (CSFV). It was found that, of 44 lots of challenged pigs, only 28 (64%) had 100% protection. The vaccine induced lower herd immunity than expected (Morilla 1991, 1994).

In response to these results, immediate measures were taken to reduce vaccine failure. Based on the aforementioned study, the animal health authorities allowed the continued use of PAV-1, PAV-250, and GPE strains and removed the Chinese strain from the market. In addition, each lot of vaccine was officially tested for efficacy. This requirement had not previously been in place. The efficacy testing procedure was to immunize five pigs with a 1:100 dilution of the vaccine and then challenge them 15 days later with a virus challenge strain, such as AMES or ALD, at a virus concentration of 10^6 lethal dose 50% (LD_{50}). If the test were valid and the vaccine efficacious, the expected outcome was that all five control animals would die, but none of the vaccinated animals would show clinical signs and all must survive. Thereafter, only vaccine lots with 100% of efficacy were marketed.

IMPACT OF THE COLD CHAIN ON VACCINE EFFICACY

Martínez-Jáuregui et al. (1992) analyzed the CSF vaccine cold chain for possible problems related to vaccine failure. This study included an evaluation of the time and temperature parameters under which vaccines were held during the packaging process at the laboratory, during transportation, and in storage at the veterinary pharmacies. The investigators subsequently followed the transport of the vaccine to the farms by pig producers and documented the vaccination of animals. The results were that the vaccine had occasional periods of a few hours at room temperature.

Several experiments were then conducted to determine the rate of inactivation of the PAV-1 vaccine strain under various environmental conditions (Morilla 1994):

1. The stability of the vaccine under proper storage conditions was examined. The lyophilized vaccine had an expiration date of 1 year after production when maintained at 4°C. Eleven lots of vaccine were tested 1 year beyond the expiration date, and all passed the efficacy test.
2. A study of accelerated shelf life was carried out. Six lots of vaccine were stored at 37°C for up to 2 weeks. At the end of the first week, three lots were tested and passed the efficacy test. At the end of 2 weeks, two (66%) of three passed the efficacy test.
3. The stability of the rehydrated product on the farm was considered. When vaccinating a large number of animals on the farm, it was not uncommon for producers to use an automatic syringe attached to a 500-ml bottle of vaccine carried in the rear pocket. To evaluate the stability of the vaccine conditions simulating body temperature, the diluted vaccine was placed in a water bath at 37°C. Three animals were vaccinated after 30 minutes in the water bath and three after 60 minutes. Two weeks after vaccination, the pigs were challenged. None became sick or died of CSF. Therefore, once reconstituted, the vaccine remained efficacious at 37°C for 1 hour. Subsequent information indicated that vaccine virus would be inactivated after 2 hours under such conditions (C. Terpstra, personal communication).

On the basis of field and experimental observations, it was concluded that the cold chain and on-farm usage did not negatively affect the efficacy of the PAV-1 vaccine and was an unlikely cause of vaccine failure.

IMMUNOSUPPRESSION

Aflatoxin intoxication has been reported as a cause of immunization failure with *Erysipelothrix rhusiopathiae* bacterins (Cysewski et al. 1978). To demonstrate a causal link between CSF vaccine failure and mycotoxin intoxication, five pigs were fed aflatoxin B1 for 21 days at a rate of 1.0 part per million and five pigs at 0.6 parts per million, and then vaccinated and fed aflatoxin for an additional 15 days. Although animals lost weight on the aflatoxin-contaminated diet, none of the experimental animals developed fever or died after challenge with a virulent CSFV strain. In contrast, the five pigs in the control group died (Izeta et al. 1990). It was concluded that aflatoxin B1 intoxication did not in-

terfere with the development of a vaccine-induced protective immune response.

MATERNAL ANTIBODY INTERFERENCE

Pigs without maternal antibodies can be vaccinated with attenuated strains as early as 1 day of age. However, in herds where vaccine is routinely used, piglets have circulating maternally derived antibodies that block the immune response against the vaccine virus. As shown in Figure 5.4.1A, in herds where only sows were vaccinated, maternal antibodies were present until pigs reached approximately 3 months of age (Morilla 1997).

Under such conditions, vaccination of animals at 3 to 4 weeks of age produces an irregular antibody response (Figure 5.4.1B). In contrast, vaccination of pigs at 7 weeks of age induced antibody response in at least 90% of the pigs when measured 2 to 3 months after vaccination (Figure 5.4.1C).

Corona et al. (1996) also found that the age at which piglets should be vaccinated is related to the disappearance of maternal antibodies. When pigs were vaccinated at 3 weeks of age, 60% (12 of 20) were seropositive 3 months later; at 5 weeks of age, 62% (53 of 85); at 6 weeks of age, 79% (103 of 130); at 7 weeks of age, 96% (47 of 49); at 8 weeks of age, 100% (10 of 10); and at 9 weeks of age, 87% (33 of 38). Thus, pigs should be vaccinated at 6 weeks of age or older to avoid maternal antibody interference.

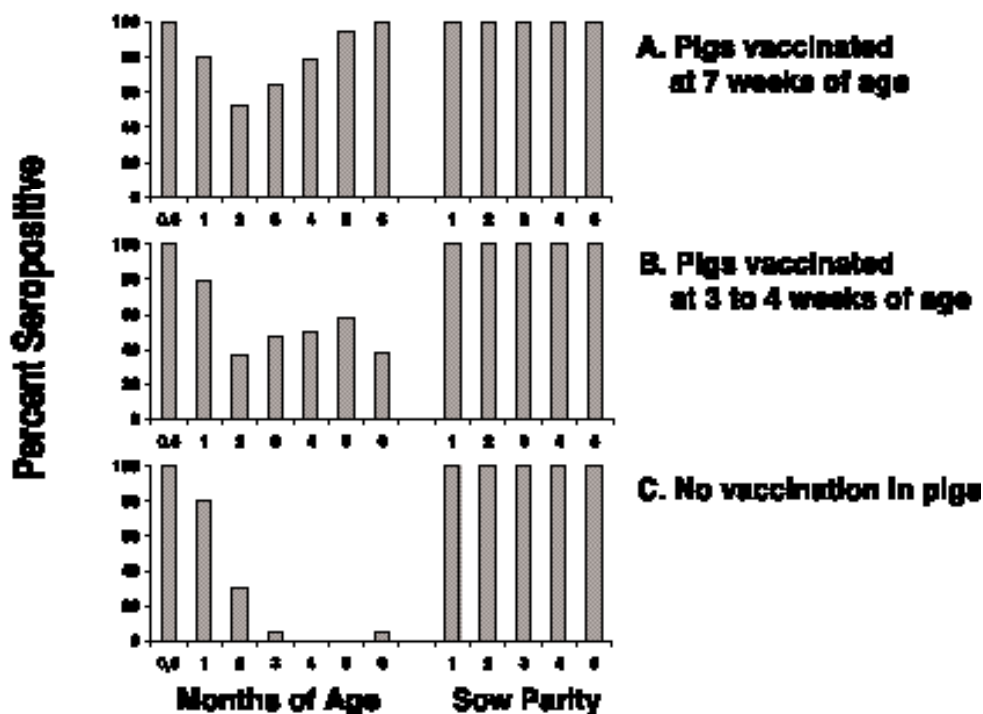
Herd animals were sometimes vaccinated two or more times during the weaning and growing periods. A survey by Corona et al. (1996) found that 77% of the herd piglets were vaccinated once and 23% were vaccinated two or more times. However, serologically it was found that 84% of 5- to 6-month-old pigs that had been vaccinated once were seropositive versus 79% of pigs in herds where animals were vaccinated two or more times. Thus, there was no benefit to vaccinating pigs twice.

From these studies, it was concluded that the principal cause of vaccine failure was vaccination of piglets before 6 weeks of age.

ADVERSE EFFECTS OF VACCINATION

Respiratory Signs

CSF vaccine virus multiplies in actively growing cells, such as fetal and reticuloendothelial cells. Pijoan et al. (1980) reported that animals had more respiratory infections due to concurrent *Pasteurella multocida* infection after vaccination with Chinese strain virus. However, systemic immunosuppressive effects resulting from CSF vaccination of healthy animals have not been demonstrated when compared with pigs inoculated with virulent CSFV (Martínez-Sosa et al. 1986, 1993, 1995).



5.4.1. Serological profile (enzyme-linked immunosorbent assay) of herds vaccinated against classical swine fever. **A:** Sows vaccinated 1 week before weaning, and pigs vaccinated at 7 to 9 weeks of age. **B:** Sows vaccinated 1 week before weaning, and pigs vaccinated at 3 to 4 weeks of age. **C:** Sows vaccinated 1 week before weaning and pigs not vaccinated.

Decline in Production Parameters

Vaccine virus may induce embryonic death and myoclonia congenita when administered to pregnant sows. When herds are vaccinated for the first time and pregnant sows are included, a fall in sow fertility has been reported in the 6 weeks following vaccination, after which fertility returns to normal. To evaluate the possible effects of vaccine strain in herds, production parameters in three farrow-to-finish farms using vaccine were compared with three farms not using vaccine. Parameters statistically different ($p < 0.05$) were the following: (1) return to estrus (18.8% in vaccinated herds vs 12.2% in non-vaccinated herds); (2) litters with fewer than seven piglets born alive (17.7% in vaccinated herds vs 10.8% in nonvaccinated herds); and (3) preweaning mortality (14.8% in vaccinated herds vs 10.9% in nonvaccinated herds). It was concluded that vaccination lowered herd productivity (Morilla 1994).

Adverse Reactions to Vaccination

In the 1980s and early 1990s, during the time lapinized Chinese strain was used in Mexico, vaccinated pigs occasionally exhibited adverse reactions and some died (Cervantes et al. 1987). Clinically, the response resembled anaphylactic shock due to previous sensitization, but it

was frequently observed in pigs that were vaccinated for the first time and, if the recovered animals were revaccinated, they did not exhibit the same response. Therefore, it was postulated that the response was due to endotoxins present in the vaccine because of bacterial contamination, rather than anaphylactic shock due to allergens.

To investigate this possibility, 0.1 ml of the vaccine causing adverse reactions was injected subcutaneously in the ears of ten 6-week-old pigs that had never been vaccinated. Within 5 minutes, the animals developed hematomas at the application site. In addition, abundant bacterial growth was observed when an aliquot of vaccine was placed in thioglycolate broth. It was concluded that some lots of lapinized vaccine were contaminated with bacteria and the response in vaccinated pigs was endotoxic shock (unpublished results).

CSF Following Vaccination

Another side effect of vaccination was postvaccinal CSF (Ramírez 2000). In farms where a field strain of CSFV was endemic, a few sick pigs were sometimes observed within 2 weeks of vaccination, some of which would die with clinical signs and pathologic lesions compatible with CSF. These episodes were usually blamed on insufficient attenuation of the vaccine strain. However, when these

herds were inspected closely, it was found that farmers used one needle to vaccinate large groups of pigs. In persistently infected herds, when viremic pigs were vaccinated, the field virus was transmitted on contaminated needles, and susceptible animals became sick.

POSITIVE EFFECTS OF VACCINATION

The best way to eliminate CSFV when there is an outbreak is to quarantine the herd and kill all the animals (Van Oirschot 1999; Moennig 2000). However, in countries where the virus is present and elimination of herds is not economically feasible, vaccination is commonly practiced to minimize economic losses. Vaccination usually reduces the number of sick animals and the number of outbreaks in a region, and there are several examples of the successful control of CSF through vaccination. In the Department of Rivas in Nicaragua, after 2 years of intensive vaccination of the swine population together with other control measures, the disease was eradicated (Zepeda 2000). In Mexico, after extensive vaccination that reached more than 90% of backyard pigs and animals in commercial farms during 1994 through 1996, outbreaks of CSF were eliminated from the eradication area (Morilla 2000; Morilla et al. 2000). In Japan, a 90% of vaccination coverage with the GPE-vaccine strain and other control measures has resulted in no outbreaks since 1992 (Edwards et al. 2000).

ADVANTAGES AND DISADVANTAGES OF MODIFIED-LIVE VIRUS VACCINES

The advantages and disadvantages of vaccination during an outbreak have been described by Terpstra (1992) and Hamdy (2000). Together with our observations, these could be summarized as follows:

Advantages of vaccination:

- It is easy to perform.
- The cost is low.
- It is the only method currently available to prevent and stop outbreaks of CSF when slaughter is not possible.
- It induces life-long immunity with one dose.
- It is acceptable to producers; particularly, compared to the slaughter of animals.

The disadvantages of vaccination:

- There is a false perception by farmers that vaccination protects herds against infection with a field strain of CSFV. Under this false sense of security, biosecurity measures are relaxed.
- In an outbreak, farmers believe that vaccination will cure sick animals and the virus will be eliminated from the herd.

- Vaccination in infected herds helps spread field virus. This is primarily due to the use of one needle to vaccinate groups of animals. This results in the transmission of virus from viremic, field strain-infected animals to others in the herd. At the same time, vaccines, antibiotics, and injectable iron may become contaminated with field virus.
- In endemically infected, vaccinated herds, there is a selection for low-virulent CSFV strains. These infections may pass unnoticed until producers become complacent and stop vaccination, at which time herd immunity wanes and new outbreaks appear.

OBSERVATIONS IN VACCINATED, INFECTED HERDS

In regions where CSF outbreaks appeared in susceptible herds, vaccinated herds also became infected. When this happened, pig producers usually attributed the clinical signs to acute porcine reproductive and respiratory virus infection. They did not suspect CSF because the herd was vaccinated and, therefore, "it could not be infected with CSFV."

Clinical signs in these herds included abortions, a drop in fertility, the birth of mummified fetuses and pigs of low birth weight, and increased mortality, especially 2 weeks after weaning and, to a lesser degree, during the suckling and fattening periods. Less frequently, clinical signs included sudden death, fever, cyanosis of the skin and tips of the ears, nervous signs such as paddling and profuse salivation, palpebral edema of the eyelids, mucopurulent secretions from the eyes, and coughing starting 2 weeks after weaning and continuing for at least 2 months afterward. An increase in the number of long-haired, runted animals was also a common finding. A cyclical pattern was observed in which a few pigs would become sick, followed by periods of clinical normalcy. In dead animals, it was uncommon to find pathognomonic lesions suggestive of CSF. The most common lesions were marbling of lymph nodes, pneumonia, petechia on serosa, but rarely in kidney and spleen, and intestinal lesions compatible with salmonellosis. Ultimately, CSFV diagnosis was done by means of a fluorescent antibody test, antigen-capture enzyme-linked immunosorbent assay (ELISA), reverse transcriptase polymerase chain reaction, and virus isolation from organs of animals that were sick or died before vaccination.

To control the disease, producers were advised to kill all animals with clinical signs and fever above 40.5°C (104.9°F), as well as underweight and long-haired pigs. This was to be done every time that sick animals appeared. Sows that returned to estrus, aborted, or gave birth to small litters, mummies, and/or weak piglets were removed from the herd. All bottles of injectable

medicines on the farm were destroyed because of the likelihood of contamination with CSFV. For the future, producers were advised to use one needle per animal. The goal was to eliminate infected animals, avoid needle-borne transmission, and build up an immune population that would resist viral replication. Once these measures were in place, it took at least 4 months for a farm to return to previous production parameters.

We concluded that when the virus began infecting herds in a region, it easily entered into vaccinated herds, reaching the few partially immune or susceptible animals of the herd. Those animals became viremic and, as discussed earlier, were the source for further needle-borne transmission. Infected pregnant sows became carriers and gave birth to infected, immunotolerant animals that broke with CSF at 1 to 2 months of age, further spreading the infection. This established a cycle wherein sick animals were treated and/or vaccinated, followed by further needle-borne spread of virus. In this manner, CSF field virus was maintained endemically in vaccinated herds.

SUBUNIT VACCINE

There are currently two commercially available vaccines based on the E2 glycoprotein. The main advantage of these vaccines is that it is possible to differentiate vaccine from field virus-induced serum antibodies in animals by means of ELISA E2 and the E(RNS) tests (Moormann et al. 2000).

To evaluate the efficacy of the vaccine, seven animals were vaccinated twice 1 month apart and challenged intramuscularly with 1 ml of inoculum containing 10^6 LD₅₀/ml of the official challenge virus ALD strain 3 weeks after the last vaccination. None of the vaccinated animals died following challenge, and only one animal had a rectal temperature above 40.5°C (104.9°F) on 1 day. In contrast, all control animals died. In a second experiment, seven 2-month-old vaccinated pigs were randomly chosen from a farm routinely using subunit vaccine and challenged intramuscularly with 1 ml of inoculum containing 10^6 LD₅₀/ml of ALD strain. None of the vaccinated animals died or had a rectal temperature above 40.5°C (104.9°F), and all control animals died. Therefore, the subunit vaccine passed the official challenge test satisfactorily (Diosdado et al. 2000).

Due to the appearance of CSF outbreaks in the eradication area in 1999, animal health authorities allowed the use of subunit vaccine in several municipalities in the state of Jalisco. In a limited field trial, it was found that in municipalities where 100% of pigs were vaccinated outbreaks stopped, but when herd immunity became lower, outbreaks continued (Diosdado et al. 2000). These results suggested that the subunit vaccine could induce a sufficient level of herd immunity to stop the clinical CSF in a region, as was suggested by Moorman et al. (2000).

Serological studies were done in three healthy herds where animals were vaccinated twice with subunit vaccine. It was found that all animals tested had developed E2 antibodies against the vaccine, and none of them had E(RNS) antibodies, indicating that they were not infected with field virus. When vaccine was used in two herds with clinically affected pigs, clinical signs of CSF and mortality stopped in few days. These two herds were then quarantined, and pigs were tested for antibodies against CSFV. In one farm where 22 animals had died of CSF, 100% (30 of 30) had antibodies against E2, indicating that they had developed antibodies against the vaccine, but 13% (4 of 30) also had E(RNS) antibodies, showing that they were also infected with field virus. In the second herd, where 440 animals had died of CSF, it was found that 100% (30 of 30) of the animals tested had E2 antibodies from the vaccine and 37% (11 of 30) also had E(RNS) antibodies due to field virus (Diosdado et al. 2000). In addition, subunit vaccine did not affect the reproductive performance of pregnant, susceptible sows, as occurs with modified-live virus vaccines.

It was concluded that the subunit vaccine in combination with the differential ELISAs make it possible to determine the degree of CSF field virus infection within herds. The fact that vaccination with the subunit vaccine did not affect pregnant sows is an additional advantage. However, these observations are based on limited field trials, and further studies are required to determine its role in the prevention and control of CSF in the field.

CONCLUSIONS

To control CSF outbreaks, vaccination with attenuated strains gives good results when used extensively and systematically. Elevated herd immunity will stop viral circulation in an area. In developing countries, vaccination is the only way to stop the economic losses from CSF and is the first step in any eradication program. Vaccination must also be accompanied by producer education. However, it must be recognized that, when used in infected herds, vaccination masks CSFV infection, and the virus continues to circulate among susceptible and partially immune animals. In a limited field trial, subunit vaccine stopped clinical signs and allowed for determining the degree of infection with field virus within the herd.

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5.5

An Update on Classical Swine Fever Virus Molecular Epidemiology

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SUMMARY

Phylogenetic analysis performed using the sequences of different regions of the classical swine fever virus (CSFV) genome showed that the CSFV strains and isolates can be divided into three groups and several subgroups. Group 1 is represented by the Brescia and Alfort/187 strains and includes old vaccine and laboratory strains isolated until the 1980s in Europe and the United States and newer isolates from Asia, South America, and Russia. Group 2 includes almost all newer viruses isolated after 1985 in Western and Eastern Europe and some Asian isolates. Up to the present, viruses of group 3 seem to be restricted to Asia. Excepting Russia, viruses of group 1 seem to have disappeared in Europe and were replaced by isolates of group 2. Independent of the movement of pigs, certain genetic subgroups were found to be associated with specific regions and do not occur at random. The importance of this is that in regions where the virus is endemic, such as the Caribbean or in the wild boar population in some regions in Europe, these viruses sporadically cause local outbreaks.

INTRODUCTION

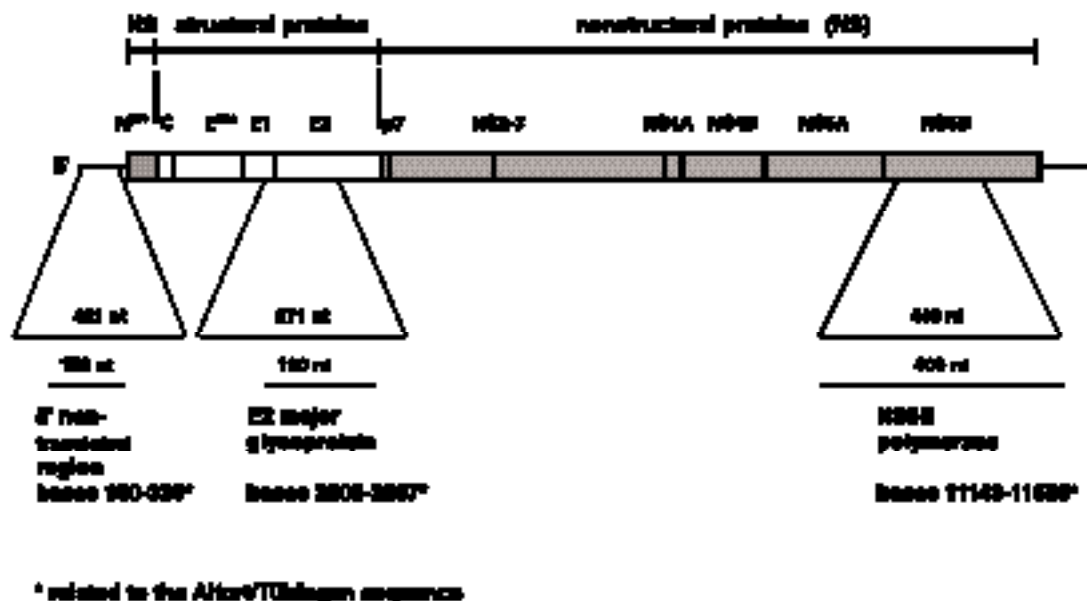
Classical swine fever (CSF) is an infectious disease of viral origin, which due to its high degree of contagiousness and its worldwide distribution is considered the most important pig disease. For this reason, it is included in List A of Obligatory Notification Diseases of the Office International des Epizooties (Moennig 1992). The causative agent of this disease is a small enveloped RNA virus that belongs to family Flaviviridae, genus *Pestivirus*, along with bovine viral diarrhea virus (BVDV) and border disease virus (BDV) (Wengler 1991). With regard to host range, virion structure, and antigenicity, all pestiviruses are closely related (Darbyshire 1960; Dinter 1963; Donis and Dubovi 1987).

To assist in epidemiological studies, modern techniques like genotyping are being applied to supplement clinical observations and serological data. These new approaches have been made possible by a greater knowl-

edge of the molecular structure of the pathogen and are based on the ability to amplify and sequence nucleic acids. The study of the variable regions of different isolates have made it possible to establish a phylogenetic relationship among them in order to obtain information concerning their origin and route of dissemination (Lowings et al. 1994, 1996). These methods have made it possible to generate epidemiological information on a number of different viruses, such as the human immunodeficiency virus (Salminen et al. 1993; Rolo et al. 1996), dengue (Guzmán et al. 1995; Rico-Hesse 1990), influenza B (Rota et al. 1992), and foot-and-mouth disease virus (Beck and Strohmaier 1987; Piccone et al. 2000).

There are several reports on the use of genetic typing of CSF virus (CSFV) in combination with epidemiological studies (Björklund et al. 1999; Díaz de Arce et al. 1999; Edwards and Sands 1990; Greiser-Wilke et al. 2000a; Harding et al. 1994; Lowings et al. 1996; Paton et al. 2000; Ward and Lubroth 2000). It was found to be a potent tool for characterizing strains involved in individual outbreaks, as well as for understanding the dissemination and evolution of the virus. It is also useful for uncovering gaps in control strategies. In addition, phylogenetic analyses performed in the last few years clearly showed that CSFV isolates that differ by genetic typing seem to be characteristic of certain geographical regions (Bartak and Greiser-Wilke 2000; Stadejek et al. 1997; Vilcek et al. 1997).

Several procedures have been used for the differentiation of individual CSFV strains and isolates, including the analysis of the binding patterns of monoclonal antibodies (Edwards and Sands 1990; Kosmidou et al. 1995), restriction data (Harding et al. 1994; Vilcek et al. 1994), and nucleotide sequences (Hofmann et al. 1994; Lowings et al. 1994, 1996; Stadejek et al. 1996; Vilcek et al. 1996). Among these procedures, the greatest discrimination has been obtained from sequence analysis in combination with phylogenetic studies (Lowings et al. 1996). These studies have been based on different regions of the viral genome (Figure 5.5.1), namely, fragments of the 3' end of the polymerase gene (NS5B) (Björklund et al. 1999; Lowings et al. 1994), the 5' nontranslated region (5' NTR)



5.5.1. Schematic representation of the classical swine fever virus genome, indicating the genomic regions proposed to be used for genotyping of isolates. The top bars refer to the regions amplified by reverse transcriptase-polymerase chain reaction, and the bottom bars indicate the regions sequenced (Paton et al. 2000).

(Greiser-Wilke et al. 1998; Hofmann et al. 1994; Stadejek et al. 1996), and the gene coding for the main glycoprotein E2 (gp55) (Díaz de Arce et al. 1999; Lowings et al. 1996; Vilcek et al. 1996).

STANDARDIZATION OF THE PHYLOGENETIC ANALYSIS OF CSF VIRUSES

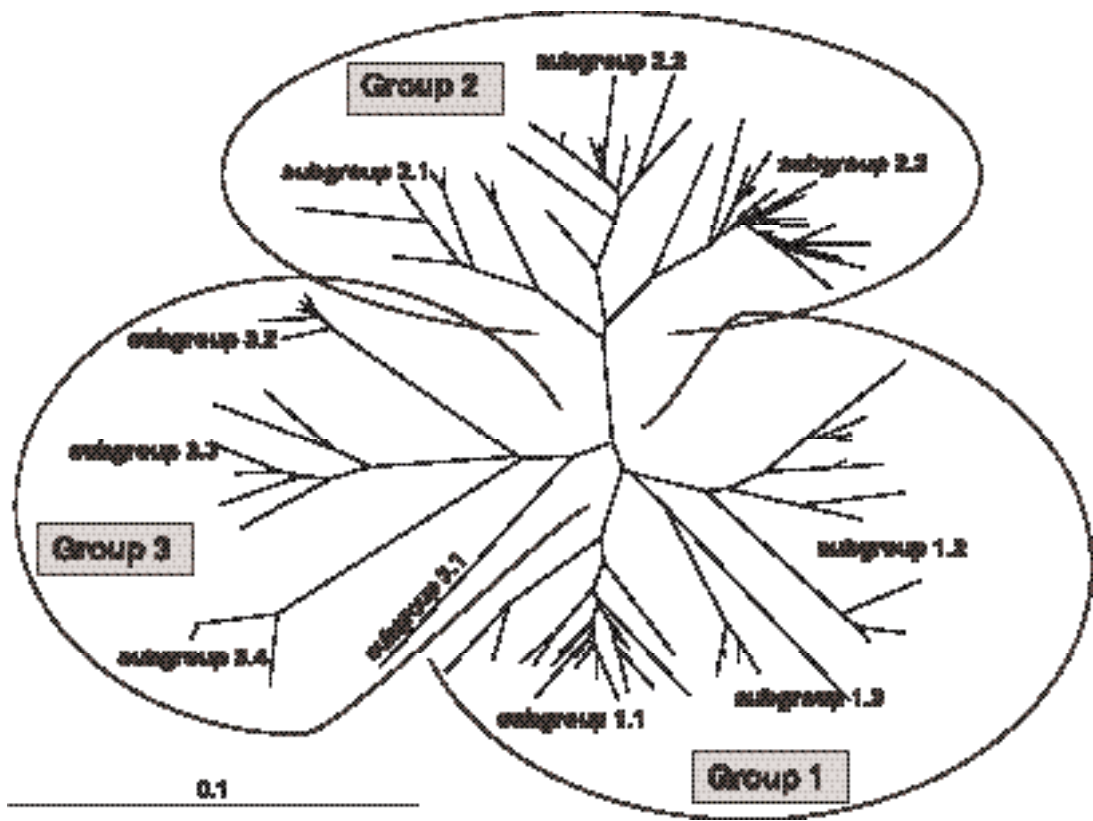
Most of the previous phylogenetic analyses of CSF viruses not only used different regions of the genome, but also differed in the algorithms applied for calculation of the phylogenetic trees and the nomenclature of genetic groups and subgroups. This greatly hampered comparison of data between different studies. To improve this situation, a working group comprising laboratories in the European Union and Asia worked on a standardized approach (Greiser-Wilke and Paton 2000; Paton et al. 2000). As a basis, three regions of the CSFV genome, which had been widely sequenced and which had been found useful for segregating viruses into genetic groups and for discriminating between isolates, were evaluated, namely, 150 bases of the 5' NTR, 190 bases of the E2 gene, and 409 bases of the NS5B gene. For calculation of the phylogenetic trees, it was agreed to suggest a protocol that uses a set of free-ware programs available from the World Wide Web (WWW), mainly the Clustal program (Thompson et al. 1994) to generate the alignments, and the DNAdist and Neighbor programs from the PHYLIP package

(Felsenstein 1989) to calculate and illustrate the phylogenetic relationship (Paton et al. 2000).

It was found that the three fragments analyzed led to essentially the same grouping, but, for closely related viruses, the larger data sets gave better discrimination. The most reliable classification was obtained with the sequence data from the NS5B region (Paton et al. 2000). In spite of this, and because extensive data sets exist for the 5' NTR and E2 gene fragments, further analyses were performed using these two fragments. To make the epidemiological and the sequence data available for other studies through the WWW, a searchable web interface that can be accessed using an Internet browser was developed (Greiser-Wilke et al. 2000b).

Nomenclature of the genetic groups and subgroups was adapted to the previously defined nomenclature based on analysis of the E2 gene fragment (190 bases) (Lowings et al. 1996). The study included European, Asian, and South American CSFV isolates, and ancient laboratory and vaccine strains. This made it possible to discriminate between two genetic groups. Group 1, represented by strains Alfort (subgroup 1.1) and Brescia (subgroup 1.2), comprised all viruses isolated before 1964 in Europe and some recent Asian and South American isolates.

The more recent (since about 1980) European isolates and isolates from Malaysia were in group 2, which formed three different clusters (subgroups 2.1, 2.2, and 2.3). Inclusion of additional Asian and South American isolates made it necessary to update the previous nomenclature (Paton et al. 2000). While isolates



5.5.2. Phylogenetic tree generated with 190 nucleotides of the E2 region from 100 classical swine fever viruses from the Americas, Asia, and Europe isolated between about 1946 and 2000. Calculation of the phylogenetic tree and nomenclature of virus groups and subgroups is based on Paton et al. (2000). The bar represents the nucleotide substitutions per site.

from Honduras, Thailand, and Malaysia formed a new subgroup in group 1, tentatively designated as subgroup 1.3, other Asian isolates, i.e., from Korea, Thailand, and one previously ungrouped ancient isolate from the United Kingdom (congenital tremor), generated a new group comprising three subgroups (3.1, 3.2, and 3.3) (Figure 5.5.2).

MOLECULAR EPIDEMIOLOGY OF CSF VIRUSES IN ASIA

Reports from China (Chundi and Yinguo 2000), Indonesia (Satya et al. 2000), Lao People's Democratic Republic (Lao PDR) (Vongthilath and Blacksell 2000), Thailand (Damrongwatanapokin et al. 2000), Vietnam (Dzung 2000), and Hong Kong (Ellis et al. 2000) show that CSF has become a major problem in Asia. Epidemiological data are available from Thailand, Korea, Malaysia, and Lao PDR. In Thailand, CSF was first reported in 1950 and has gradually become endemic. It was declared a notifiable disease in 1954 (Damrongwatanapokin et al. 2000). At

present, no strict regulation is placed on farms where the disease is diagnosed. Culling and massive vaccination are the common control measures applied. Genetic typing was carried out with isolates collected in the 1990s. The results indicated that there may have been a new introduction of an isolate of subgroup 2.2 in 1996, possibly from Europe (Italy) (Parchariyanon et al. 2000), which seemed to be responsible for most outbreaks in recent years. In addition, Thailand was found to have a unique cluster of CSFVs that, up to the present, have been detected only in Asia. One of these new groups was designated as subgroup 3.3 within the new Asian group 3 (Parchariyanon et al. 2000; Paton et al. 2000). Several Korean isolates clustered in subgroup 3.2, and the congenital tremor virus that was isolated in 1964 in the United Kingdom is presently the only representative of subgroup 3.1 (Paton et al. 2000). In addition, many representatives of Malaysian and Thai isolates were in subgroups 1.1 and 1.2, with the remaining isolates forming a new subgroup (1.3) within group 1. Up to now, only one isolate from

Malaysia has been placed in subgroup 2.1 and no isolates belonging to subgroup 2.3 have been detected in these two countries (Parchariyanon et al. 2000).

Lao PDR has a population of about four million people, and 64% of the families are involved in pig production (Blacksell 2000). Pig raising is generally performed by farmers with small holdings, and pigs are kept in free-ranging fashion. CSFV is endemic, with many outbreaks reported annually (Vongthilath and Blacksell 2000). Genetic typing of the Lao PDR isolates showed that they fall into two distinct groups. Although the northern regions of the country are very mountainous and isolated, so that unique viruses would be expected, the isolates from this region cluster together with European viruses of subgroup 2.1. In contrast, isolates from the southern parts of Lao PDR fall into subgroup 2.2 (Blacksell 2000). There is no information concerning the relationship between the Italian, Thai, and Lao isolates of this subgroup.

MOLECULAR EPIDEMIOLOGY OF CSF VIRUSES IN AMERICA AND THE CARIBBEAN

Canada and the United States have been free of CSF since 1963 and 1976, respectively. The disease is enzootic in South and Central America and Mexico, with recent outbreaks in Argentina, Costa Rica, Cuba, the Dominican Republic, and Haiti (Lubroth 1999). In Cuba, CSF was reported for the first time in the 1940s and was probably introduced by pigs imported from the United States. It was kept under control until 1993 through a National Control Program with vaccination campaigns and strict epidemiological surveillance. Between 1993 to 1997, a new epizootic of CSF occurred (Anonymous 1997) concurrently with a worsening economic situation on the island. The first case in Haiti was reported in 1996 and in the Dominican Republic in 1997, but the origin of the infection is not known (Edwards et al. 2000).

The reemergence of the disease in Cuba made us consider the possibility of an external reintroduction of virus, followed by spread to Haiti (Frías 2000). This evidenced the need to develop new tools for diagnosis and for molecular characterization of regional isolates in order to aid epidemiological studies. In addition, it was necessary to obtain information concerning the origin of the epidemic in order to develop a more accurate prevention and control program.

The results of the phylogenetic analysis of 18 Cuban isolates, the vaccine strain LABIOFAM, and the "Margarita" strain (the challenge strain used for testing the efficacy of the LABIOFAM vaccine) using a fragment of the E2 gene showed that they were all in subgroup 1.2. They did not distort the Lowings dendrogram, and a common origin, almost identical to the Margarita strain, was established. We concluded that it was not likely to be an external reintroduction (Díaz de Arce et al. 1999). In ad-

dition, the comparison of the Cuban isolates with strains isolated in Haiti until 1997, in the Dominican Republic until 1998, in Honduras in 1998, and in Mexico in 1998 showed that the latter were not related to the previous ones and that they formed an independent cluster (Ward and Lubroth 2000).

MOLECULAR EPIDEMIOLOGY OF CSF VIRUSES IN EUROPE

In the European Union (EU), the goal has been to eradicate the disease, and legislative control for CSF was introduced in 1980. In the case of an outbreak of CSF, the eradication measures (Council Directive 80/217/EEC; Anonymous 1980; Edwards et al. 2000) are based on the *stamping out* of infected pig herds and possibly infected contact and neighbor herds; epidemiological, clinical, and virological investigations; and movement restrictions for live pigs and pig products from zones surrounding the infected farms (i.e., restriction zones). Vaccination against CSFV has been forbidden since 1991 (Moennig 2000).

In some regions, CSF eradication is greatly hampered because CSFV became endemic in the wild boar population (Laddomada 2000). From there, the infection sporadically spreads to domestic pigs by either direct or indirect contact (Edwards et al. 2000).

In the last decade, domestic pig populations in Austria, Belgium, France, Germany, Italy, Spain, the Netherlands, and the United Kingdom have been affected (Anonymous 1999; Sandvik et al. 2000).

The most extensive retrospective study concerning the epidemiology of CSF using genetic typing as an additional tool to supplement epidemiological data from field investigations was performed in Germany (Fritzemeier et al. 2000), where, besides a great number of cases among wild boar, 424 outbreaks were recorded between 1990 and 1998 in domestic pigs. Field isolates were available from more than 150 outbreaks. Genetic typing based on 150 bases within the 5' NTR of the virus genome (Greiser-Wilke et al. 1998) revealed the existence of seven regional groups of CSFV isolates within group 2: one within subgroup 2.1, two within subgroup 2.2, and four within subgroup 2.3 (Fritzemeier et al. 2000). The virus was found to be endemic in wild boar populations in eastern Germany and in northern Germany, with the regional subgroups 2.3*Guestrow/Rostock and 2.3*Uelzen, respectively (Fritzemeier et al. 2000). In combination with epidemiological findings, genetic typing greatly helped to find relations between outbreaks, and it was concluded that in the last decade several virus types were newly introduced into German domestic pigs and wild boar, respectively. As for the other members of the EU, CSF viruses of subgroup 2.3 are endemic in Sardinia (De Mia 2000; Lowings et al. 1994) and caused epidemics of CSF in Belgium in 1990 and 1993–1994

(Vanderhallen and Koenen 1997). Interestingly, Sardinia seems to be the only region in the EU where three outbreaks with viruses of subgroup 1.1 occurred (Rutigli 1999).

In Northern Italy, viruses of subgroup 2.2 are endemic in the wild boar population (Lowings et al. 1994, 1996), sporadically causing outbreaks in domestic pigs (De Mia 2000). The same virus caused CSF among wild boar in Switzerland and was first detected in May 1998 in the southern part of the country, near the border of Italy (Hofmann et al. 1999). The outbreaks in domestic pigs caused by isolates of subgroup 2.2 in Austria formed a different cluster (Bartak and Greiser-Wilke 2000; Lowings et al. 1996).

Viruses of subgroup 2.1 only sporadically occurred in the EU in 1989 and 1993. In this last case, the virus was detected in wild boar meat illegally imported from China to Austria (Hofmann 1996). Another CSFV isolate of this subgroup that was also most likely newly introduced into Germany caused the first outbreak in domestic pigs in January 1997. From there, it seems to have spread to The Netherlands, where it produced a devastating epidemic that caused 429 outbreaks until January 1998 (Widjoatmodjo et al. 1999). By genetic typing, it was verified that the same virus was responsible for outbreaks in Italy, Spain, and Belgium. The last epidemic outbreak by the same virus was recorded in June 1998 in Spain (Greiser-Wilke et al. 2000a).

Finally, a virus isolate of subgroup 2.1 caused the first outbreaks in the United Kingdom in 14 years. Genetic typing showed that the East Anglian CSFV could be distinguished from all previous European subgroup 2.1 isolates; the source of infection could not be determined (Sandvik et al. 2000). The first of the 16 outbreaks was recorded at the beginning of August 2000 and the last one in November 2000 (Anonymous 2000).

Whereas most neighbors of the EU follow a nonvaccination policy, vaccination is allowed and generally routinely used in many central and eastern European countries (Edwards et al. 2000). In some of these countries, only sick or clinically suspect animals are destroyed in an outbreak, whereas the other animals in the herd and other contact herds are vaccinated (Moennig 2000).

Genotyping of virus isolates from different countries in Eastern Europe showed that they mostly belonged to subgroups 2.2 or 2.3, respectively (Bartak and Greiser-Wilke 2000; Stadejek et al. 1997). It also became evident that the individual clusters within the subgroups largely coincided with their regional origins in Poland, Slovakia, Hungary, Estonia (Stadejek et al. 1997), and the Czech Republic (Bartak and Greiser-Wilke 2000). As expected, a close relation was found between the Czech and the Austrian isolates in subgroup 2.2, which occurred in the border regions of both countries. The Czech isolates in subgroup 2.3, which originated from different parts of the country, formed a very homogeneous cluster of

closely related—yet not identical—viruses. Although two of them seemed identical to Slovakian isolates from 1998, they all clearly differed from Hungarian and Polish isolates (Bartak and Greiser-Wilke 2000).

Interestingly, all of the field isolates collected in different regions of Russia analyzed up to now were found to belong to subgroup 1.1. They clearly differ from the vaccine strain CS, which belongs to subgroup 1.2. The isolates were characterized by reverse transcriptase polymerase chain reaction and restriction analysis (Grebennikova et al. 1999; Zaberezhny et al. 1999) and genotyped using the E2 gene fragment (Vlasova et al. 2000).

CONCLUSIONS

In conclusion, harmonization of phylogenetic analyses in combination with epidemiological surveys will greatly enhance the possibility of tracing the origin and spread of the disease in different regions of the world. In addition, progress in molecular biological methods and the development and affordability of equipment will make it possible to perform genetic typing in any laboratory. The CSFV database held at the European Community Reference Laboratory for CSF in Hannover, Germany, which is accessible by the WWW, will help to establish phylogenetic relationships among the different isolates from various parts of the world, and thus aid in setting up more effective control strategies on an objective and scientific basis.

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6 FOOT-AND-MOUTH DISEASE AND SWINE VESICULAR DISEASE VIRUSES

6.1 Foot-and-Mouth Disease in Taiwan

Ping-Cheng Yang

SUMMARY

Before March 1997, outbreaks of foot-and-mouth disease (FMD) had been documented twice in Taiwan (Ryu 1984), the first between 1913 and 1916, and the second between 1924 and 1929. Since vaccines were unavailable, both of these early epidemics of FMD were eradicated by slaughtering infected herds and movement restrictions around infected premises. Following the eradication of the second FMD epidemic in 1929, Taiwan remained free of the disease until mid-March 1997, when a pig-adapted type O FMD virus (FMDV) invaded Taiwan's pig population (Chang et al. 1997; Donaldson 1997; Shieh 1997; Yang et al. 1999). This virus genetically belonged to the Hong Kong topotype (Kitching 2000), and sporadic outbreaks continued after the initial epidemic wave, most recently on March 9, 2001. Moreover, a novel type O FMDV was isolated from clinically normal Chinese yellow cattle in May 1999 in Kinmen (Huang et al. 2000; Lin 2000), one of Taiwan's offshore islands, located close to Mainland China. This FMDV was genetically within the PanAsia topotype, which differs from the Hong Kong topotype (Kitching 2000). The virus entered Taiwan and caused clinical FMD in dairy cattle and goats in early 2000 (Huang et al. 2000; Lin 2000). Taiwan is currently suffering endemic type O FMDV, and its government is endeavoring to eradicate the disease.

SUMMARY OF THE 1997 FMD OUTBREAK

The first suspected case of foot-and-mouth disease (FMD) appeared on a pig farm on March 14, 1997, in Hsinchu County. Samples of vesicular fluid, vesicular epithelium, and serum from clinically sick pigs were collected and sent to the National Institute for Animal Health (NIAH, Tamsui, Taipei County, Taiwan), formerly the Taiwan Animal Health Research Institute, for diagnosis, and FMD was diagnosed on March 19 (Huang et al. 2000; Shieh 1997; Yang et al. 1999). The outbreak was then reported to the Office International des Epizooties (OIE, Paris) on March 20, 1997 (Donaldson 1997; Shieh 1997). Meanwhile, samples of vesicular epithelium were swiftly sent to the World Reference Laboratory (WRL) for FMD at Pirbright, UK, for further confirmation.

Serotype O, referred to as O Taiwan/97 (O/TW/97), was identified and reported on March 25 (Donaldson 1997).

By the time the central government confirmed the outbreaks of FMD in Taiwan on March 20, the disease had been identified on 28 pig farms in 10 counties or cities along Taiwan's western coast. The epidemic curve (Figure 6.1.1) peaked during week 5, when 1113 newly infected farms were identified. Control measures became effective by week 9, a week after a second dose of FMD vaccine had been administered to all cloven-hoofed animals. The two-dose blanket vaccination program markedly reduced new infections, with only five new cases being reported in June, followed by just two during the first week of July. The epidemic lasted approximately 4 months, and the last case was reported on July 15 (Yang et al. 1999).

Outbreaks caused by the same strain of FMD virus (FMDV) spread throughout the island of Taiwan. A total of 6147 pig farms, 24.2% of such farms in Taiwan, or over 4 million pigs were either infected or in close contact with infection. Approximately 38% of Taiwan's pig population either died (0.18 million) or was culled and slaughtered (3.85 million). Except for two cities, Taipei City and Keelung City, the whole of Taiwan was declared an FMD-infected zone. However, besides the blanket vaccination, a shipping ban on all cloven-hoofed animals and their meat products from the island of Taiwan successfully protected the offshore islands of Penghu, Green Island, Orchid Island, Kinmen, and Matsu (Yang et al. 1999). The geographical distribution of the 1997 FMD epidemic in Taiwan is shown in Figure 6.1.2.

CONTROL STRATEGIES

A policy of depopulating all farms where the infection was confirmed and vaccinating all pigs on farms with a high risk of becoming infected was adopted as soon as the epidemic was discovered. As the disease continued to spread, the decision to vaccinate all of Taiwan's pig population was made in late April. FMD vaccines made from strains of O 4174, O₁ Campos, and O₁ Manisa were recommended by the WRL for FMD based on the evaluation of r_1 values (Kitching 1992). Blanket vaccination then began from late April, immediately after the arrival

of a shipment of 15 million doses of O₁ Campos vaccine from Argentina, and a booster was administered 2 to 3 weeks later. Once every pig in Taiwan had received at least one dose of the FMD vaccine, the eradication policy was changed, shifting from the complete depopulation of infected farms to destroying only clinically ill pigs (Shieh 1997).

The movement of pigs from infected farms was restricted from March 20 to halt the spread of the disease. Additionally, on March 22, the central government announced a ban on shipping all cloven-hoofed animals and related meat products to the three counties along the eastern coast of Taiwan and to all offshore islands, including Penghu, Kinmen, Matzu, Green Island, and Orchid Island (Shieh 1997). Although the local county governments rigorously enforced movement controls on animals and animal products, outbreaks of FMD eventually occurred in the three counties of eastern Taiwan (Shieh 1997), and the only areas to remain free from FMD were offshore islands (Yang et al. 1999).

To minimize the spread of FMD in eastern Taiwan, a 3-km-radius protection zone, along with a surveillance zone with a radius of another 3 km, were declared around each infected premises within the three counties of eastern Taiwan. Owing to the shortage of vaccine between April and early May, it was decided to vaccinate all animals in the surveillance zone first. Later, when more vaccine became available, animals in the protection zone were vaccinated, with the order of priority being sows and piglets, followed by the nearly finished fattening pigs and, finally, weanling pigs and animals of other susceptible species. Control through the establishment of protection and surveillance zone measures certainly helped minimize the transmission of FMDV within eastern Taiwan, and only one outbreak was identified in each of the three counties (Yang et al. 1999).

O TAIWAN/97 FMD VIRUS

Under experimental conditions, The O Taiwan/97 FMDV (O/TW/97) was shown to exhibit a natural adaptation to pigs (Dunn and Donaldson 1997). Huang and his colleagues (2000) at the NIAH further corroborated this porcophilic phenomenon. During the epidemic, the lack of field reports of the virus spreading from infected pigs to ruminant animals in close proximity supported this finding. No clinical cases were reported in cattle, water buffalo, or goats (Chang et al. 1997; Huang et al. 2000; Yang et al. 1999). A routine monitoring program, including clinical examinations plus probang samples of esophagopharyngeal fluid for isolation of FMDV, was implemented in vaccinated cattle and goats from late 1997 until the end of 1998. Meanwhile, the results from virus isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) assays indicated that no cases of FMD had occurred in any vaccinated ruminant species (Huang et al. 2000).

Nucleic acid sequence analysis on the VP1 gene of O/TW/97 showed that, genetically, the virus was closely related to strains found in mid-1990 in Hong Kong, the Philippines, and Russia (Kitching 1998), and this finding was mirrored by Tsai and his colleagues (2000). Virus O/TW/97 was, therefore, categorized as a member of the Hong Kong topotype (Kitching 2000). Based on molecular epidemiological studies, O Taiwan FMD viruses isolated from clinical specimens during the 1997 epidemic were genetically highly homogeneous, as evidenced in the minute divergence of 0.2% to 0.9% revealed in 20 variants. In conclusion, the causative agent responsible for the 1997 epidemic presumably originated from a single common source of type O FMD viruses prevalent in neighboring areas, including the Philippines and Hong Kong (Tsai et al. 2000). The entry of the O/TW/97 was strongly suspected to have been associated with the smuggling of pigs or swine products from Mainland China via fishing boats (Shieh 1997), although the precise origin of the causative virus has never been confirmed.

CLINICAL CHARACTERISTICS

The clinical disease generally started in the farrowing house, with sows becoming depressed, pyretic, and anorexic. This was followed by the formation of vesicles on snout, lips, tongue, coronary bands of the feet, and teats and udders. Once a lactating sow developed vesicular lesions on its udders and teats, the entire litter of suckling pigs rapidly developed similar signs and lesions, and often died within 24 hours. Older pigs, including growers and finishers, showed signs and vesicular lesions similar to those seen in sows. Some finishers lost their claws owing to ruptured vesicles on the coronary band of their feet, which led to extensive bleeding (Chang et al. 1997; Yang et al. 1999).

On average, 21.7% of pigs on farms showed clinical signs, and mortality was close to 4%. The overall case fatality rate for pigs with the disease was 18.2%. Suckling pigs had the highest mortality, reaching 100% during the initial stage of the epidemic when the entire population lacked immunity against FMDV. In contrast, older pigs generally had a much lower mortality, ranging between 5% and 20% (Yang et al. 1999).

The clinical course of the disease ranged from 10 to 38 days, with a mean of 22 days. The incubation period was relatively short, i.e., 24 to 48 hours after exposure. Notably, the first animals to show clinical signs on farms were generally larger pigs, either finishers or sows, followed by younger pigs (Yang et al. 1999). It has been suggested that the reason that clinical disease appeared first in larger pigs and later in younger pigs may be related to the higher air-intake rate of larger pigs, causing them to be exposed to larger doses of aerosolized FMDV (Donaldson 1997).

ECONOMIC IMPACT

Yang and his colleagues (1999) investigated the economic impact of the 1997 FMD epidemic. The Taiwanese government bore approximately 49.5% of the total financial cost of the epidemic, providing \$187.5 million (US) to compensate pig producers for culling their pigs. The total cost of 21 million doses of vaccine was \$13.6 million (US), representing 3.6% of the total financial cost of the epidemic. Meanwhile, the cost of carcass disposal and environmental protection was estimated at \$24.6 million (US), or 6.5% of the total financial cost of the epidemic. Finally, other miscellaneous expenses, such as disinfectants, disposable coveralls, boots, gloves, syringes, electrocution devices, lime, rental of bulldozers, and transportation, were estimated at \$27.9 million (US), or 7.4% of the total financial cost of the epidemic. The loss of market value to the pig industry during the 4-month epidemic was estimated at \$125 million (US), or 33% of the total financial cost. The total financial cost of the 1997 FMD epidemic in Taiwan was estimated at \$378.6 million (US) (Table 6.1.1).

Besides the direct economic losses sustained at the time of the epidemic, pork production and related industries continue to suffer severe financial losses today. The ban on the export of pork to Japan has caused an estimated loss of \$1.6 billion (US) to the Taiwanese pig industry, as well as other businesses, such as feed mills, pharmaceuticals, meat packers for exportation, farm equipment manufacture and supply, livestock sales, and transportation. Over 65,000 jobs were lost in these affected businesses either during or after the epidemic (Yang et al. 1999).

A cost-benefit analysis was performed to assess the FMD control and eradication programs. Under the sce-

narios of FMD control or eradication, social welfare during the adjustment period did not differ significantly with either control or eradication programs. However, the social cost suggested that a disease eradication program is necessary and that consumers may gain from the eradication of FMD (Tsai and Yang 1998).

ENVIRONMENTAL IMPACT

Of the 4.03 million pig carcasses disposed of by the government, 80% were buried, 15% were rendered, and 5% were either incinerated or burned in open fields (Table 6.1.2). Analysis of the cost effectiveness of these three disposal methods revealed that burying was the most inexpensive and easiest means of handling large quantities of animal carcasses, with 32.5% of the total disposal costs covering 80% of the carcasses. Rendering was more expensive, with 26.1% of the total expense used to dispose of 15% of the carcasses, and incinerating or burning was the most expensive, with 41.4% of the total expense used to dispose of only 5% of the carcasses. Carcass disposal methods were selected according to the following considerations: landfill availability (either private or public), level of the water table, proximity to human residences, incinerator availability, and other environmental factors (Yang et al. 1999). Since Taiwan is a country with a very high population density, locating either landfills or open burning sites without provoking protests from local citizens was very difficult. To resolve fears that decomposed animal carcasses might contaminate underground water, the government has continued to monitor the quality of underground water at landfills that were used to bury large quantities of animal carcasses.

Table 6.1.1. Distribution of costs of Taiwan’s 1997 foot-and-mouth disease epidemic

Items	Million (\$US)	Percent of Total
Indemnity for pigs culled	187.5	49.5
Cost of vaccines	13.6	3.6
Carcass disposal and environmental protection	24.6	6.5
Miscellaneous expenses	27.9	7.4
Loss of market value	125.0	33.0
Total cost	378.6	100.0

Table 6.1.2. Carcass disposal by various methods and associated costs

Carcass Disposal Method	Million (\$US)	Percent of Total
Burying (80%)	8.0	32.5
Rendering (15%)	6.4	26.1
Burning or incinerating (5%)	10.2	41.4
TOTAL	24.6	100.0

SPORADIC FMD OUTBREAKS AFTER JULY 1997

The last case of the original epidemic was reported on July 15, 1997, but cases of pig-adapted FMD have been detected occasionally since December 1997. As of March 2001, sporadic outbreaks have involved 21 pig farms and three abattoirs, and required the slaughter of 1735 pigs. The causative FMDV was confirmed as O/TW/97 in all these cases, with lack of proper vaccination as the main cause of these outbreaks.

FMD OUTBREAKS IN RUMINANT ANIMALS

As mentioned earlier, FMDV O/TW/97 is a pig-adapted strain that is harmless to ruminant animals. In May 1999, a serological surveillance program was launched on Kinmen, an island a few kilometers off the coast of the Chinese mainland, in response to a Wall Street Asia report regarding FMD outbreaks in southeastern China. The surveillance program produced a novel type O FMDV isolate from clinically normal Chinese yellow cattle (Huang et al. 2000; Lin 2000). This type O virus, referred to as O/TW/99, was confirmed to be FMDV by the WRL for FMD at Pirbright, United Kingdom. The entry of this new FMDV was determined to have occurred through the smuggling of live cattle from China (Lin 2000). However, immunological studies suggested that the vaccine strains currently used in Taiwan, namely, O/TW/97, O₁ Manisa, and O₁ Campos, would provide cross-protection against FMDV O/TW/99. This finding may explain why O/TW/99, shown to be pathogenic in pigs under experimental conditions, caused no clinical cases in the pig population.

Epidemiological investigation and further serological tracing found that five cattle farms on Taiwan property had been infected through the purchase of clinically normal but infected yellow cattle from Kinmen. By July 9, 1999, five beef-cattle farms in Kinmen and five more in Taiwan were diagnosed as infected with type O FMDV, with the diagnosis being based on seropositive results on nonstructural protein, virus isolation, or RT-PCR of esophagopharyngeal fluid samples. Subsequent nucleic acid-sequencing results indicated that the virus was O/TW/99. A total of 663 cattle were killed to stop the spread of this FMDV (Lin 2000).

During January and February 2000, clinical FMD broke out at two goat farms and three dairy-cattle farms in Taiwan, causing many deaths among goat kids and vesicular lesions in dairy cattle (Lin 2000). A total of 474 goats and 262 dairy cattle on the five infected premises were destroyed, and the causative FMDV was again confirmed to be O/TW/99 (Huang et al. 2001).

THE O TAIWAN/99 FMD VIRUS

Phylogenetic analysis of the nucleic acid sequence of the VP1 gene revealed that O/TW/99 FMDV shared 95% to 97% homology with the virus strains isolated from the Middle East and India (Huang et al. 2000) and was classified as a PanAsia topotype (Kitching 2000). The species susceptibility of O/TW/99 was studied experimentally in several susceptible animal species, and it was determined that the virus caused typical generalized vesicular lesions in dairy cattle and pigs. Although the virus did not cause vesicular lesions in Chinese yellow cattle and adult goats, it was recovered from feces and esophagopharyngeal fluid samples from experimentally infected animals (Lee et al. 2000).

CURRENT POLICY FOR CONTROLLING FMD

To achieve FMD-free status without vaccination, the Taiwan government launched a three-stage eradication program at the beginning of 2001. A surveillance program that uses a peptide enzyme-linked immunosorbent assay (Shen et al. 1999) to detect nonstructural protein antibodies from vaccinated animals has been implemented. Surveillance will be continued throughout the three-stage eradication program to verify the prevalence of FMD in the field. The first stage—the control stage—will focus on compulsory vaccination of all cloven-hoofed animals with the aim of reducing the incidence rate of FMD to zero by the end of 2002. The second stage will involve compulsory vaccination for an additional 2 years while continuing to prevent any further FMD outbreaks. The objective of the second stage will be to free the country of FMD through vaccination by the end of 2004. Finally, vaccination will be banned in the third stage. The ultimate goal is to be FMD-free countrywide without the practice of vaccination by the end of 2005.

DISCUSSION

Four major factors were considered responsible for the rapid spread of the 1997 epidemic of FMD in Taiwan: the inability of the government to shut down livestock auction markets, the long delays before infected farms were depopulated during eradication, the high density of pig farms, and the initial shortage of vaccine (Yang et al. 1999).

First, livestock auction markets were found to have been a major source of FMDV transmission. Although the government implemented health requirements for market-weight hogs entering auction markets immediately after the first outbreak, the movement of subclinically infected pigs between farms may have spread the virus. A complete shutdown of all livestock markets

was infeasible because of the popularity of fresh pork in Taiwan. Fresh pork is obtained through daily shipments of live market-weight pigs to 21 livestock markets spread throughout Taiwan. A move early in the epidemic to shut down all livestock markets for 5 days in an attempt to stabilize plummeting prices did not halt the spread of the epidemic.

Second, infected farms waiting to be depopulated during the early stages of the epidemic were sources of the virus. Infected pigs can each shed $10^{8.6}$ TCID₅₀ (50% tissue culture infectious doses) of FMDV per day (Donaldson et al. 1982), and FMDV repeatedly entered new herds during the first month of the epidemic, despite the strict biosecurity measures practiced by most producers.

Third, the pig population density in Taiwan was extremely high during early 1997. Roughly, 83% of the entire pig population was concentrated in the southwestern region of Taiwan: approximately 1922 pigs/km² of cultivated land. This region was the area hit hardest by the epidemic (Figure 6.1.2).

Finally, the vaccine imports were insufficient to create an immune blanket during the first month of the epidemic when the decision was made to carry out mass vaccination. On most farms, only a partial vaccination was implemented, with the emphasis on sows and boars, until early May; that is, for roughly 6 to 7 weeks after the outbreak. Consequently, many partially vaccinated farms became infected. However, the huge supply of 15 million doses of O₁ Campos vaccine that arrived between late April and early May allowed further transmission to be halted, and the epidemic was eventually brought under control.

The market value of pigs sharply declined due to two major factors: the loss of the export market led to an immediate 40% oversupply on the domestic market, and consumers temporarily rejected pork because of the heavy publicity surrounding the epidemic. Fear that FMD might be a zoonotic disease and that pork might therefore be unsafe, and ethical concerns about the destruction of healthy pigs, became major consumer concerns. Domestic demand for pork decreased 28% during the first month of the 1997 epidemic, but the market gradually recovered and was back to normal by August 1997, 5 months after the government announced the outbreak (Yang et al. 1999).

Analysis of the direct economic losses due to the 1997 FMD epidemic clearly shows that blanket vaccination was the cheapest method of controlling the spread of the disease. Vaccine costs accounted for only 3.6% of the total costs, compared with 49.5% for indemnity. In the long term, however, since vaccination continues to be so widely practiced, Taiwan will encounter difficulties in regaining its status as an FMD-free country without vaccination (Yang et al. 1999).

RECOMMENDATIONS

Conventional control methods should be sufficient to adequately control the Hong Kong topotype FMDV. Particularly, control over the feeding of untreated swill to pigs, a common practice in Chinese communities, should be emphasized and enforced. Since the Hong Kong topotype virus infects only pigs, the control and eradication program should focus on restricting or licensing the movement of pigs and ensuring proper inspection prior to slaughter. Adequate vaccination covering large pig populations is very difficult and expensive owing to the relatively high turnover rate of animals. However, since this topotype does not appear to spread over more than a few meters as an aerosol, on-farm biosecurity measures should adequately prevent the entry of this virus (Kitching 2000).

Control of the PanAsia topotype is likely to be more difficult because of this strain's ability to infect all cloven-hoofed animals, particularly without causing recognizable clinical signs in some cattle breeds. Movement control is crucial, especially for illegal cross-border trade. Vaccination and serological surveillance are also essential components of the control program (Kitching 2000).

Although FMD vaccines are always tailor-made for endemically infected countries, the 1997 FMD epidemic experience has established that large quantities of vaccines made from the proper strains might require as long as 4 to 5 weeks for delivery after an order is placed. Therefore, FMD-free countries that do not practice vaccination should consider establishing an adequate supply of FMDV antigen reserve, which can serve as an insurance policy to guarantee a sufficient supply of vaccine in a few days should an epidemic break out and either ring vaccination or mass vaccination to be the elected course of action.

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6.2 Foot-and-Mouth Disease in Japan

Toshiyuki Tsutsui and Kenichi Sakamoto

SUMMARY

The first outbreak of foot-and-mouth disease (FMD) after 92 years of absence occurred in a beef-fattening farm in Kyusyu Island, the southern part of Japan, on March 25, 2000. Reverse transcriptase-polymerase chain reaction and antibody detection enzyme-linked immunosorbent assay confirmed type O FMD virus (FMDV). Intensive clinical and serological surveillance detected another two positive cattle farms in the movement control area in Kyusyu, and one cattle farm in Hokkaido, the northern part of Japan. As a result of epidemiological investigations, imported wheat straw was suspected as the most likely source of the primary infection. Erosions in the muzzle and mouth were confirmed in some infected Japanese black cattle, but no vesicles were observed. Laboratory experiments suggested that the virulence of the isolated virus was lower for cattle than with conventional types of FMDV. However, as inoculated pigs showed typical clinical signs with vesicles and transmitted the disease to in-contact pigs, it was suggested that this virus could spread easily within the pig population. Conclusively, Japan succeeded in eradicating FMD within a short period. Several reasons for this achievement are raised and discussed.

INTRODUCTION

Foot-and-mouth disease (FMD) is a highly infectious disease caused by FMD virus (FMDV), which belongs to the genus *Aphthovirus* in the family Picornaviridae. This disease affects cloven-hoofed animals, including important domesticated animals, such as cattle, swine, sheep, and goats. FMD has great potential to bring huge economic losses to the livestock industry due to its high degree of contagiousness. In addition, the presence of FMD leads to restrictions in the international trade of animals and animal products. Therefore, FMD is regarded as one of the most important infectious diseases of animals and is classified as a List A disease by the Office International Des Epizooties (OIE).

Characteristic clinical signs of FMD are pyrexia, salivation, and lameness, with formation of vesicles and erosions in the mouth, on the feet, and on the mammary

glands. Production losses mainly occur because of the retardation in rate of growth, drop in milk production, and/or death of young livestock. In some countries, field crop production is also affected by the loss of draft animals and the power needed to work the farms. In general, cattle are sensitive to FMDV, whereas swine are less sensitive and require a larger inoculum of virus to become infected (Donaldson et al. 1987; Suttmoller and Vose 1997). When pigs are infected, they excrete larger quantities of virus through the respiratory route compared to cattle and sheep (Sellers 1971) and become a source of infection to other susceptible animals. Gloster et al. (1981) reported that a pig could emit around 10^8 infectious units of virus per day at the time of peak excretion, whereas a steer or a sheep excreted around 10^5 . Therefore, an area where pig husbandry is concentrated and practiced on a wide scale presents a great danger for the spread of FMD.

It should be noted that there are strains of FMDV showing species adaptation. For example, the strains isolated during the outbreak of FMD in Taiwan in 1997 caused typical lesions in pigs and spread rapidly among them. However, laboratory experiments showed that neither disease nor a subclinical infection was transmitted from infected pigs to cattle placed in close contact with them. This strain did not cause disease even when inoculated intradermally into cattle (Dunn and Donaldson 1997). It is obvious that the diversity of FMDV makes FMD control difficult.

Until March 2000, Japan had been free from FMD since 522 cases were reported in 1908. This is mainly because Japan has stringent border controls in place to prevent the entry of FMD through infectious materials from overseas. Japan is one of the biggest importers of livestock products in the world. A total of more than 1.3 million tons of beef and pork were imported in 1999, while 4.5 million cattle and ten million pigs were raised domestically to meet 35% of beef and 60% of pork consumption. Livestock production is still a significant industry in Japan. In particular, it plays an important role in the rural economy. Therefore, protecting domestic animals from malignant infectious diseases, like FMD, has been crucial to sustaining the livestock industry in Japan.

DETECTION OF FMD

The first outbreak of FMD after 92 years of absence occurred in a beef-cattle-fattening farm in Miyazaki City on Kyusyu Island, the southern part of Japan (Figure 6.2.1). This is one of the most prominent beef cattle production areas in Japan, although most production is on small farms holding fewer than 20 cattle. The affected farm held ten Japanese black (JB) cattle, the most popular beef breed in Japan. The farmer observed pyrexia, anorexia, and coughing in some cattle on the farm on March 8, 2000. At the farmer's request, a veterinarian treated the cattle with antibiotics and agents to promote peristalsis on March 12. Despite these treatments, the anorexia and erosions in the muzzle and mouth continued to spread among cattle on the farm. On March 21, the veterinarian reported the case to a Livestock Hygiene Service Center, i.e., the prefectural government's institution for prevention and control of animal infectious diseases in the field. Veterinary inspectors of the center visited the farm, suspected FMD, and brought specimens to the Department of Exotic Diseases, National Institute of Animal Health (NIAH), which has a high biosecurity laboratory for the diagnosis of exotic diseases. Reverse transcriptase-polymerase chain reaction (RT-PCR) on epithelial tissues from ulcers and erosion lesions confirmed the presence of genomic material from the FMDV, although the enzyme-linked immunosorbent assay (ELISA) and complement fixation (CF) test for antigen detection showed negative results. The serum samples from nine cattle tested for antibody by ELISA were all positive with high antibody

titers against type O FMDV. In light of these results, the outbreak of FMD was announced on March 25, and the application of contingency measures started immediately in the region.

CLINICAL SIGNS

Spreading pyrexia, salivation, and erosions in the mouth and on the muzzles of JB cattle were observed in the first case. In the third case, in which the FMDV was isolated, the farmer had observed anorexia and salivation before FMD was detected. Importantly, no vesicles were observed in either case, and no clinical signs were observed in two other herds, including the case in Holstein cattle.

These field observations were consistent with results of laboratory experiments lately conducted at the NIAH. JB calves inoculated with $10^{6.5}$ TCID₅₀ (50% tissue culture infectious doses) of FMDV (O/JPN/2000) showed clinical signs of pyrexia, salivation, and erosions in the mouth and on the muzzle, but no clear vesicles. By histopathology, microscopic lesions were found widely in the stratified squamous epithelium of digestive organs in JB calves. Holstein calves inoculated with 10^6 TCID₅₀ showed neither clinical signs nor presence of detectable antibody, although slight pyrexia was confirmed. Piglets inoculated with 10^6 TCID₅₀ developed typical clinical signs of FMD, with pyrexia and lameness caused by erosions and vesicles on the feet. Transmission studies showed that JB calves transmitted the disease to in-contact JB calves, but Holstein calves failed to do so to other Holsteins. Transmission between pigs was also established, and infected piglets showed typical clinical



6.2.1. Location of foot-and-mouth disease outbreaks in Japan

signs with viral excretion and the presence of antibody. Infected JB calves failed to transmit FMD to piglets. From these results, it was suggested that JB cattle were more susceptible than Holstein cattle, but did not excrete enough virus to infect pigs. In addition, O/JPN/2000 caused the typical clinical disease in swine, and the virus could spread in pig populations.

CONTROL MEASURES

In the case of an FMD outbreak in Japan, emergency measures are to be taken in accordance with the Malignant Exotic Animal Diseases Control Guidelines prepared by the Ministry of Agriculture, Forestry and Fisheries (MAFF): the strategy against FMD has been based on immediate eradication by *stamping out*. On March 25, FMD eradication teams were established at MAFF, three prefectural governments (Miyazaki, Kumamoto, and Kagoshima), and the livestock hygiene service center covering the infected farm. All susceptible animals on the infected farm were destroyed, and the premises were disinfected. The movement of all animals and materials that may transmit FMDV were prohibited within a radius of 20 km of the infected farm (the *movement control area*). In this area, artificial insemination was suspended, and slaughterhouses and livestock markets were closed. Outside of the movement control area, a *surveillance area* was established in a radius of 50 km from the infected farm. Movement of all susceptible animals out of the surveillance area was prohibited, and livestock markets were closed. Checkpoints were set up on the main roads at the border of these areas to monitor compliance with movement restriction and to disinfect vehicles moving in and out of the areas. Within these two areas, intensive clinical inspections were carried out on all livestock farms. Given that the FMD observed was atypical and might not create clear lesions in cattle, serum samples were taken in all cattle herds for antibody detection (ELISA). Nationwide clinical and serological investigations were conducted, in particular on farms that had introduced cattle from these areas or fed imported hay or straw from non-FMD-free countries.

At the beginning of April, during the survey, two positive herds were detected at a distance of 2 and 7 km from the first positive herd. Both farms had kept JB cattle, 9 and 16, respectively, for calving purposes. High antibody titers were found by two sequential ELISAs on both farms, and FMDV (O/JPN/2000) was recovered from two cattle on one farm from probang samples by using primary bovine kidney cells.

On April 23, a month after movement control was begun, the areas subjected to movement restrictions were reviewed, and the area reduced to a 10-km radius around the infected farms. At the same time, the surveillance-area restriction was discarded because the transmissibility of the FMDV in this outbreak was considered lower

than that of conventional FMDV. In this outbreak, there was no evidence of airborne transmission, as no positive animals were found on the farms bordering or near the infected farms. After disease on the two farms was confirmed, no clinically or serologically suspicious cases had been found within the movement control area. In addition, based on clinical and serological examinations, farms that had indirect contact with the first infected farm were all found to be uninfected.

During the nationwide survey, seropositive Holstein steers were found at a beef-fattening farm with 705 cattle (Holstein, JB, and Holstein-JB crossbreeds) in Hokkaido, the northern part of Japan. Two sequential serum samples indicated that the number of seropositive cattle had increased, and RT-PCR on probang materials confirmed two cattle as infected, although no clinical signs were observed. The VP1 genome sequence obtained was the same as that of the FMDV isolated in Kyusyu. Consequently, all cattle on the farm were subjected to stamping out, and 10-km-radius movement control area was enforced around the infected farm on May 11. All farms within this area were subjected to clinical and serological inspection.

Ultimately, since no additional cases were detected, the movement restrictions were lifted in Kyusyu on May 2 and in Hokkaido on June 9. Since the controls had been established, 93,000 farms had been clinically inspected and 53,000 serum samples serologically tested. On September 26, the OIE recognized that Japan had regained FMD-free status without vaccination, in accordance with the relevant provisions of the international animal health code.

ROUTE OF INTRODUCTION

The World Reference Laboratory for FMD (Pirbright, UK), which serves to group strains of FMDVs into topotype according to the closeness of nucleotide sequencing, confirmed that the type O virus isolated in Japan belonged to the PanAsia topotype, which originated from a virus first isolated in India in 1990 and, since then, has spread from the Middle East to East Asia. Viruses classified in this topotype caused the FMD outbreaks in cattle in Taiwan in 1999 and Korea in 2000 (Knowles et al. 2000). Control of this topotype might be difficult because of its ability to infect cattle, sheep, pigs, and goats—in some breeds, without causing easily recognizable clinical signs.

The type of FMDV strain isolated in Japan (O/JPN/2000) suggested that it was introduced from an Asian region. Actually, wheat straw imported from China was used as a feedstuff in the first farm detected, although 23 farms using wheat straw derived from the same import lot were uninfected. In China, three outbreaks of FMD with O-type virus were reported in May 1999. Although the details of these virus strains were not

known, this is considered the most likely source of the FMD outbreak in Japan. If the origin was contaminated animal products or garbage, it would be plausible that pigs were infected first. No evidence suggested other possible routes of infection, i.e., imported animals, persons, vehicles, or airborne. FMDVs of this topotype caused the FMD outbreak in Far-East Russia and Mongolia in April 2000, as well (OIE 2000). Both outbreaks occurred near the boundary of China. Regardless of epidemiological studies in Kyusyu and Hokkaido, no convincing routes of transmission between infected farms were found (Sakamoto et al. 2000).

CONCLUSIONS

The recent outbreak of FMD in Japan in 2000 was successfully eradicated within 80 days of the confirmation of the first case. There were several reasons for successful eradication within such a short period. Firstly, it should be mentioned that control measures against FMD—such as movement control, surveillance, destruction of animals, and disinfection—were successfully carried out without confusion and with the cooperation of farmers, veterinarians, and related organizations. The guidelines prepared for the FMD outbreak were considered to have worked well generally, although they should be reviewed in the context of recent experiences. Secondly, the initial case was found before FMD had spread widely in the area. In particular, the clinical signs were not characteristic of conventional FMD, so the disease would have escaped notice if the cattle were not carefully examined. The veterinarian who reported the first case was officially commended for his service. This incident illustrated the important role that field veterinarians play in emergency preparedness against foreign animal diseases. Thirdly, the transmissibility of the FMDV between cattle farms was not high. This observation was supported by laboratory experiments and may be related to the mild clinical signs observed in cattle in the field; that is, the mild clinical signs suggest that the replication of O/JPN/2000 in cattle is low as well and that infected cattle excrete less virus into the environment. Farm size was also considered associated with successful eradication. Most cattle farms in the infected area in Kyusyu were small-scale operations. Therefore, the outbreak may have been restricted to sporadic occurrences because both the excretion of virus and the movement of infected cattle per single farm were minimal. On the other hand, if FMD had entered pig farms, the situation may have been quite different. Pigs excrete a large amount of virus, which may result in airborne transmission. In addition, swine farms are considerably larger than cattle farms, and the movement of animals is more frequent due to their short life cycle. Therefore, FMD might have become epidemic and seriously damaged the livestock industry in Japan if it had entered the swine population. One rea-

son why FMD did not enter pig farms may be that infected cattle did not excrete enough virus to infect pigs, as the laboratory experiments suggested. Another factor is that mixed farming of pigs and cattle is quite unusual in Japan. Finally, many farmers, in particular pig farmers, are aware of FMD and have been alert for possible problems since the FMD outbreak in Taiwan caused catastrophic damage to the pig industry in 1997.

In conclusion, Japan succeeded in eradicating FMD in a short period by stamping out the disease at four farms. However, contingency management plans need to be reviewed in a timely fashion taking into account the diversification of FMDV observed worldwide. In particular, caution should be exercised against the invasion of the FMD viruses that can transmit and spread unnoticed.

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6.3 Foot-and-Mouth Disease: Preventive Measures in the Republic of Korea

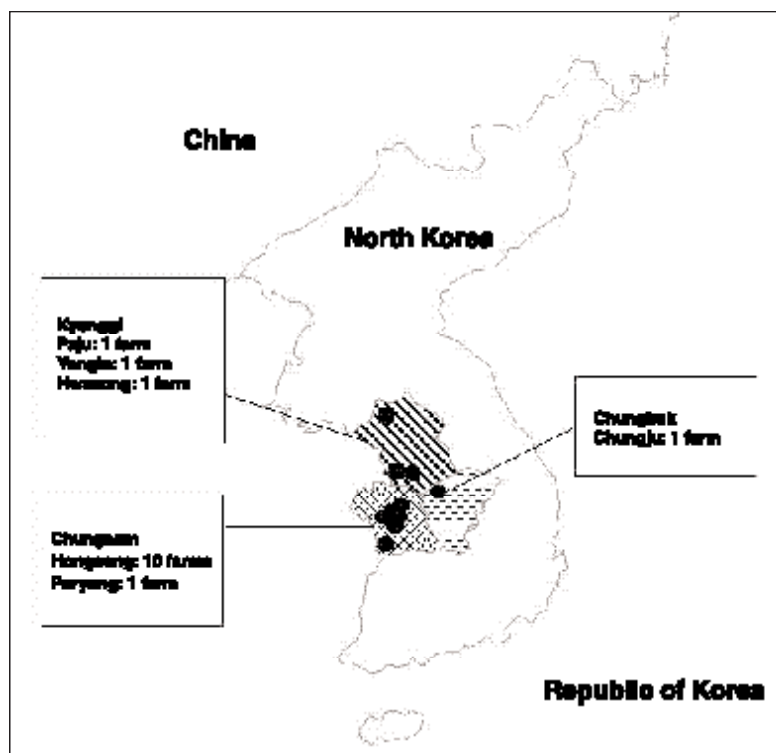
Soo-Hwan An

SUMMARY

The Republic of Korea had been free of foot-and-mouth disease (FMD) for 66 years until 15 outbreaks were reported in the year 2000. Various control measures were implemented, including stamping out of animals on infected and neighboring farms, movement controls, emergency vaccination, surveillance, and epidemiological investigations. These measures were effective in limiting the number of outbreaks to 15 and containing the FMD outbreak within a month. Preventive measures such as increased quarantine measures at ports, education of personnel involved in the livestock industry, and public information programs were also implemented to prevent possible reentry of FMD virus.

FOOT-AND-MOUTH DISEASE OUTBREAK

A total of 15 outbreaks in the three provinces of Kyonggi, Chungnam, and Chungbuk in the Republic of Korea (ROK) were reported between March 24 and April 15, 2000. Altogether, six foci of infection in the regions of Paju, Hwasong, Yongin, Hongsong, Poryong, and Chungju were identified, with one outbreak reported in each region, except Hongsong, where 10 outbreaks were reported (Figure 6.3.1). The outbreaks involved only cattle, and other animals were not affected. Previous to these outbreaks, the last recorded outbreak of foot-and-mouth disease (FMD) in the ROK was in 1934, which was limited to the northern parts.



6.3.1. Foot-and-mouth disease outbreaks in Korea.

DIAGNOSTIC TESTS

For screening tests, antibody detection using enzyme-linked immunosorbent assay (ELISA) was used. Non-vaccinated animals were tested by liquid phase blocking (LPB) ELISA, obtained from the World Reference Laboratory (WRL) for FMD at Pirbright (UK) and checked by virus neutralization (VN) tests. Vaccinated animals were tested using the nonstructural protein (NSP)-ELISA (3ABC-ELISA) such as provided by the Foreign Animal Disease Diagnostic Laboratory (FAD-DL) at Plum Island (USA), and 3D-ELISA developed by the National Veterinary Research and Quarantine Service (NVRQS), ROK (Choi et al. 2000). For positive diagnosis of FMD, tests were performed to demonstrate FMD viral antigen from samples such as blood, vesicular fluids, and tissues from lesions, nasal secretions, and esophageal-pharyngeal fluid (probang sample) collected from suspect animals. The samples were tested by antigen-ELISA (Pirbright, UK), polymerase chain reaction using primers for IRES and VP1 gene regions, and by virus isolation using primary fetal lung cells from Korean native goat. All suspect samples were tested in a high-containment facility.

CHARACTERISTICS OF FMD VIRUS ISOLATES

Clinical lesions in dairy cattle included severe salivation and vesicles on the mouth, teats, and hooves, whereas, in Korean native cattle, lesions were largely restricted to the oral mucosa. Although only cattle were affected during the outbreak in 2000, experiments carried out at WRL, Pirbright, and FADDL demonstrated that pigs were fully susceptible to the Korean FMD virus (FMDV) isolate and that clinical signs were more severe in pigs than in cattle. A cow that was inoculated with the Korean isolate showed severe salivation and vesicles on the tongue at day 2 post inoculation (PI), whereas a cow and a pig placed in the same stall showed similar signs between days 2 and 3 PI. Other pigs that were placed in the same stall, but were prevented from having direct physical contact with the inoculated cow, also showed signs between days 3 and 7 PI (Sur et al. 2000), which indicated the possibility of aerosol infection, although field evidence suggested that most infections within farms were through direct contact, and the spread of disease between farms was limited. Histological lesions in the hoof included intercellular edema and separation of epithelial cells, and in the tongue included vesicle formation and necrosis of the stratum spinosum. Keratinocytes floating in the vesicular fluid were also seen. DNA sequence analysis of VP1 gene showed the Korean field isolate to be an FMD type O virus closely related to O/TAW/1/99 and O/Kinmen/TAW/99 strains isolated in Taiwan (Shin et al. 2000).

CONTROL MEASURES

Stamping-out measures were applied to all infected and neighboring farms within a 0.5-km radius. A total of 2492 susceptible animals were destroyed from 192 farms. *Protection zones* (areas within a 10-km radius of a farm with an outbreak) and *surveillance zones* (areas between a 10- and 20-km radius of a farm with an outbreak) were declared, and movement restrictions were applied to these zones. Checkpoints placed along the border of these zones were enforced by government workers, local police, and the military. Vehicles passing through the checkpoints were disinfected. As of July 19, 2000, after extensive serological surveillance, all movement restrictions were lifted. However, restrictions have been applied to vaccinated animals so that these animals are slaughtered only at designated slaughterhouses, and any sales or movement must first be reported to the county officer and tests conducted for FMD.

Vaccination is a control option that requires much caution because it can delay declaration of freedom from FMD, is labor intensive, and presents problems with surveillance. However, the decision to implement emergency vaccination was made because the lesions observed on the first outbreak farm in Paju indicated that infection had been present on the farm for some time (about 3 weeks), and windy conditions during the period made rapid spread of this highly contagious disease more likely. For these reasons, susceptible animals within the protection zones were clinically examined and vaccinated. Before the FMD outbreak, the ROK maintained a vaccine reserve of 300,000 doses and an antigen bank reserve of 2,000,000 doses. The vaccines were trivalent, double-oil-emulsion vaccines using strains O1 Manisa, A22 Iraq, and Asia1 Shamir. After the first outbreak, additional vaccines were obtained from the antigen bank. During the FMD outbreak, all susceptible animals within the protection zones were vaccinated. A total of 860,700 animals on 13,038 farms were vaccinated during the first round of vaccination and 661,770 animals on 12,876 farms during the round of booster vaccination. After the completion of vaccination, the animals were branded (cattle) or identified by ear punching (pigs) to prevent problems later with surveillance.

Reporting of suspected cases by an owner or veterinarian is important in surveillance, but requires organization, legislation, and an effective system for reporting clinical disease activity. In 2000, a total of 89 suspected cases were reported and investigated. Except for the 15 outbreak cases, the remaining 74 were diagnosed as infectious bovine rhinotracheitis, bovine viral diarrhea, bovine papilloma, pseudocowpox, contagious ecthyma, etc. Since the outbreak, cash incentives and education have been used to encourage reporting.

SEROEPIDEMIOLOGICAL SURVEY DURING THE OUTBREAK

Extensive serological surveillance of the protection zones, surveillance zones, and the rest of the regions was conducted from the time of the first reported outbreak until July 19, 2000, when all movement restrictions were removed. A total of 8863 vaccinated animals in the protection zones were tested using 3D- or 3ABC-ELISA. Also, a total of 5400 animals from the surveillance zones and 3568 animals from the free regions were tested using LPB ELISA. Several farms had one or two animals that were seropositive on ELISA and were placed under quarantine while an appropriate number of additional serum and probang samples were acquired and tested. All samples were negative for FMDV. Heat treating the positive serum samples at 64°C for 30 minutes eliminated many of the positive reactions, which supported the view that these were actually false positives.

EDUCATION AND INFORMATION

Gaining the understanding and support of people in the livestock industry and the general public is essential for the success of any eradication program. Educational meetings and seminars for veterinary service personnel, livestock traders, practicing veterinarians, and producers were held periodically on subjects such as prevention, the importance of reporting diseases, surveillance of animal diseases, and the proper use of disinfectants. Leaflets, booklets, posters, and videos were distributed, as well as TV documentary specials and newspapers articles to inform the public of the disease.

SURVEILLANCE PROGRAM

The purpose of the FMD Surveillance Program in the ROK is to define the current disease status of the country, maintain an effective system of detecting disease activity for rapid containment of the disease, identify possible primary index farms or other high-risk farms that should be quickly removed, and provide sufficient evidence that the country is FMD free.

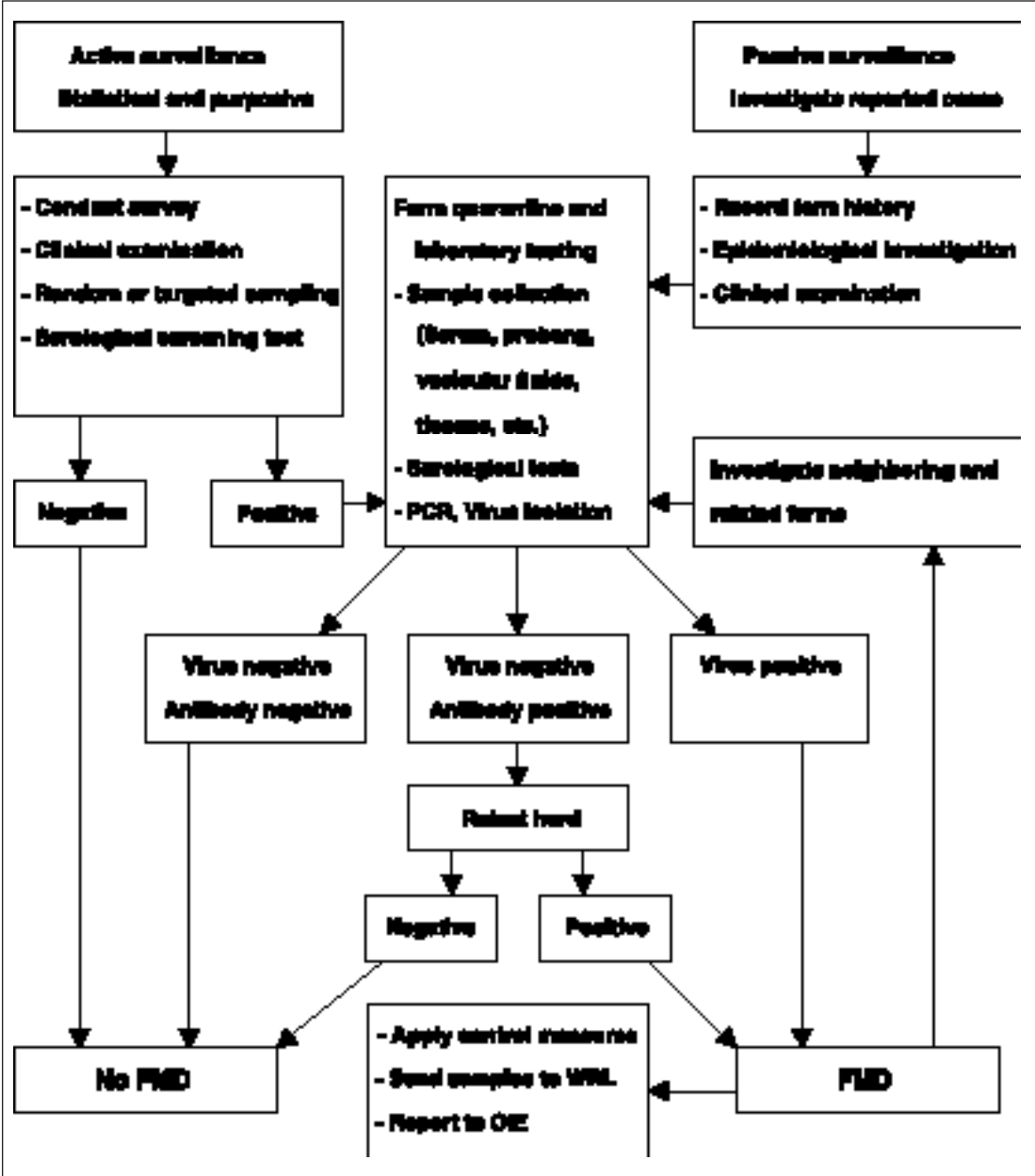
Surveillance is defined by the OIE International Animal Health Code as the continuous investigation of a given population to detect the occurrence of disease for control purposes, which may involve testing of a part of the population. Surveillance activity in the ROK consists of *passive surveillance*, i.e., investigating reported disease activity, and *active surveillance*, which means testing statistically selected and targeted samples from within host populations. Any suspicion of FMD is followed by quarantine, confirmatory diagnostic tests, and control measures (Figure 6.3.2).

INTRODUCTION OF THE FMD VIRUS

Although there is insufficient evidence to establish definitively how the FMDV was introduced into the ROK, the following routes have been considered and investigated:

1. Movement of people and vehicles. There is evidence to support that the movement of people and vehicles was the source of spread among farms, and the possibility that FMD has been introduced into the ROK by people who have traveled to countries with FMD cannot be excluded.
2. Imported hay. This was used throughout the country and, because only a low dose of FMDV is needed for transmission by the respiratory route among ruminants, this could explain why only cattle were affected. However, none of the infected farms or neighboring farms reported using imported hay.
3. Wind-borne spread and “yellow sand.” All farms with outbreaks were on the western or windward side of the Korean Peninsula. Wind-borne spread is also supported by the fact that cattle are more susceptible to aerosol infection. Weather conditions were suitable for the survival of the virus during some of the outbreak period. However, the distance from China and Mongolia to the ROK is quite long (400 to 1500 km), and aerosol transmission does not explain why larger cattle farms were not affected. The argument is the same for the transport of *yellow sand*, an annual spring event in which sand originating from the deserts of China and Mongolia is carried on strong westerly winds to the Korean Peninsula. Although several of the affected areas reported heavy yellow sand during periods just before the outbreaks, the long distance would make survival of FMDV questionable.

Epidemiological studies were conducted to investigate the possible source or sources of FMDV in the ROK. Imported hay samples and yellow sand samples collected from across the country were tested by polymerase chain reaction. Although all samples were negative for FMDV, more studies will be needed before these can be ruled out as possible sources. Clinical investigations and serological examinations of epidemiologically significant farms—such as those using imported hay, those that had contact through milk pickup trucks and feed delivery trucks, and those that had previously reported signs similar to early signs of FMD—were also investigated. However, no source of FMD was definitively identified by the investigations.



6.3.2. Surveillance protocol for detecting foot-and-mouth disease (FMD) virus-infected herds. PCR, polymerase chain reaction.

CONCLUSIONS

In recent years, there have been an increasing number of FMD outbreaks in Asia, including previously FMD-free countries, such as the ROK and Japan. Although current disease information is lacking in some parts of Asia, FMD is believed to be widespread in China and Mongolia. Thus, the ROK faces further risks of FMD, so

increased vigilance will be essential in preventing further outbreaks.

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6.4 Swine as the Origin of the Foot-and-Mouth Disease in the United Kingdom, 2001

Paul Kitching

SUMMARY

The available evidence indicates that the 2001 outbreak of foot-and-mouth disease in the United Kingdom started in a large, swill-fed farm close to the city of Newcastle, in the north of England. Subsequent investigations by the customs authorities identified an established illegal trade of pig meat coming into the country with false documentation. The supposition is that some of this material was collected as waste from a restaurant and fed untreated on the index farm. The initial spread was by aerosol to a nearby sheep and cattle farm, from where it was taken by sheep through two major markets, effectively spreading the virus countrywide. Pigs were only occasionally affected during the outbreak, and both experimental and field evidence indicated that this virus was not produced as an aerosol by pigs at the same level as previous outbreak viruses. The susceptibility of pigs to aerosol infection is also approximately 100 times less than for sheep and cattle. This chapter relates the course of events up to the time of writing (August 2001) and addresses some of the issues raised during the outbreak.

INTRODUCTION

Foot-and-mouth disease (FMD) is a highly infectious viral disease of cattle, pigs, sheep, goats, and wild cloven-hoofed animals, characterized by fever, loss of production, and vesicles on the mouth, feet, and teats. In young animals, the virus attacks the developing heart muscle, resulting in death without other clinical signs. It is caused by a small RNA virus within the family Picornaviridae. There are seven immunologically distinct serotypes of FMD virus (FMDV): types O, A, C, Asia1, and SAT1, SAT2, and SAT3. It is considered the most contagious of all animal viral diseases and is the single most important constraint to international trade in live animals and animal products.

The United Kingdom had been free of FMD since 1981, when there had been a single outbreak in a dairy herd on the Isle of Wight, off the coast of southern England. On February 19, 2001, pigs waiting slaughter in an abattoir in southern Essex, to the north of London, were identified by the on-duty veterinarian to have feet lesions

consistent with FMD. The animals were confirmed as FMDV positive the following day at the high-security Institute for Animal Health laboratory at Pirbright. This laboratory is also the World Reference Laboratory for FMD and, apart from having the largest research group working on FMD, also maintains a library of FMDV isolates collected from around the world during the last 60 years.

The virus strain was quickly identified by nucleotide sequencing to be the PanAsia strain of serotype O FMDV. This strain was known to be present throughout most of Asia and had recently caused new outbreaks in Japan (free since 1908), South Korea (free since 1934), and South Africa (this was the first outbreak of serotype O ever recorded), Mongolia, and eastern Russia. The pigs affected in the abattoir had been held over the weekend before developing signs, and it was assumed that their farm of origin was the source of infection. However, tracing back showed no evidence of infection, so it was concluded that they had acquired infection in the abattoir. Other farms supplying pigs were then visited, and, on February 23, a farm in northeast England, close to Newcastle, was found with evidence of FMD. On February 24, a movement ban on all FMD-susceptible species was applied to the whole of the United Kingdom, which included markets, but even affected race meetings and dog shows. The affected farm contained over 500 adult, mostly cull, sows and boars. Following clinical examination, it was apparent that the disease had probably been introduced as early as the beginning of February, as most of the pigs had lesions approximately 10 days old. The farmer fed almost exclusively a diet of waste food (swill) collected from nearby schools, hospitals, and restaurants. Regulations relating to swill feeding make it compulsory to boil all waste food before feeding to pigs, but this is difficult to enforce.

Transmission of FMD follows the movement of infected animals, the feeding of animal products contaminated with FMDV, by contact with mechanically carried FMDV (on vehicles, clothes, hands, instruments, etc.), or as an aerosol produced by infected animals. Persistently infected (carrier) cattle can also precipitate new outbreaks, although this is rare. It is likely that the pigs had been infected from the swill

feed, and subsequent seizure of illegally imported animal products by the customs authority supported the hypothesis that this farm was the index case. Infected pigs produce a large amount of aerosol virus, up to 3000 times more than infected sheep or bovines, and farms immediately neighboring the premises were examined for evidence of disease. A number of nearby farms had been exposed to virus, and one in particular, on which there were infected sheep and cattle, had earlier sold sheep through two markets to a farm in south-west England, in Devon. When this farm was visited, it also was identified as infected with FMDV. Tracing back through the markets revealed a very large number of potential contacts, and visiting these contact premises quickly exhausted the veterinary resources of the Ministry of Agriculture, Fisheries and Food (MAFF), now the Department for Environment, Food and Rural Affairs (DEFRA). Only 200 veterinarians were employed by MAFF, and, for disease security reasons, veterinarians that had visited an infected farm could not go on another farm for 3 days. However, the potential animal contacts from the markets and their subsequent contacts numbered many thousands. This resulted from the methods of sheep trading used by the animal dealers whereby some animals could change owner up to seven times in as many days.

FMD outbreaks started appearing throughout the country, particularly in the west of England, southern Scotland, and Wales. Disease had also spread by the movement of infected sheep to Northern Ireland, the Republic of Ireland, France, and the Netherlands.

Control of the outbreaks was taken over from MAFF by the Chief Scientist, supported by four teams of mathematical modelers and other involved groups, who reported directly to the Prime Minister's Office. The slaughter policy was extended to all neighboring farms and those within a 3-km radius of the infected farm. Slaughter of animals on the infected farm was to be completed within 24 hours of diagnosis and on neighboring farms within 48 hours. This became the responsibility of the Army.

Clinical disease in cattle and pigs is relatively easy to diagnose, but FMD in sheep is frequently mild and can easily be confused with other common conditions, such as foot rot and/or nonspecific mouth ulcers. The majority of suspect cases were in sheep and, while samples were not collected from all of the suspect farms, many of those submitted for laboratory confirmation were negative. The rate of FMD transmission in sheep is also much lower than in cattle or pig herds, so the potential for infected sheep flocks to transmit the virus as an aerosol to neighboring farms was considerably lower (Sorensen et al. 2000). Consequently, many uninfected animals were slaughtered, which added to the problem of carcass disposal.

During the last major outbreak of FMD in the United Kingdom, in 1967-1968, approximately 2500 farms were diagnosed infected and 500,000 animals were slaughtered. During the 2001 outbreak, over 1900 farms have been diagnosed infected at the time of writing (August 2001) and over 4,000,000 animals have been slaughtered. The policy of slaughtering animals on neighboring farms and those within a 3-km radius was modified once it was shown that this particular strain of type O virus did not spread significantly as an aerosol (Donaldson et al. 2001) and that most transmission that occurred after the movement ban was a consequence of illegal movements, direct contacts, or farmers not applying strict disinfectant procedures when visiting other livestock. It should be noted that farmers in the north of England frequently have several livestock holdings on different premises. The use of vaccination was considered, but would not have significantly altered the disease distribution or the outcome and was likely to confuse subsequent reestablishment of disease-free status.

The total cost of the outbreak has not been calculated, but, including the effect on tourism, will exceed \$30 billion (Canadian).

DISCUSSION

Resources

Although the lessons to be learned from the recent FMD outbreak in the United Kingdom await to be fully documented in a government inquiry, there were a number of problems that arose during the outbreak that would have been better addressed by contingency planning beforehand. Some of these were predictable, but most only became apparent once the outbreak had started. Most important was the availability of resources such as veterinary staff, livestock appraisers, and slaughterers. The United Kingdom, like all other European countries, had been reducing its civil service numbers, including those employed by MAFF. While it was always considered that an outbreak of FMD was possible, it was never envisaged on the scale that occurred. The concept that the first case would immediately be identified and subsequent spread prevented was clearly wrong. As was the assumption that other European countries would be infected before the United Kingdom and, therefore, that there would be ample warning of its possible introduction. However, no government in the world would have been able to maintain the personnel resources required for an event that might never happen. Arrangements were in place to bring in veterinarians trained in disease control from around the world, but the manner in which the disease became established throughout the United Kingdom before its presence was even realized made it impossible to bring in the necessary help sufficiently quickly.

The Sheep Industry

Although the disease started in pigs, its spread and establishment in the sheep population also delayed and confused the control program because of the difficulty of recognizing clinical signs in sheep. Farmers did not immediately recognize infection and, even after the declaration of the outbreak, many were not taking sufficient precautions when moving between flocks because they thought their own animals were free of FMD. Consequently, when disease was recognized, either in the sheep or because it had spread to in-contact cattle or pigs, the virus was well established in a number of flocks owned by the same farmer, and possibly in neighboring farms. On quite a large number of occasions, the disease had been present for many weeks, having been introduced before the movement ban. Added to this was the very large volume of sheep movements that were routine at that time of year. There were also a large number of sheep markets taking place. It is normal for farmers to inspect the mouths and teeth of any sheep they intended to buy, which is an ideal way of spreading infection among sheep and contaminating the farmers themselves. There were even instances of the handlers in the markets taking infection back to their own farms. Most of the transactions that occurred in the markets were recorded, but those occurring outside the market, for instance, in the car park, were not registered. This made tracing even more difficult, particularly as the sheep were not individually identified. There was legislation put in place following the outbreaks of swine vesicular disease in the 1980s in the United Kingdom that prevented the movement of pigs off a farm for 21 days after the introduction of any additional pigs from another premise. This did not apply to sheep, so it was frequent to discover that sheep had been moved from one farm to another over a relatively short time, particularly through the premises of the sheep dealers.

Swill Feeding

The FMDV had almost certainly been introduced with infected meat, imported illegally. There had been an outbreak of classical swine fever (hog cholera) during the previous year in the United Kingdom, also thought to have been introduced through infected meat products. This event had already alerted MAFF to the dangers of this route of introduction. The volume of illegal movement of all products across international borders is impossible to quantify, but is likely to be extremely large, involving drugs, cars, and even people. The ability of any country to control this traffic is limited by staff resources and the desirability not to disrupt legal movements. Since it is likely that no country can prevent the occasional introduction of a foreign animal pathogen, the only defense is to prevent it from making contact with a susceptible species. The feeding of waste food to pigs is an obvious method by which this contact can occur, in

spite of any legislation in place to render the material sterile by boiling or other treatment. In the United Kingdom, approximately 1% of the pigs were fed some swill in their diet, making it a marginal market of little economic significance to the pig industry. However, the consequences of the introduction of a disease such as swine vesicular disease, hog cholera, pseudorabies, or FMD affect all producers, not only pig producers. Many more could be added to this list of pathogens, such as strains of *Salmonella*, *Arterivirus*, *Parvovirus*, and *Circovirus*. The European Union has now banned the use of swill in pig feed throughout Europe.

Carcass Disposal

Because of the large numbers of animals being slaughtered, carcass disposal became a prominent issue in the media, and the pictures of large piles of dead animals clearly affected the decision of many tourists to visit the United Kingdom. While it remains the duty of the government inquiry to establish whether the size of the cull was necessary, the consequence of the control strategy employed and the use of the army to carry it out resulted in an accumulating number of carcasses that could not be immediately buried or burnt. Because of environmental considerations, such as proximity to housing and water catchment areas, it was not usually possible to bury or burn carcasses on the infected farms. Many were transported in leak-proof vehicles to rendering plants, but these were quickly overwhelmed. Other problems encountered included the following: cattle over 30 months of age could not be buried because of the possible contamination of the ground with the agent of bovine spongiform encephalopathy, leakage from burial sites could contaminate local streams, and funeral pyres could give off dioxins, as well as acrid smoke. Together these considerations made decisions on whether to bury, burn, or transport extremely difficult and complex, involving many different agencies.

Public Footpaths

The decision to close all footpaths across nonurban land also severely affected the tourist industry, making it pointless for visitors to take holidays in some of the most scenic areas of the United Kingdom. A risk assessment had been carried out as to the dangers associated with free movement of people across land on which there could be susceptible animals, and while the risk of mechanically carrying virus from an infected farm would have been small, the decision was made. It can be appreciated that farmers worried about virus getting onto their land would be very concerned about strangers using footpaths in proximity to their animals.

Wildlife

At no time during the outbreak was there any evidence of the involvement of susceptible wildlife species, such as

deer or feral pigs. The diagnostic laboratory received samples from farmed deer close to infected farms and from deer killed by hunters or in road accidents. All results were negative. This is consistent with FMD outbreaks in other regions of the world, except southern Africa, where the African buffalo is often associated with outbreaks in domestic stock. Generally, if wildlife species are affected, they are unable to maintain the disease because of low stocking density or lower susceptibility and the virus dies out. Even if they were affected, it would be inadvisable to attempt control because it would likely scare the wildlife involved out of the area and, thereby, possibly introduce FMD into a previously free area.

Vaccination

Considerable discussion took place concerning the use of vaccination, and vaccine was formulated from the bank held in the United Kingdom for use in the north of England. The situation was different from that in the Netherlands, where vaccination was used. Unlike the Dutch outbreak, which was focal and could be surrounded by a barrier of vaccinated animals, the outbreak was well distributed around the United Kingdom before FMD was even recognized. This made the choice of where to vaccinate impossible. The total sheep population exceeded 20 million and cattle over 10 million and, because the distribution of the disease was not known, vaccination could have actually spread the disease.

None of the animals in the United Kingdom had previously been vaccinated and, therefore, would not have become protected for at least 5 days. If already infected animals were vaccinated, there was the danger of needle spread of the virus, as well as the danger that bringing the animals together for vaccination would encourage contact spread.

Vaccination would not prevent disease in infected animals, and even those animals that were vaccinated would not be saved from infection should they have subsequently contacted active virus. It is a characteristic of FMDV that it will persistently infect ruminants following recovery from clinical disease—even animals that have been protected by vaccination. Cattle can remain carriers for up to 3 years and sheep for up to 9 months. There is a small risk that these infected animals can precipitate new outbreaks of disease.

Not only does vaccination not prevent infection, but the duration of immunity is generally less than 6 months and, depending on the virus exposure dose, not all vaccinated animals are completely protected. Pigs cannot be fully protected by vaccination and, if one of a group develops disease, it will overwhelm the immunity of the others. One option was to vaccinate the cattle coming out of winter accommodation in the north of England onto land on which there may have been infected sheep. If they had become infected, they would have consumed

considerable resources in their disposal. The idea was discarded after the Food Standards Agency insisted (although later changed its mind) that milk from vaccinated animals would have to be labeled. The farmers rightly said that this would reduce the value of the milk and could even prevent its sale. They were also concerned that they would have to slaughter their animals because they were vaccinated, and if that was likely, they preferred to have the compensation paid at once.

Reestablishment of FMD-Free Status

The decision to slaughter vaccinated animals, as occurred in the Netherlands, was determined by the need to reestablish freedom from FMD so that trading in live animals and animal products could be resumed. Free status can be obtained 3 months after the slaughter of the last infected animal or the last vaccinated animal, whichever is the later, in addition to a surveillance program to show that the disease has been eradicated. The presence of vaccinated animals makes this more difficult because of the possibility of carrier animals and the problem of vaccinated animals having antibodies to FMDV. If vaccinated animals are not slaughtered, a minimum of a year is required before the country can be recognized as disease free. The United Kingdom is currently undertaking a serum survey, testing up to 160,000 sera a week, to prove to its trading partners that it has the disease under control and, ultimately, eradicated.

CONCLUSIONS

At the time of writing (August 2001), sporadic outbreaks of FMD are still occurring in the United Kingdom. Most of these had been hidden by their association with sheep. This situation is quite normal in any large outbreak and can be expected to cease over the next few months. Many questions have been raised concerning the management of the control program, some of which have been discussed in this chapter, and an inquiry will undoubtedly take place. The results of the inquiry will be of considerable interest and value to all FMD-free countries, particularly those with an economically important and flourishing export trade in live animals and animal products.

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6.5 Application of Assays Based on Foot-and-Mouth Disease Nonstructural Proteins to Epidemiological Surveillance

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SUMMARY

Foot-and-mouth disease (FMD) is a highly contagious and economically devastating viral disease of cloven-hoofed animals, including pigs, cattle, and sheep. In ruminants, especially cattle and sheep, the disease can be very mild or even subclinical, so FMD can sometimes spread unnoticed. Furthermore, once infected, ruminants become carriers of the virus and consequently may become the source of new outbreaks of disease. Therefore, the identification of FMD virus-infected animals is of considerable importance for the control and eradication of the disease. Consequently, efforts have been directed to the development of diagnostic tests that can distinguish infected animals from those that have been vaccinated.

Eradication programs are based on a combination of vaccination programs and slaughter policies. So far, the only vaccines available for control of FMD are based on semipurified, chemically inactivated virus. These vaccines elicit antibodies principally to structural proteins whereas field virus-infected animals develop antibodies against both structural and nonstructural proteins. Therefore, the approach has been to identify antibodies against viral nonstructural proteins that are present only in infected animals. This chapter describes and discusses the techniques developed to identify antibodies against virus-specific proteins that are present only in infected animals, with special emphasis on the more recent advances in assay development.

INTRODUCTION

Foot-and-mouth disease (FMD) has an economically devastating impact on affected countries, primarily because of trade barriers that are imposed wherever the disease occurs. The distribution of FMD virus (FMDV) is extensive, including parts of Africa, Asia, the Middle East, and South America (Kitching 1998). FMD is difficult to control because it is perhaps the most highly transmissible

viral disease of animals (Pereira 1981). Recently (February 2001), FMD appeared in Great Britain and spread rapidly throughout the country (Ferguson et al. 2001). This and other recent epidemics, e.g., in Japan and Korea, exemplify the potential threat that FMDV holds even for countries that have long been free of the disease.

The FMDV produces an acute, systemic disease characterized by the appearance of vesicles on the feet, in and around the oral cavity, and on the mammary glands of females (Mackay 2000). In ruminants, an asymptomatic, persistent infection can be established (Salt 1993), even in vaccinated animals (Sutmoller and Gaggero 1965). Although definitive confirmation of FMDV transmission from carrier to susceptible animals has not been reported, there is epidemiological evidence to support this (Barnett and Cox 1999).

DIFFERENTIATING VACCINATED FROM INFECTED ANIMALS

The control of FMD in endemic areas involves regular immunization with a vaccine composed of semipurified, chemically inactivated virus (Barteling and Vreeswijk 1992). Within the European Union (EU), a nonvaccination policy has been in place since January 1992. Within the EU, prevention of the disease relies on stringent controls regarding the importation of animals from affected areas. In the event of an outbreak, the response would involve *stamping out* (slaughter of infected and contact animals), restrictions on animal movement and, eventually, emergency ring vaccination around the outbreaks.

Accurate diagnosis of infection with FMDV is of great importance both as a supportive measure to the stamping-out policy in FMD-free areas and in control and eradication campaigns in FMD-endemic areas. In countries where vaccination is carried out, the majority of animals are seropositive by current serological techniques; that is, these assays cannot discriminate between animals that were infected with FMDV and those that were only vaccinated. This distinction is important

because asymptomatic carrier animals can be found in vaccinated herds (Doel et al. 1994).

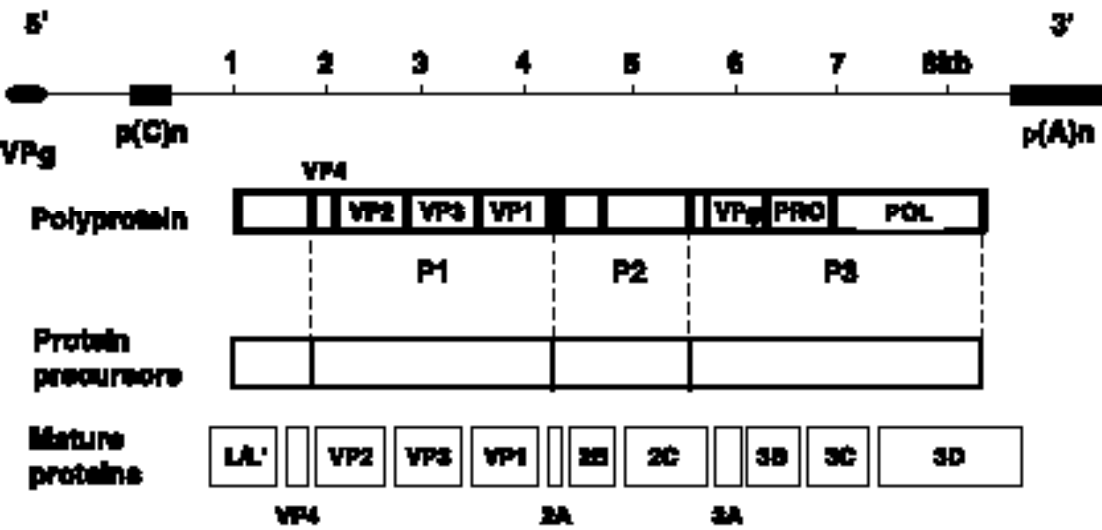
However, understanding the biology of the virus suggests possible solutions. The FMDV is a positive-sense, single-stranded RNA virus. During viral replication, the viral RNA is translated into a single polypeptide in the cytoplasm of the infected cell and then cleaved into structural and nonstructural proteins (Figure 6.5.1). During infection, animals produce antibodies against both structural and nonstructural proteins. In contrast, conventional FMD vaccines based on chemically inactivated virus primarily induce the production of specific antibodies against structural proteins. Therefore, efforts to develop assays able to differentiate infected from vaccinated animals have focused on the detection of antibodies against nonstructural proteins.

According to the Office International des Epizooties (OIE) International Animal Health Code rules, countries can be declared either *FMD free* or *free with vaccination*. Countries wishing to be declared FMD free have the obligation to conduct surveillance to demonstrate freedom from infection. In nonvaccinating countries, this can be done using conventional serological assays that detect antibodies against structural proteins. In countries that are free with vaccination but wish to become FMD free or in those wishing to obtain free-with-vaccination status, the detection of antibodies against nonstructural proteins could be used as a direct measure of viral activity in the population.

The availability of such tests would provide for a number of important applications, including (1) detection of evidence of infection, (2) follow-up to ring vaccination in FMD-free countries, (3) analysis of serum from import/export cases, and (4) prevalence surveys in endemically infected countries. The overall effect of these assays would be to reduce the extent and duration of the restrictions imposed on the vaccinated areas.

Identification of asymptomatic FMDV replication is today as important as the diagnosis of acute infections because of its relevance for programs of prevention, control, and eradication of FMD. Suttmoller et al. (1968) defined persistent infection as animals being virus positive for at least 28 days after infection. Persistent infection can be induced in cattle, sheep, and goats, and its detectable form is limited in time and in virus level. The carrier state in cattle can be up to 3.5 years and in sheep and goats up to 9 and 4 months, respectively (Barnett and Cox 1999; McVicar and Suttmoller 1969).

However, it is important to take into account some of the limitations of this test related to the detection of persistent animals. One limitation is that not all persistently infected animals seroconvert to nonstructural proteins. Another is that the level of antibodies to nonstructural proteins is not correlated to the carrier state, and animals that have eliminated the virus may remain seropositive for a long time. In spite of these limitations, the detection of antibodies against nonstructural proteins can provide useful information in



6.5.1. Schematic of foot-and-mouth disease virus genome showing the single encoded polypeptide and the different viral mature proteins. The capsid proteins VP4, VP2, VP3, and VP1 are also termed 1A, 1B, 1C, and 1D, respectively. The nonstructural proteins are located on regions L, P2, and P3.

epidemiological surveys to detect persistently infected animals, particularly if the results are interpreted in combination with additional indicators, i.e., animal age, vaccination history, previous infection, etc. (Bergmann et al. 2000).

As has been mentioned previously, the severity of the lesions caused by FMDV can vary between viral strains and viral hosts. The pathogenicity of FMDV infection in small ruminants is characterized by mild or subclinical presentation (Barnett and Cox 1999). Therefore, the detection of antibodies against FMDV nonstructural proteins in this situation can be an alternative diagnostic tool to identify subclinical or silent infection. During the 1999 FMD epidemic in Morocco, almost 600 sera collected from the zones where outbreaks occurred were tested by the enzyme-linked immunosorbent assay (ELISA) based on the use of 3ABC recombinant protein purified by excision from PAGE (3ABC-ELISA) standardized at the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Centro de Investigación en Sanidad Animal (CISA), in Valdeolmos, Spain. The test was able to detect asymptomatic infections that had occurred in unvaccinated sheep in the field (Blanco et al. 2002b) (Figures 6.5.2 and 6.5.3). It follows that this test could also be very useful for the detection of subclinically infected animals in serological surveys, resulting in enhanced control of the disease.

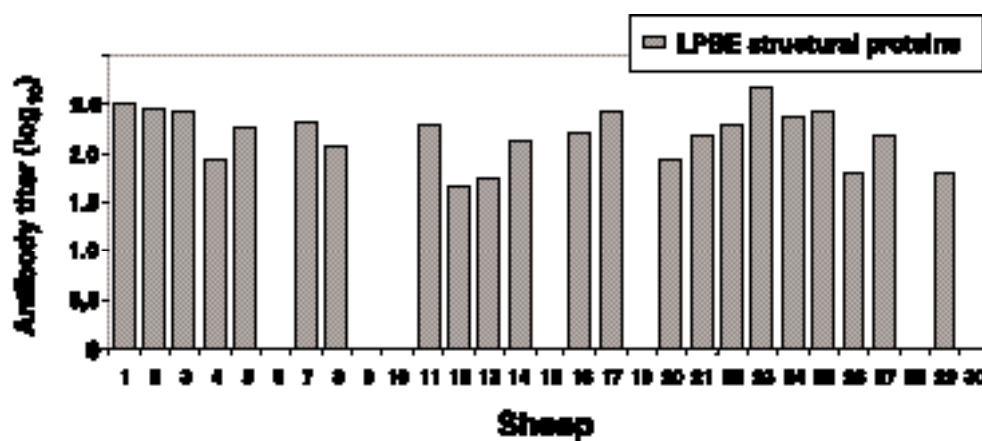
WHICH NONSTRUCTURAL PROTEIN CAN BE USED AS INFECTION PARAMETER?

The antigenic variation exhibited by FMDV has largely conditioned the strategies followed for its diagnosis, in-

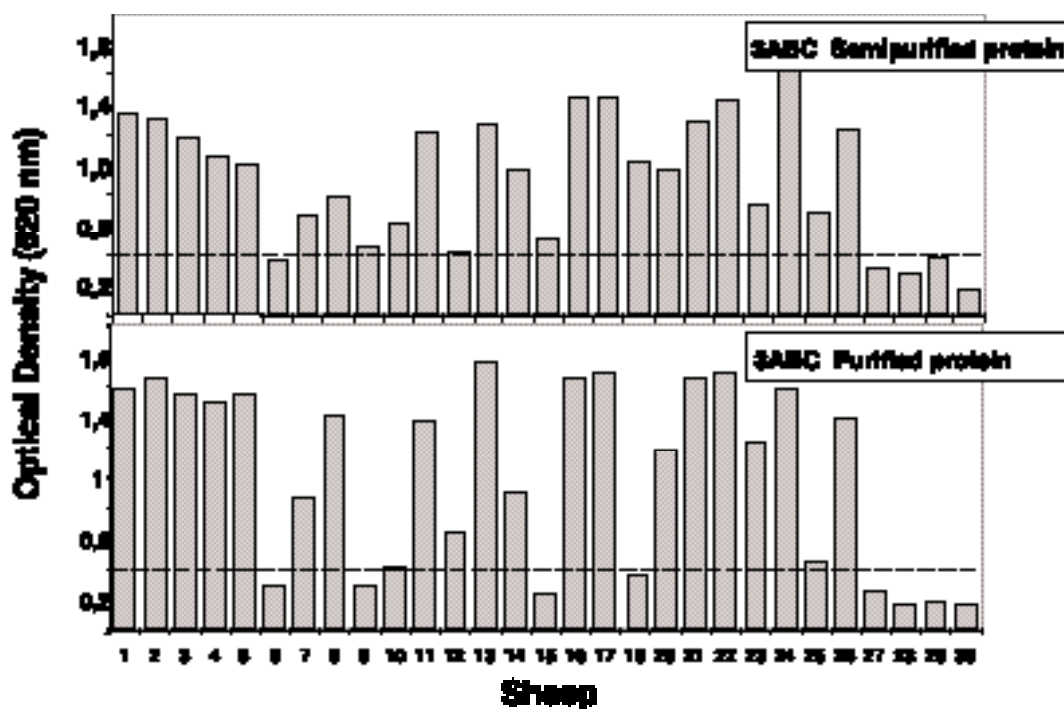
cluding serological procedures. The FMDVs are classified into seven distinct serotypes (A, O, C, Asia 1, SAT1, SAT2, and SAT3), among which there is no cross-protection. Conventional serological assays (serum neutralization and ELISA) are mostly based on the detection of antibodies against capsid proteins and, consequently, are serotype specific. However, the low degree of amino acid variation in nonstructural proteins among different FMDV serotypes allows for the detection of antibodies against the virus regardless of the serotype causing the infection (Blanco et al. 2001a). This feature presents the opportunity for extensive technical simplification in FMD serology by reducing the number and type of viral reagents. Furthermore, the use of bioengineered antigens eliminates the need to manipulate FMDV and eliminates the biosafety issues represented by live virus.

The RNA from FMDV consists of a single open reading frame (ORF) flanked by two noncoding regions. The regions L, P2, and P3 encode eight different mature nonstructural proteins (Porter 1993) (Figure 6.5.1).

The viral RNA polymerase protein 3D, also known as *virus infection associated antigen* (VIAA), has long been used for the detection of anti-FMDV antibodies by agar gel immunodiffusion (AGID). Protein 3D is one of the most immunogenic of the nonstructural proteins, but does not reliably allow differentiation between infected and vaccinated animals. The association of small amounts of 3D with purified FMD virions has been reported (Newman et al. 1994) and may contribute to the immunogenicity of 3D in vaccinated animals. In spite of this, the detection of antibodies against the 3D protein constitutes an additional parameter of great interest if



6.5.2. Antibody titers to foot-and-mouth disease virus (FMDV) type O detected by liquid phase blocking ELISA in a flock of 30 sheep exposed to FMDV during an outbreak in Oujda, Morocco. Samples were tested in duplicate by twofold serial dilutions (1:25 to 1:3200). Endpoint titers are expressed as the inverse of the log of the serum dilution that gave the same OD₆₂₀ response as the negative control at 1:25 dilution. Although these animals did not display clinical signs, they were in contact with cattle infected with FMDV and, in the absence of vaccination, the antibody-positive results to FMDV structural proteins confirm the infection. (OD, optical density.)



6.5.3. ELISA response in the same herd as Figure 6.5.2 using either 3ABC protein semipurified or protein purified. The response is expressed as the absorbencies measured at 620 nm testing the sera at a dilution of 1:25. The cutoff was established as an absorbance of 0.4. The results obtained using the purified protein provided greater differentiation than those obtained against foot-and-mouth disease virus structural proteins (Figure 6.5.2). When semipurified protein was used, some samples required further testing (immunoblotting) to confirm the result.

its result is combined with those obtained against other nonstructural proteins.

The nonstructural protein Lb appears to be the least immunogenic of the proteins, as shown by the failure of some animals such as pigs (Rodriguez et al. 1994) or cattle (Mackay et al. 1998b) to respond to Lb and a weak, transient response in others.

Lubroth and Brown (1995) proposed that antibody to 2C could be used as an indicator of infection. However, results reported by Mackay et al. (1998a) have shown that not all animals exposed to FMDV under field conditions seroconverted to 2C. Moreover, antibodies to 2C were detected in both experimentally and naturally infected cattle for a shorter time than antibody to other nonstructural proteins.

Examination of both bovine (Mackay et al. 1998a) and porcine sera (Rodriguez et al. 1994) indicated that the detection of antibody to the polyprotein 3ABC was the most reliable single indicator of infection relative to the immunogenicity of all nonstructural proteins. Of its derived proteins, 3A generally induces a similar response; some animals fail to react against 3B, whereas 3C alone is a very weak immunogen (Brocchi 2001). The detection of antibody to one or more of the nonstructural

proteins 2C, 3A, or 3AB, in addition to those against 3ABC, provides further confirmation of prior infection.

ASSAYS TO DETECT ANTIBODIES AGAINST FMDV NONSTRUCTURAL PROTEINS

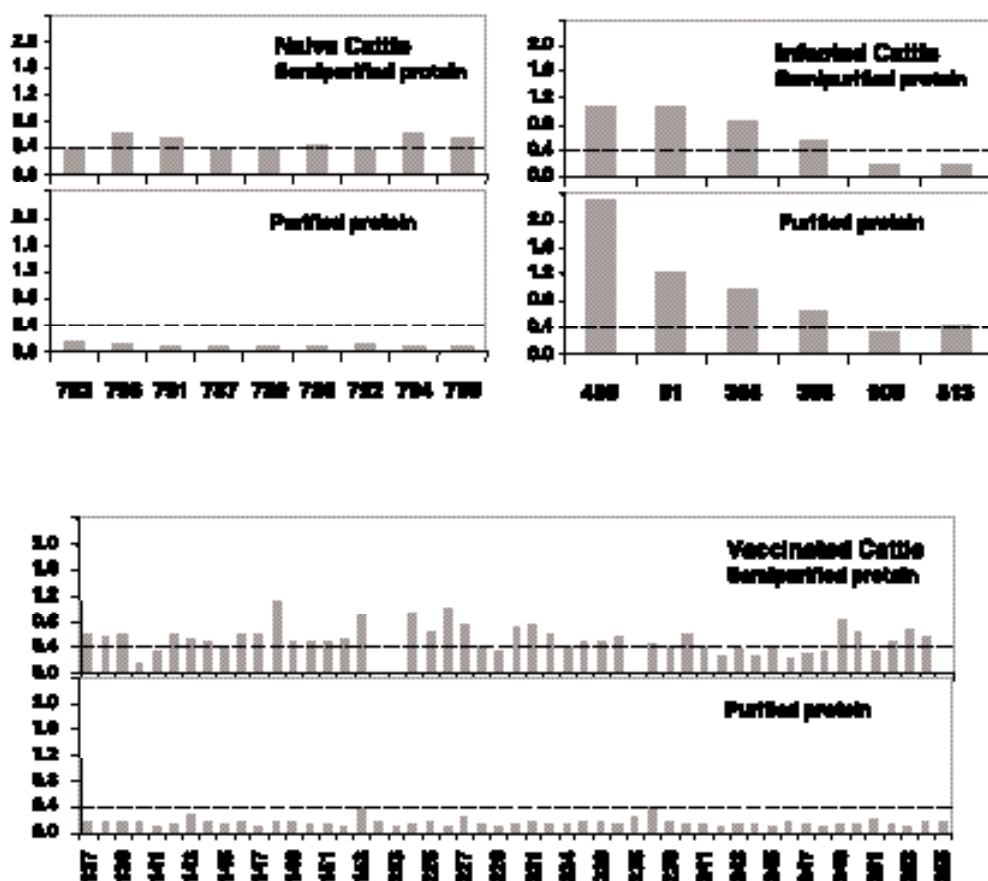
The potential use of measuring antibodies to FMDV nonstructural proteins to differentiate infection from vaccination has been adapted to several diagnostic techniques, including radioimmunoprecipitation, immunoblotting, and different ELISA formats. As the ELISA technique is the more suitable test for screening large numbers of sera, considerable effort has been focused on developing sensitive, specific, and reproducible measurement of antibodies against nonstructural proteins by this test.

The 3ABC virus-specific protein has been expressed in several heterologous systems (i.e., *Escherichia coli* and insect cells) and used for the development of ELISAs to discriminate vaccinated and infected animals serologically. These tests present specificity problems that make interpretation of results difficult, thus limiting their efficacy. It has been shown that in many cases the specificity

problems are associated with the presence in the sera samples of antibodies against expression vector antigens (proteins from *E. coli* or insect cells) that copurify with recombinant products.

In an attempt to circumvent the aforementioned specificity problems and develop a convenient, fast, and simple test, several approaches have been explored. De Diego et al. (1997) developed an indirect-trapping ELISA based on the capture of semipurified 3ABC recombinant protein by the monoclonal antibody 2C2. Mackay et al. (1998a) reported results obtained testing cattle sera by a monoclonal antibody indirect ELISA for the detection of bovine antibody of immunoglobulin G1 isotype specific for FMDV nonstructural proteins. The strategy followed by Sorensen et al. (1998) was based on the development of a competitive ELISA that measured the ability of test sera to block the binding of an immune serum from infected guinea pigs to the antigen labeled with biotin. In this system, the recombinant nonpurified protein was captured by specific guinea pig immunoglobulins.

Recently, promising results have been obtained at the CISA laboratory in Valdeolmos in a study that included experimental and field sera from noninfected, vaccinated, and infected animals of the primary FMDV hosts (cattle, pigs, and sheep) tested with a simple, indirect ELISA based on a purified recombinant 3ABC protein (Blanco et al. 2002a). As a part of this study, we analyzed field sera from sheep collected in Morocco during the 1999 FMD outbreak (Figure 6.5.3), pig sera from China and the Philippines, and cattle sera from Argentina (Figure 6.5.4). Modifying the purification protocol for the 3ABC protein enabled the development of a simple, rapid ELISA that did not require the use of monoclonal antibodies. Furthermore, the interpretation of the results was easier, clearer, and reduced the necessity of confirmation of results by immunoblotting assays. Therefore, this ELISA would be suitable to large numbers of samples and could be very useful in serological surveys.



6.5.4. ELISA response detected in naive, infected, or vaccinated cattle using either semipurified or purified 3ABC protein. As illustrated, greater protein purification improved the sensitivity and specificity of the ELISA, i.e., all naive and vaccinated cattle are under the cutoff whereas the infected animal signal increased using the purified protein. Infected cattle 305 and 513 represent sera collected 1 year after infection.

An ELISA based on a 3B long synthetic peptide has been described by Shen et al. (1999). This ELISA has the advantage of avoiding nonspecific reactions caused by antibodies against host cell-derived proteins (*E. coli* or insect cells). Despite this advantage, the test misses some infected animals that consistently react positively with 3ABC (Brocchi 2001).

The detection of antibodies to recombinant non-structural proteins by radioimmunoprecipitation (Berger et al. 1990) or immunoblotting (Bergmann et al. 1993) has been described for the identification of FMDV-infected animals, although the tests are not suited to routine diagnosis. Extensive field validation of immunoblotting test has been carried out in South America (Bergmann et al. 1998). The strategy followed consisted of the development and standardization of an immunoblotting assay capable of identifying serum antibodies against nonstructural proteins 3A, 3B, 2C, 3D, and 3ABC in a single test. In this test, a sample is considered reactive if all four antigens (3A, 3B, 3D, and 3ABC) have reactivity equal to or higher than the cutoff control (Mackay 2000). The immunoblotting test has comparable sensitivity to ELISA but higher specificity; thus it has been proposed as a confirmatory test for suspect or positive ELISA responses (Blanco et al. 2002b; Malirat et al. 1998).

CONCLUSIONS

The potential use in routine serological surveys of a test based on FMDV nonstructural proteins is of great interest, particularly because of the new and increasing risks of introduction of the infection in FMD-free areas, as have occurred recently in several parts of the world. During the last 2 years, several countries that were free of FMD for many years suffered dramatic outbreaks: Great Britain, Ireland, the Netherlands, and France in Europe; Japan, Korea, and Mongolia in Asia; and Argentina and Uruguay in South America. There is evidence that current surveillance tests have some deficiencies. The utilization of technology based on nonstructural proteins can assist in FMD diagnostics and improve the control of the disease.

Summarizing published data and the results reported in this chapter, the following conclusions can be drawn:

1. The detection of antibodies to polyprotein 3ABC or 3AB is the single most reliable indicator of FMDV infection.
2. In animals seropositive to structural proteins and/or to 3ABC/3AB, the detection of antibodies against one or more nonstructural proteins 2C, 3A, 3B, or 3D provides further confirmation of previous infection.
3. The assay (i.e., ELISA and/or immunoblotting based on 3ABC recombinant protein) can be

used on a herd basis to detect FMDV infection in vaccinated and unvaccinated populations.

4. The assay can be used to detect FMDV infection in animals with few or no clinical signs.

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6.6

Swine Vesicular Disease

Paul Kitching

SUMMARY

Although swine vesicular disease (SVD) is of little direct economic consequence to pig production, its clinical similarity to foot-and-mouth disease and its presence on the Office International des Epizooties List A ensures its prominence as a constraint to international trade in live pigs and pig products. Its known distribution includes Italy, Taiwan, and Hong Kong, but the existence of strains that cause only subclinical disease, the trade consequences of reporting its presence, and the reluctance of some countries to maintain a surveillance program make it likely that the virus has a far greater global distribution. Control of SVD is by slaughter of affected pigs and by disinfection, which is both expensive and difficult to carry out because of the resistance of the virus to inactivation. It is possible that SVD could be removed from List A, but, by reducing its importance, the pressure to control and eradicate it from affected countries is removed, resulting in the probability of its spread and its establishment in previously free areas. Should this happen, its effect on the control of foot-and-mouth disease, from which it is impossible to distinguish clinically, could be dramatic.

INTRODUCTION

Swine vesicular disease (SVD) is a contagious viral disease of pigs that is characterized by mild fever and vesicles on the feet and pressure points of the legs, snout, lips, and tongue. The clinical signs are more severe and obvious on pigs kept on concrete than on straw or in fields, and some strains of SVD virus (SVDV) fail to cause any clinical disease whatsoever. Recovery is usually rapid after a few days, and the disease is not associated with death. Human infection has been reported, and in one case caused meningitis, from which recovery was complete, but it is rare and has been identified only in laboratory personnel working with high concentrations of the virus. A complete review of current knowledge on the epidemiology and molecular characteristics of SVDV has recently been published (Lin and Kitching 2000), to which readers are referred. This chapter discusses aspects of SVD that relate to trade and only briefly summarizes

the epidemiology, molecular biology, diagnosis, and control of the virus.

EPIDEMIOLOGY

Swine vesicular disease was first diagnosed in Italy in 1966 and originally called *porcine enterovirus infection*. It was not recognized in Hong Kong until 1971, but, in retrospect, it had almost certainly been present there before spreading to Europe. The virus quickly moved into most of the other European countries and became the subject of an intense eradication campaign, which was successful for all western European countries by the early 1980s, except for Italy. In Italy, it persists in the south, occasionally spreading to the north and into other European countries. It was also reported in Poland, Romania, and Bulgaria, associated with trade with the Far East. It is likely present in mainland China, from where it periodically spreads into Taiwan and Hong Kong. Although most European Union countries maintain a constant surveillance program of variable intensity, other countries worldwide do not report the results of any seromonitoring that may be taking place and testing is restricted to pigs being moved for export.

The epidemiology of SVD is dominated by the extreme resistance of the virus to environmental inactivation. It is stable in the pH range 2.5 to 12.0, can withstand drying, and can remain viable in pig slurry for over a year. Spread is by ingestion of contaminated feed or, more usually, through abrasions on the skin and mucous membranes consequential to fighting or damage caused during transport. Vehicles can become contaminated with the virus unless properly cleaned with a detergent and a strong alkali such as 1.5% (wt/vol) sodium hydroxide. Aerosol transmission is not a feature of SVD, as it is for foot-and-mouth disease (FMD), and infection on a farm can remain restricted to single pens if the slurry does not move from one pen to another. Spread of SVDV into northern Europe from southern Italy, where the virus is still widespread, has been associated with the movement of contaminated vehicles. Outbreaks in the last decade—i.e., in Holland (1992 and 1994), Portugal (1995), Spain (1993), and Belgium (1992–1993)—have been linked to the movement of infected pigs and

contaminated vehicles, although it is not certain that Italy is the only reservoir of SVDV in Europe.

The incubation period for SVD is between 2 and 7 days, depending on the infecting dose and the strain of virus. Even before the development of clinical signs, the infected pig starts shedding virus from its nose and mouth and in its feces. This declines rapidly after 7 days as the antibody response eliminates the virus, but virus can still be detected in ruptured lesions on the legs and feet. There have been reports of the virus continuing to be shed after 14 days (Gourreau et al. 1975; Lin et al. 1998), but this is rare and thought to be of little epidemiological significance compared with the general persistence of the virus in the environment. Some pigs shed virus in their feces without developing a detectable antibody response, suggesting local gut infection without a systemic reaction; this may particularly be true for some of the milder strains of virus.

The meat of pigs slaughtered during the viremic phase of the disease will contain SVDV that can survive most of the processing procedures traditionally used to preserve pork products. Should any of these subsequently enter the pig food chain, for instance, in inadequately boiled swill, infection can reestablish itself.

MOLECULAR BIOLOGY

Swine vesicular disease virus is classified as a porcine variant of human coxsackievirus B5 (CVB5) and a member of the genus *Enterovirus* in the family Picornaviridae. The structural proteins of SVDV are genomically and antigenically very similar to those of CVB5, although the nonstructural proteins are no more like those of CVB5 than any of the other CVB serotypes, leading to the hypothesis that the SVDV was derived from a recombination event between the human virus and an as-yet-unidentified pig virus. It has been further speculated that the conditions frequently found on farms in mainland China, where livestock and their human owners live in close association, would predispose to such an event. Considerable research has been carried out to identify the genomic differences between the virulent and non-virulent strains of SVDV and, by exchanging individual amino acid sequences between them, those changes associated with virulence have been identified, at least in those strains chosen (Lin and Kitching 2000).

Individual strains of SVDV can be identified by sequencing the nucleotides in the VP1 gene or the 3BC gene. Because the virus has a relatively high mutation rate (frequently seen in RNA viruses such as SVDV), as strains separate in space and time, more sequence differences accumulate. By comparing the sequences of strains in a database held at the Institute for Animal Health (Pirbright Laboratory) at Pirbright, England, the possible origin of new outbreaks can be identified. In this way, the close association between Italian strains of virus and

those causing outbreaks in other parts of Europe during the last 10 years was identified. Different groups of virus are circulating in Hong Kong and Taiwan, but sequencing results indicate that the virus was introduced into Europe more than once. The reappearance in Italy and Holland in 1994 of a strain found in Romania in 1987 suggested that the virus may be persisting in some Eastern European countries.

DIAGNOSIS

Swine vesicular disease is often first suspected from serological evidence. Recent strains of the virus have frequently failed to cause fever in affected pigs, and the milder clinical signs are often missed. However, serological surveillance for SVD is complicated by the presence of *singleton reactors*. These are individual pigs that are serologically positive, but that have shown no clinical signs and for which there is neither a history of the disease on the holding nor contact with a known outbreak. There are two serology tests in common use: the monoclonal antibody competition, enzyme-linked immunosorbent assay (MAC-ELISA) and the virus neutralization test (VNT). The MAC-ELISA detects singleton reactors at approximately 0.45%, whereas the VNT detects them at 0.2%. The cause of these reactors is not known, but it is suspected that they may have been infected with the human CBV5, which is considered not to spread between pigs, although no work has been carried out using recent strains of CBV5. Singleton reactors are characterized by the following:

- They have a low VNT titer.
- They are negative for SVDV in feces.
- There is only one present per herd.
- Pen mates do not seroconvert.
- Most are negative when re-bled 20 days later.
- Immunoglobulin M is responsible for the positive result.
- Western immunoblotting of the serum produces inconsistent results.

Swine vesicular disease virus will grow well on pig kidney cells, but not on cells of bovine or ovine origin frequently used to isolate FMD virus (FMDV). The virus can be isolated from blood during the viremic phase of the disease, from lesion material, from feces collected directly from the suspect animal, or from swabs used to sample material from a market or vehicle as part of a surveillance program. Procedures for using polymerase chain reaction have been well documented (Callens and De Clercq 1999; Lin et al. 1997).

It is essential when considering a diagnosis of SVD that the samples collected are also examined for the presence of FMDV, and that the more stringent requirements for the collection and preservation of the FMDV

are followed. In particular, the collecting media must be well buffered to maintain the specimens at pH 7.2 to 7.4, because FMDV, unlike SVDV, is extremely sensitive to pH.

CONTROL

The necessity to control SVD is linked to the indistinguishable clinical signs from those of FMD and the presence of SVD on the Office International des Epizooties (OIE) List A, which automatically gives the disease and the virus causing it considerable significance in international trade. No matter that the strain present may not actually produce clinical signs, the presence of antibody-positive animals is sufficient to prevent trade. A consequence of the economic penalties of having the virus and/or disease present is that reporting its presence can become a political decision. There are recent examples where the disease has been in the national pig herd, but because of the effect that acknowledging this would have had on trade, its existence was ignored and, when FMD was introduced, there was a delay in the response because it was originally thought to be SVD.

The control of SVD is frustrated by the ability of the virus to persist on infected farms and the cost of totally decontaminating an infected premise or vehicle. In southern Italy, the last remaining area of the European Union where the virus remains, the inability to control movement of pigs between the very large number of small holdings, often involving dealers, probably accounts for its survival. The situation in Eastern Europe is not clear, but few countries in that region have the resources to embark on a slaughter program. Similarly, it is unlikely to be eliminated from eastern Asia.

One option being promoted by some countries is to remove SVD from List A. This would certainly reduce its profile, and those in the Americas with similar feelings toward vesicular stomatitis (VS) would support a reciprocal arrangement by which both diseases would be removed together. Certainly, this would relieve some of the pressure to commit significant resources to a SVD (or VS) control program, but the consequences should be considered. In the European Union, for example, after many years and a considerable amount of money, SVD is restricted to southern Italy. If the current level of control were removed or reduced, would the virus spread back into Europe? Of course, merely removing SVD from List A does not mean that European trading partners would also ignore its presence; the OIE only produces guidelines for bilateral trade agreements. But if SVDV became widespread and, bearing in mind its often mild nature, would trade eventually learn to live with it?

The original reason for putting SVD (and VS) on List A was their clinical similarity to FMD, which all nations

agree is a serious disease. SVD is now a less virulent disease than before, and it could be argued that it is, therefore, less easy to confuse with FMD. However, while it can change its virulence profile one way, it can also potentially do the reverse. But if it did so when the virus had become widespread, its eventual control would be too expensive to contemplate. If it did continue to produce FMD-like disease in pig populations around the world, what effect would this likely have on FMD control? Many developing countries do not have their own FMD diagnostic facility and rely on sending material to the regional or world reference centers for diagnosis. Sending pathological material by airfreight is expensive and time consuming, and even for the few FMD samples submitted, this can exceed a limited budget. If SVD were also present, expanding the number of potential submissions, many FMD outbreaks probably would be missed, particularly since there are now some very pig-specific strains of FMDV circulating in Southeast Asia, making it additionally difficult to distinguish FMD from SVD.

Even developing countries with their own FMD diagnostic capabilities have difficulty in maintaining surveillance for FMD, and the presence of SVD would introduce further complications. It is, however, possible that SVD is already present in some of these countries and diagnosis is only used to confirm the presence of FMD and not look for any differential diagnoses. But how would a country in Europe or the Americas that has extensive trade commitments respond to increasing numbers of suspect FMD cases due to the presence of SVDV in the national pig herd? Each time a suspect was identified, the herd would have to be quarantined until shown to be negative for FMD; this could take a week on each occasion and would have financial consequences for the farmer involved. It would not take long for farmers to anticipate that the vesicular disease in their pigs was SVD and, to avoid the restrictions consequent to a disease investigation, nothing would be reported to the veterinary authorities. This situation would continue until FMD did eventually arrive in the country.

It is possible that the option to tolerate SVD could be reassessed when rapid and reliable pen-side diagnostic kits are readily available to distinguish SVD from FMD on a suspect farm, but not yet.

CONCLUSIONS

SVD continues to cause problems in differential diagnosis from FMD, and although the strains now circulating appear less virulent than those previously seen, it is my opinion that present control programs should be maintained to eliminate the virus from Europe and identify it in any country involved in regular trade in live pigs or pig meat.

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7 PORCINE HERPESVIRUSES

7.1

Pseudorabies: A Century of Learning

George W. Beran

SUMMARY

Pseudorabies (Aujeszky's disease) was first described 100 years ago. The century since Aladar Aujeszky made his initial observations has been one of learning. Ultimately, this process of learning has led us to understand the means by which this disease could be controlled. The latter part of the 20th century has seen us apply this learning effectively to the control and eradication of pseudorabies in vast areas of the swine-producing world. In many ways, this effort has served as a model for the correct use of basic and applied science.

1900–1910

Pseudorabies was first described as an infectious disease in cattle, cats, and dogs in Hungary in 1902 by Aladar Aujeszky (Aujeszky 1902); thus the name, Aujeszky's disease. Aujeszky learned much about the infectious agent by experimental studies in rabbits, guinea pigs, and mice and discerned that it was transmitted by direct or airborne contact.

THE 1910s

The viral etiology of pseudorabies was identified by passage through bacteria-holding filters.

THE 1930s

Richard F. Shope established in 1931 that the virus causing "mad itch" in cattle in Iowa, and which had been clinically described in Ohio as early as 1813, was immunologically identical to the virus causing pseudorabies in Hungary (Shope 1931). He learned much about the pathogenesis of the infection in swine: It occurred as an acute clinical disease in baby pigs, but all recovered swine, including older animals in which infections were mild or inapparent, remained as carriers. Transmission among swine was recognized by aerosol or through milk, and from carrier swine to cattle by traumatic contact or, hypothetically, by rats as vectors.

THE 1940s

Ray, and McNutt and Packer (R. A. Packer, personal communication), described two outbreaks of pseudorabies in baby pigs, mortality of 84 of 190 and 105 of 176 pigs under 2 weeks of age. Weaned pigs and sows in contact or proximity to the dying baby pigs remained clinically normal. Pseudorabies antibodies were demonstrated to be widespread in normal swine and in anti-hog-cholera serum.

THE 1950s

Reports were frequent in the European literature, especially from Russia and the Balkan countries, of a fatal disease in young weaned pigs and clinical illness with some deaths among older swine.

THE 1960s (ACTUALLY, BEGINNING IN 1958)

I unexpectedly learned about the pseudorabies virus in 1958 when I was in graduate school, doing my graduate studies on enteroviruses at the Missouri Welfare Farm in Kansas City, where about 1400 pigs were marketed annually. The immunization practice at the farm was to give modified-live hog cholera vaccine and antiserum to piglets at 5 to 7 weeks of age and a booster dose of the vaccine without antiserum at 3 months of age, at which time they were moved to the feedlots, where they were fed out on cooked municipal garbage. During March of that year, two groups of 215 and 200 grower pigs, respectively, were moved to the feedlots within 3 weeks. Five days after arrival of the second group, acute central nervous system illness, principally with flaccid paralysis, coma, and death, appeared and, during the next 17 days, 159 of the pigs in the two pens had died. Experts were called in, in various ways involving the US Department of Agriculture (USDA) Hog Cholera Research Station, the US Army Biological Warfare Laboratory, the Walter Reed Army Institute of Research, Virginia Polytechnic Institute, Jensen-Salsbery Laboratories, Affiliated Laboratories Corporation, and Dr. Shope of the Rockefeller Institute for Medical Research. Two viruses were

identified in the same young pigs in the outbreak. At the USDA Hog Cholera Research Station in Ames, Iowa, a hog cholera virus was recovered from blood and in experimentally inoculated pigs that produced, not the acute encephalitic disease of the outbreak, but classical hog cholera. In the small laboratory in which I was working, pseudorabies virus was recovered from the brains. Experimentally, it reproduced a clinical disease indistinguishable from that of the outbreak in young pigs. The best expert conclusions were that the outbreak had been caused by synergistic infections by two viruses, one of which was present in a carrier or chronic state and the other of which was introduced into the pigs and spread rapidly through the two pens. At least 20 pigs affected in the farm outbreak recovered, some additional pigs acted sluggish for a few days, and none of the other swine in the feedlots developed the clinical syndrome. It would be about three decades before we would gain a better understanding of synergistic infections involving pseudorabies virus in swine.

THE 1960s

In 1961 and 1962, pseudorabies epidemics began to be reported in herds of swine in Indiana that were clinically and pathologically different from the occasional outbreaks previously observed in this country. Similar reports of acute pseudorabies outbreaks in Illinois and other Midwestern states followed in the late 1960s, as well as reports from Europe. Outbreaks were characterized by rapid intraherd spread and severe losses among suckling pigs, clinical illness with sequelae in grower pigs, reproductive disease in gilts and sows, and lesions observed at necropsy, particularly herpetic yellow-white foci of necrosis scattered through the spleen and liver. This picture differed dramatically from our previous concept of pseudorabies as an endemic, subclinical infection.

THE 1970s

The new pseudorabies became an epidemic in the concentrated swine-raising areas of the United States during the early 1970s, and we learned a lot about the epidemiology of the infectious disease in swine. We puzzled over whether the new pseudorabies represented an introduction of more virulent strains, presumably from Europe, whether mutations were taking place in the pseudorabies strains endemic in the United States, or whether our new confinement hog-raising systems were changing the susceptibility of our swine. Strain differences in pseudorabies viruses were being recognized, both in pathogenesis and in virulence. A general view prevailed that more virulent strains had been introduced into the United States, possibly through importation of boar semen or by inadvertent human transport. This is still a legitimate but unprovable conclusion.

All hypotheses are considered today to be components of actuality. The hosts, the etiologic agents, and the environment all play their roles in the epidemiology of disease. In the past, slow transmission of the virus through dispersed outdoor herds led to low-exposure doses of environmentally attenuated viruses with inapparent and incomplete herd infections. However, this management style was disappearing rapidly. Now, in large, concentrated swine populations occupying enclosed environments, infected swine excreted high quantities of virus that moved rapidly along airflow patterns and heavily contaminated the environments in both farrowing and breeding units, changing the strain selection pressure to the more rapidly infecting and excreted viruses. Anti-hog-cholera serum was no longer in use. Unintentionally but commonly containing anti-pseudorabies antibodies, the use of anti-hog-cholera serum had inadvertently provided a level of passive protection to young pigs. Now, rapid dispersal of strains occurred by swine movement between herds, the stress that accompanied movement inducing shedding by swine that were inapparent carriers of the virus (Davies and Beran 1980). This came to be recognized as a new source of exposure and one that fostered clinically severe disease compared with earlier decades (Beran 1991; Davies and Beran 1981).

The spread of pseudorabies in swine-raising areas in the early 1970s prior to the use of widespread vaccination provided opportunities, however undesirable, to learn much about the disease. Serological surveys on market hogs identified about a 0.5% prevalence of antibodies against pseudorabies. The first clinical appearance of pseudorabies in a herd was frequently rough-haired, listless, neonatal pigs less than 3 weeks old that stopped nursing, developed central nervous systems signs, and died in 24 to 36 hours, with mortality rates of 90% or higher. In other herds, the first clinical appearance was in the breeding herd, with gestating sows and gilts aborting or farrowing stillborn or weak pigs that often died within 1 or 2 days. Respiratory disease, listlessness, and going off feed for 3 or more days often accompanied the reproductive failures or were the only clinical signs observed. In open breeding stock, failure to conceive, or in early gestation, resorption of fetuses and return to estrus, were seen. During farm outbreaks, weaned pigs frequently went through clinical disease with listlessness, anorexia, and rhinitis with dyspnea and severe cough, with full recovery within 1 week or, in those that developed neurological signs, convalescence and sequelae. In grower-finisher swine, depression, anorexia, and mild-to-severe respiratory disease with weight loss, but with rapid recovery, frequently occurred. In herds harboring clinically inapparent *Actinobacillus pleuropneumoniae* or *Pasteurella multocida*, infection with pseudorabies virus in pigs from weaning age to breeding stock could result in exacerbated or

synergistic clinical pleuropneumonia or pasteurellosis, hearkening back to a synergistic pseudorabies-hog cholera outbreak recognized two decades earlier (Iglesias et al. 1992).

Very important information was gained during this decade regarding the excretion of pseudorabies virus by infected swine. The incubation period was commonly 2 to 5 days, with nasal and oral excretion and, in adult swine, with vaginal, preputial, and/or milk secretion coincident or just preceding primary symptoms. Lifelong latent infection commonly followed clinical recovery or inapparent infection in trigeminal ganglia and tonsils. Recrudescence shedding by latently infected swine followed stress of subsequent disease, farrowing, crowding or commingling with unfamiliar animals, or transport. Shedding in primary infection persisted for 1 to 3 weeks and in recrudescence for 3 to 4 days. Long-term or recrudescence shedding was a common source of viral transmission into previously uninfected herds or portions of herds (Cheung 1995; Wheeler and Osorio 1991).

Entry of pseudorabies virus into susceptible swine was commonly via nasal mucosa by inhaled virus or via tonsillar or oral/digestive tract mucosa following oral exposure. Virus-contaminated semen could infect gilts and sows during breeding; virus was not transmitted to embryos during early gestation, but fetuses could be infected in utero, with outcomes dependent on the stage of gestation. Area serosurveys of market hogs estimated the prevalence of pseudorabies at 5.8% in 1977 to 1978 and 13% in 1981 to 1982.

Whole virion attenuated live and inactivated pseudorabies vaccines for swine began to come into wide application in the 1970s. Vaccinated swine resisted higher doses of virus than unvaccinated swine exposed to the virus. They were protected against clinical disease and, if infected, did not transmit the virus transplacentally and shed less virus over a shorter time. However, infected vaccinated swine still developed latent infections, still recrudescence, and still shed virulent virus. Nonetheless, the epidemiology of pseudorabies was changed by vaccination: viral levels were lowered in the air and on fomites in infected production units, both intra- and interherd transmission was reduced, and total infected-herd losses were greatly reduced. Recognition of infected herds or individual swine by clinical histories was masked by vaccination, and serological case findings became less certain (Mengeling et al. 1992; Van Oirschot et al. 1991).

THE 1980s

Studies on the role of species other than swine in the epidemiology of pseudorabies continued into the 1980s and confirmed swine as the reservoir host. Feral swine were determined to be competent alternative hosts in certain environments, but through most of their range entered into transmission cycles without perpetually maintain-

ing the infection. All other susceptible species were found to be aberrant hosts. Cattle in direct contact with infected swine or with access to exhaust-fan airflow from confinement swine units in cold weather sometimes succumbed to mad itch in the former and encephalitic disease in the latter situation. All cases of pseudorabies in cattle were rapidly fatal. Sheep were determined to be highly susceptible to pseudorabies virus by oral or inhalation exposure and, in contact with active or reactivated infected swine, acted as inadvertent sentinels and exhibited rapidly fatal infections. Cats were shown to be highly susceptible; dogs, raccoons, and skunks moderately susceptible; and rats and mice moderately resistant to infection. Incubation periods were found to be typically less than 3 days. Infection produced encephalitis followed by death in 2 to 3 days. In dogs, pruritus also developed. Exposure of these animals was determined to be by scavenging swine carcasses, inhalation of aerosolized virus, and ingestion of contaminated feed or water. Dogs may drag carcasses of infected swine from one production site to another, susceptible swine may eat carcasses of any of these animals, and rodents may unknowingly be milled into swine feed or transferred in bedding from an infected farm. Surveys of trapped wild animals in areas of infected swine revealed no evidence of the maintenance of pseudorabies in raccoons, skunks, or opossums. Birds and insects were not shown to enter transmission cycles, although houseflies experimentally fed pseudorabies virus were found to have retained viable virus in the gut with a half-life of 3 hours at ambient temperatures, and virus-contaminated flies have occasionally transmitted pseudorabies through experimental corneal contact to swine (Vanderleek et al. 1993; Zimmerman et al. 1989).

Studies of inter- and intraherd transmission of pseudorabies, other than by animal contact, centered on air, water, and contaminated fomites. Airborne movement of pseudorabies virus was determined to be the major vehicle of short-distance transmission within and between production units or from market transport and holding units. During major atmospheric events, virus could be moved many kilometers. Despite the rapid inactivation of pseudorabies virus by sunlight or drying, or dispersal of virus suspensions, droplet nuclei were shown to transport infectious virus over both time and distance.

Environmental studies found the virus to be quite unstable at a pH below 4.3 or above 9.7 and at temperatures that fluctuated around freezing. Experimentally, pseudorabies virus suspended in porcine saliva remained infectious for less than 1 day on denim cloth, on alfalfa hay, and in pit effluent; 2 days on rubber, on green grass, in meat and bone meal, on sawdust bedding, in chlorinated water, and in anaerobic lagoon effluent; and 3 to 7 days on plastic, steel, concrete, shelled corn, pelleted hog feed, straw bedding, and in well water at ambient

temperature (Christiansen et al. 1993; Davies and Beran 1981; Schoenbaum et al. 1990, 1993).

Pseudorabies elimination studies in five states between 1983 and 1986 enhanced our knowledge of the epidemiology of pseudorabies and led to the development of effective approaches to eradication. In Marshall County, Iowa, 213 swine herds were tested serologically in a program of on-farm evaluation. Sample sizes were set to detect infected swine at 95% probability if 10% or more of the animals from weaning age to breeding stock were seropositive. In vaccinated herds, serum-neutralizing antibody titers at 1:16 cutoff levels were used on a herd basis to differentiate vaccinated uninfected herds from vaccinated infected herds. In the initial serosurvey, 14.3% of herds (30 herds) and 17.2% of swine were identified as infected. Quarterly retesting of each farm with comparison of the previous results was demonstrated to be effective in case finding. During the 18-month program, 15 additional herds were identified as infected. The testing cost per infected herd identified was \$642. Herd cleanup plans were individually developed for each infected herd based on four basic approaches: (1) depopulation and repopulation (10 herds, 90% successful), (2) depopulation without repopulation (3 herds, 100% successful), (3) test and removal (4 herds, 100% successful), and (4) offspring segregation (28 herds, 71% successful). Although eradication of pseudorabies was not fully achieved in 18 months, the program demonstrated that elimination of the infection in the Iowa setting was possible using an epidemiologically valid approach (Thawley et al. 1982).

The 1980s were also marked by major advancements in pseudorabies vaccine and in the development of serological assays. The genetically engineered vaccines were based on deletion of the thymidine kinase virulence gene for safety and the deletion of nonessential glycoprotein genes, including gI, gIII, gX, and gp63, to provide immunological markers to differentiate vaccinated uninfected swine from vaccinated infected swine in serological tests. Sensitive, reliable serum-neutralization and latex-agglutination tests and enzyme-linked immunosorbent assays were developed and put into use (White et al. 1996).

At the end of the decade, the national pseudorabies eradication program was inaugurated with the goal of eradicating pseudorabies by the end of the century. The anticipated budget was balanced with industry, state, and federal inputs. A five-stage program was developed and implemented on a state-by-state basis: (1) stage I, preparation, (2) stage II, control, (3) stage III, mandatory herd cleanup, (4) stage IV, surveillance, and (5) stage V, free status.

THE 1990s

Major progress toward achievement of eradication of pseudorabies from the United States characterized the

1990s. As of the end of 1990, a total of 10 states were in stage I, 21 in stage II, and 11 in stages III and IV, with no states in stage V because the program had not been in effect long enough to meet requirement of "one-year with no cases" mandated for stage V status.

The program has been built on, and functions based on, the epidemiological knowledge of the infection gained through the past decades. It has included a few painful realizations of the areas of epidemiology that have not yet been fully explored. Eradication has not been achieved on the target schedule, but it is close. There are epidemiological lessons for our future that must not be put aside.

Although their use is generally prohibited in the last stages of eradication in the United States, the vaccines in use are all gE, still popularly called *gI gene deleted*. Used on a whole-herd basis with quarterly revaccination of breeding stock and single vaccination of grower pigs at 8 to 12 weeks of age, effective resistance is stimulated to levels of exposure that may occur from clinically inapparent or recrudescing shedder swine. Given intranasally to pigs 3 days to 3 weeks old, the current vaccines provide a measure of prevention against latency by wild-type virus (Van Oirschot et al. 1991).

Down the road, serotesting, circle testing within a 3-mile radius of identified infected premises, and identification and testing of separate feedlots in multisite production systems will continue to be used aggressively. Slaughter surveillance with aggressive traceback and herd testing is of major importance and will be the predominant method of surveillance in the future. Individual unique swine identification, so essential for traceback, is technologically achievable, but still not adequately implemented. The surveillance index is still set at 0.08, based on 10% sampling of breeding stock, with 80% of the positives being successfully traced to the herds of origin. We need to raise the sampling level of breeding stock to 20%, with 95% successful traceback. New surveillance tools, including a "meat juice" (muscle exudate) ELISA for slaughter surveillance, are currently being tested for sensitivity and specificity.

A resurgence in herd outbreaks occurred in a number of locations during the late 1990s and 2000. The outbreaks in 1999 and 2000 were associated with a strong downturn in the hog market, during which time market prices for live animals dropped from over \$40 per hundred pounds to as low as \$8.00 per hundred pounds. Many producers stopped vaccination in order to avoid the expense, but herd immunity against pseudorabies quickly deteriorated.

In Iowa, the number of independent hog producers quickly dropped from about 32,000 to about 20,000. Many of the herds that ceased swine production were actually those infected with pseudorabies. An emergency federal program, the Accelerated Pseudorabies Eradication Program, allocated \$40 million to buy out

entire infected herds on a voluntary basis, depopulating the herds through cooperating slaughter plants, with the carcasses going into rendered products for animal feed. Nationally, approximately 5% of pseudorabies-infected herds were depopulated. In Iowa, approximately 75% of the infected herds (about 9 million pounds of pork) were removed in 3 months.

2000–2010

As of April 1, 2001, no states remain in stage I, one state remains divided in stages II and III, three states are in stage 3, five states are divided in stages III and IV, three states are in stage IV, and 40 states are in stage V. As the eradication of pseudorabies nears achievement, the epidemiological controls that are achieving area elimination must not be relaxed. Intensive vaccination must be continued where active foci of infection or the imminent risk of entry of pseudorabies virus remains. Movement of swine, entry controls, and market controls must remain operational and monitored. If infected farms are identified, it is critical that depopulation or removal of positive animals be carried out quickly, within 10 days of collection of the blood samples that revealed the infection.

Surveillance is of critical importance and will remain of critical importance into the future. In area herd or lot testing, 95% probability of detecting infection if 10% of individual herds or lots (25 to 29 samples) have positive swine by the best tests has been effective. In slaughter-sampling surveillance, 95% probability of detecting infection if 20% of animals by lots are seropositive is adequate. In traceback herd testing, 95% probability of detecting positive animals at the 2% level is needed (78 to 149 samples). Where single reactors are recorded in herd tests, confirmatory tests or repetitive testing must be carried out. The surveillance index needs to be brought to 0.19 based on 95% successful traceback of positives in samples of 20% of breeding animals; the goal must become 100% traceback capability. The meat-juice ELISA on exudate from meat samples collected at slaughter is currently in a pilot study for detection of seropositive swine (Nielsen et al. 1995).

Surveillance of feral swine populations must be continued. Extensive serological studies in feral swine have demonstrated that they are reservoirs for pseudorabies virus and, as feral swine populations spread in the southern United States, the numbers of infected pigs are increasing. The few isolations of pseudorabies virus from feral swine have appeared to be highly adapted strains, and transmission of infections by these strains from feral to domestic swine has not been conclusively demonstrated. The epidemiological role of feral swine in pseudorabies must be continually studied and prompt response taken if transmission from domestic to feral and back to domestic swine, or primary transmission from feral to domestic swine, is identified (Muller et al. 2000).

BEYOND 2010

The pseudorabies program must not be terminated, and funding for its continuation must not be reduced below a level sufficient to sustain surveillance and emergency response. The conquest of pseudorabies has required so much valid research, so much strong scientific development, so many dedicated competent personnel, and so many meetings that brought together colleagues in production, government service, and academia in coordinated effort. We must ensure that success will be exactly 100% probability of 100% eradication for 100% of the future.

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7.2

Epidemiological Pattern of Aujeszky's Disease in a Hyperendemic Area of Mexico

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SUMMARY

Since the appearance of Aujeszky's disease (AD) in Mexico, the virus has spread to most pig-raising areas in the country. Initially, AD caused economic losses in herds due to mortality and reproductive problems, but now the clinical signs of AD are rarely noticed, even though serological surveys showed that 60.7% of farms had infected animals, with a seroprevalence in sows of more than 30% in 87.3% of the farms surveyed. Within herds, the rate of sow infection was directly related to the rate of infection of fatteners, which constitutes the amplification phase of the virus. Inactivated vaccines were used to control the disease, but when six commercial vaccines were tested for potency, only three induced antibodies in vaccinated pigs. Therefore, the animal health authorities began testing all the vaccine lots prior to sale. Because clinical AD was rarely noted, swine producers, veterinarians, and animal health authorities have paid little attention to the problem. This has allowed AD to become endemic in the swine population in Mexico.

INTRODUCTION

Aujeszky's disease (AD) has been recognized in Mexico since it was first clinically identified in bovines in 1945 (Bachtold 1945). The virus was first isolated in 1970 from an outbreak in cattle (Martell et al. 1971).

The first cases of AD in swine were reported in 1969, with the first outbreaks occurring in central Mexico: La Piedad Michoacán, the Bajío area, and in Los Altos in the state of Jalisco. These areas had the highest pig population density of the country at that time. Although it was never confirmed, those first outbreaks were believed to be associated with pig imports. Once introduced, Aujeszky's disease virus (ADV) spread easily among the highly susceptible swine population. The epidemiological pattern of the outbreaks was of a neurological disease with high morbidity and mortality of piglets and reproductive disturbances in sows (Aguirre and Garza 1980; Martell 1985). Since then, the disease has spread to almost all swine-producing areas in the country. Lately, there has been a significant reduction in the number of clinical cases, probably due to the presence of less path-

ogenic, but more infectious, ADV strains that have spread without being detected (McCullough and Todd 1988).

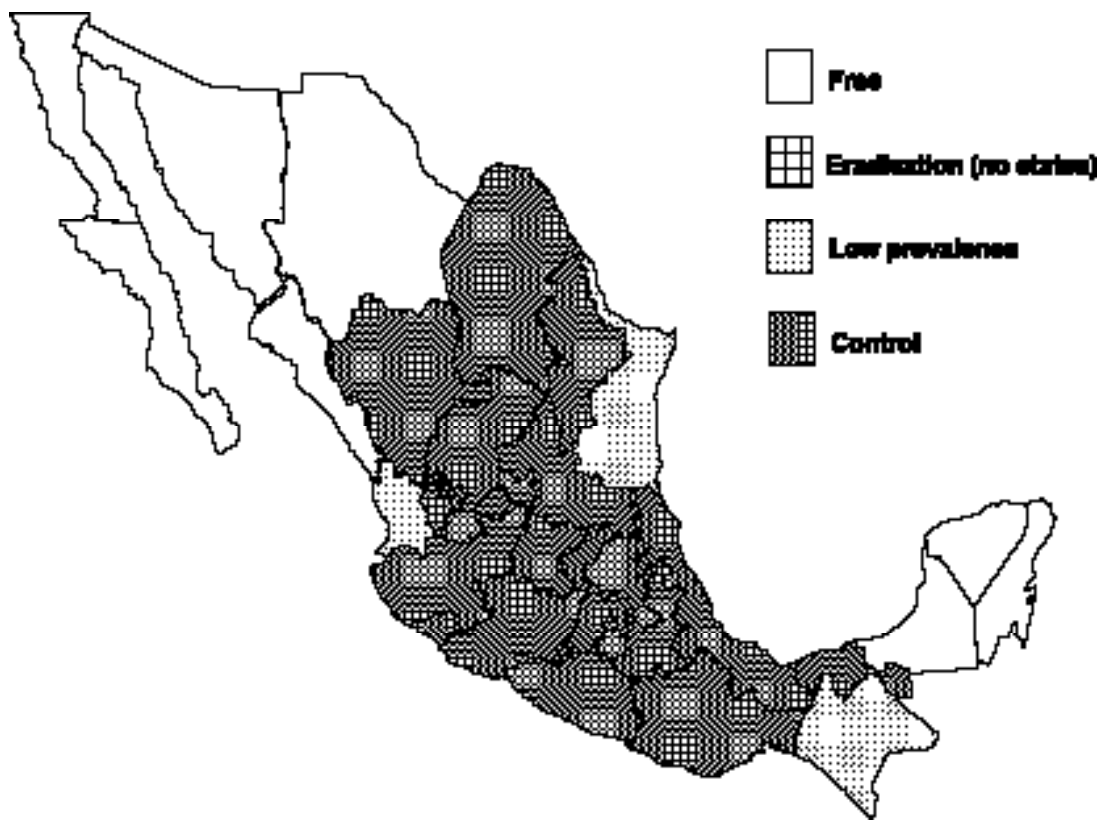
During the 1980s, AD became a significant problem—to the extent that AD and classical swine fever (CSF) became the two most important viral diseases of swine. In response, the animal health authorities established a control and eradication campaign based on serological diagnosis, elimination of infected animals, quarantine of affected premises, control of animal movement, and vaccination. The campaign included four stages. *Control*, the initial stage, applies to areas where AD is present and the prevalence of infected herds is greater than 15%. This stage includes most of the central part of the country.

Low prevalence is where the prevalence of AD-infected herds is lower than 15% in an infected area. At present, it includes only the states of Nayarit, Tamaulipas, and Chiapas. *Eradication* applies to the successful elimination of ADV from an area where no cases or serological reactors were detected during the previous year. At present, no states are in this stage. *Free* can only be attained after remaining in stage 3 with no infected herds for 1 year. The state of Yucatán, the peninsula of Baja California, and the states of Sonora, Chihuahua, and Sinaloa have attained free status (see Figure 7.2.1).

This chapter describes the epidemiological situation of AD in the hyperendemically infected area of Mexico, characterized by the infection of most premises, a high rate of seropositivity, and the absence of clinical signs. AD in Mexico can help us understand how a disease may become hyperendemic in a geographical area due to the interplay of several factors—pig producers, veterinary practitioners, and official sanitary authorities—and a lack of concern for controlling the disease.

SEROEPIDEMIOLOGY

The first serological studies (from 1982 to 1984) showed that AD was present in 12 of the 19 states surveyed. For this seroepidemiological survey, nearly 3000 serum samples from 34 farms were tested by the microimmunodiffusion test (MIDT). The results indicated a seroprevalence of 20%, with 31% of herds having



7.2.1. Aujeszky's disease (*pseudorabies*) in Mexico.

reactors (Mercado et al. 1992; Solorzano and Mercado 1985). The results of this survey may have overestimated the prevalence because the MIDT could not discriminate antibodies induced by vaccines from those resulting from field infection. Nevertheless, the results suggested that AD was widespread throughout the country.

After the advent of gene-deleted vaccines and differential serological assays, it became possible to distinguish animals immunized with the marker vaccines from those infected with field strains of virus (Van Oirschot et al. 1986). Since then, serological surveys have been done in most of the principal swine-producing areas. Several surveys were carried out in farms located in the states of México, Jalisco, Michoacán, Querétaro, and Puebla (Calderón 1995; Castro et al. 2000a; Diosdado et al. 1996; Rodríguez et al. 1993). The results of the studies were similar.

Castro et al. (2000a) took serum samples from 30 sows and 30 four- to six-month-old pigs from each of 260 herds. With this sampling design, it was possible to detect at least one infected pig with 95% of confidence when the real prevalence of AD was over 10% (Morrison and Thawley 1989). The serum samples

were tested using a blocking enzyme-linked immunosorbent assay (ELISA) gE or ELISA screen (IDEXX Laboratories), and it was found that infected pigs were present on 158 (60.7%) of the 260 farms surveyed. Infected farms were classified on the level of infection in sows. In 20 (12.7%) of 158 herds, seroprevalence in sows ranged from 1% to 30%; in 32 (20.2%) of 158 herds, sow prevalence was 31% to 70%; and, in 106 (67.1%) of 158 farms, the range was 71% to 100%. Furthermore, in 67.7% of the infected herds, ADV also circulated in fattening pigs. When the seropositive rate in sows was less than 20%, 0.5% of the fatteners had antibodies; when 21% to 50%, 33% of the fatteners were infected; and, when 51% to 100%, 79.4% of the fatteners were seropositive. Therefore, there was a direct relationship between the infection rate in breeding stock and fattening pigs.

From these serological studies, it was concluded that AD was widely distributed in the central area of Mexico, that wild ADV was circulating among herds, and that the in-herd prevalence was high. The high number of infected farms in this area could be attributed to the frequent introduction of infected animals to the farms, the lack of

preventive measures and biosecurity in many farms, and the proximity and density of neighboring swine herds.

Serological profiling has made it possible to determine how the ADV circulated among these herds. It was observed that, in those farms where sow seroprevalence rate was below 20%, ADV did not circulate among the growers and fatteners. However, as the prevalence rate of the sows increased, ADV was passed to their litters and amplified during the growing and fattening period, where most of the pigs become infected (Morilla and Rosales 1995). Also, by means of serological profiles, it was determined that maternal antibodies in piglets lasted from 2 to 3 months and, thereafter, susceptible pigs appeared that were easily infected by ADV. Usually, ADV infection in growers appeared as a respiratory disease, giving way to other secondary pathogens—like *Actinobacillus pleuropneumoniae*, *Mycoplasma*, porcine reproductive and respiratory syndrome virus, swine influenza, and/or blue eye disease virus—that increased the severity of the disease.

VACCINATION

The main control measure used in herds was test and removal, when possible, and vaccination with inactivated gE-deleted vaccines. Of the surveyed farms, 60% used vaccine, but only in breeding stock and replacement animals (Castro et al. 2000a). Presumably, this rate of vaccine coverage was sufficient only to prevent clinical disease; it could not interrupt or prevent the viral infection of pigs. It is well known that the inactivated ADV vaccines cannot prevent viral latency as the live vaccines can (Pensaert et al. 1990; Stegeman et al. 1996). In this respect, when six commercial inactivated ADV vaccines were evaluated for their immunogenicity, one dose with the vaccines prepared with Phylaxia and Bucharest, and one of the Bartha strains, elicited an antibody response that increased with the second dose. However, three other vaccines prepared with the Bartha strain of ADV failed to induce antibodies either with one or two doses. Thus, not all the vaccines commercially available in Mexico induced an antibody response, presumably due to the lack of antigenic mass or inadequate adjuvants. It was concluded that one of the reasons for vaccine failure was the lack of quality control for vaccines (Diosdado et al. 1999). The immunogenicity and infectivity of the attenuated virus gE-/Tk-Begonia strain was also evaluated. It was found to be immunogenic for all the animals tested and did not transmit to sentinels and other pigs on the farm (Castro et al. 2000b). Unfortunately, inactivated AD vaccines are only allowed for use in Mexico, and without using attenuated AD vaccines it will be very difficult to develop herd immunity sufficient to stop viral circulation in swine areas.

CONCLUSIONS

It was concluded that AD is hyperendemic in most of the swine-producing areas of Mexico because of the lack of control in the transport of infected animals, lack of proper potency of some of the vaccines, insufficient vaccination coverage, and the lack of public interest. The large number of infected herds has favored the selection of ADV strains of low pathogenicity and high infectiousness. Because clinical disease is rarely observed, pig producers, veterinary practitioners, and sanitary authorities have not been concerned with controlling the disease.

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7.3

Aujeszky's Disease in Asia

Roongroje Thanawongnuwech

SUMMARY

Aujeszky's disease is a major economic threat to swine producers all over the world. In most Asian countries, Aujeszky's disease virus is widespread among the pig population. There are variable reports from Asian countries, but in general, the incidence is high in areas with an intensive pig production system, whereas a low-to-moderate infection rate occurs in areas with less intensive pig farming. The severity of disease depends on the age of animals, strain and dose of virus, and route of exposure. Voluntary vaccination has been practiced for decades to reduce the losses of clinical outbreaks. Although vaccination is widely practiced in both breeding and fattening herds, the prevalence of Aujeszky's disease virus in some countries is still high due to lack of formal control programs. At present, there are national control and eradication programs enforced in a few countries in Asia, such as Japan, Korea, and Taiwan. This chapter focuses on Aujeszky's disease in Southeast Asia, especially in Thailand.

INTRODUCTION

Aujeszky's disease virus (ADV), also known as pseudorabies virus, belongs to the subfamily Alphaherpesvirinae within the family Herpesviridae. This virus is responsible for causing severe economic losses to the swine industry. The disease was described in cattle in the United States as early as 1813 (Pensaert and Kluge 1989), but ADV was first recognized as a nonbacterial, etiologic agent in Hungary in 1902 (Aujeszky 1902). Subsequently, ADV has emerged as an important disease in most areas of the world where pigs are raised. Clinical signs of ADV are variably characterized by central nervous system disorder in younger pigs, respiratory symptoms in older pigs, and reproductive failure in pregnant animals. Although pigs represent the only natural reservoir for ADV and serve as a source of infection for other species, most mammals, except horses and higher primates including human beings, are highly susceptible and succumb to the disease with mortality rates approaching 100% (Wittmann and Rziha 1989).

In spite of ADV eradication efforts, outbreaks of AD are still reported in some countries. The apparent increase in disease severity, prevalence, and worldwide dis-

tribution could be due to several possibilities. First, new virulent viral strains may have emerged; second, interaction between pathogens may exacerbate the disease; third, modern transportation and increased animal movement may help spread the disease; and, lastly, the dramatic change in swine management systems may have provided an ideal environment that facilitates the maintenance and spread of the virus within or among herds.

Control policies and eradication programs vary among countries. A control program is intended to reduce the prevalence of ADV-infected herds to a biologically and/or economically justifiable level. An eradication program is endorsed with an initial aim of eliminating the virus from a specific area in order to reach a final goal of an ADV-free country. Alternatively, many Asian countries choose to ignore the presence of ADV and have no official control policy. Thus, local veterinarians are responsible for implementing the control programs with the pig producers. The end result of having no formal policy could be the spread of ADV among swine herds, ongoing vaccine expenditures for an indefinite time, economic losses due to reduced productivity, and fatalities in other domestic species living in proximity to the infected herds. Therefore, guidelines must be established to control the spread of ADV among herds and to reduce the prevalence within existing infected herds.

EPIDEMIOLOGY

The first report of an ADV outbreak in Asia was in China in 1950s (Li and Guo 1994). Later, the disease gained access to other Asian countries, including Taiwan in 1971 (Lin et al. 1972), Malaysia in 1976 (Lee et al. 1979), Singapore in 1977 (Koh et al. 1979), Thailand in 1977 (Sunyasootcharee et al. 1978), Japan in 1981 (Fukusho 1982), the Philippines in 1985 (Marero 1985), and South Korea in 1987 (Kim et al. 1988).

ADV may have spread to these Asian countries through the importation of ADV-infected breeding stock. The first outbreak in Japan was associated with the importation of sows from the Netherlands (Fukusho 1982). Based on the results of the restriction endonuclease assay of the viral genome, ADVs isolated from Japan (Yamagata-S81) and Thailand (NK) are similar to the

virus found in central Europe (Nishimori et al. 1987; Yamada et al. 1992). Interestingly, the restriction fragment-length polymorphism patterns of the first Korean isolates were similar to those in Taiwan (Kim et al. 1988).

Movement of infected animals appears to be a major obstacle to disease control. Several outbreaks in Thailand were reported by local veterinarians and regional laboratories after the first outbreak in the central area (Suksaithachana et al. 1984; Sunyasootcharee et al. 1980). A similar scenario was observed in other Asian countries (Lee and Lin 1975; Lee et al. 1979; Lou and Yang 1997).

Several Asian countries have regions of high ADV prevalence intermixed with regions of low prevalence (Damrongwatanapokin et al. 2000; Jasbir et al. 1998; Liao et al. 1999; Wang et al. 1996), except Japan (Fujita 1994) and South Korea (Lyoo et al. 1997), which have a low prevalence. In Japan, the AD incidence has been limited by an official control program, but persists in certain areas. In Thailand, the use of gE-deleted vaccines and differential enzyme-linked immunosorbent assay (ELISA) kits has made it possible to determine ADV seroprevalence in Thai swine population since 1987 (Urairong et al. 1994). The ADV seroprevalence appeared to decline after more producers incorporated the attenuated gE-deleted vaccine into their vaccination program (Urairong et al. 1994). A recent report by Damrongwatanapokin et al. (2000) found that Thailand still has an ADV prevalence of over 40%, particularly in breeding stock, in some high-density pig-farming areas, but the prevalence of ADV infection in most fattening pig farms is lower than 30%. Based on information from the Veterinary Diagnostic laboratory at Chulalongkorn University, over 70% of swine herds that submitted sera for ADV gE ELISA in the year 2000 had serological evidence of infection by a wild ADV. Since a very high proportion of pigs with ADV become latently infected (Sabo 1985), latently infected gilts entering the breeding pool may serve an important role in contributing to the situation. No existent ADV vaccine can completely prevent latency in the face of a "superinfection," i.e., massive exposure, with a virulent virus. Thus, reactivation of the infection in a latently infected animal might result in a high proportion of a population becoming infected with field-strain virus or a variant virulent virus without being detected.

DIAGNOSIS OF AUJESZKY'S DISEASE IN THAILAND

Laboratory facilities in Thailand include the National Institute of Animal Health, the Regional Veterinary Research and Diagnostic Centers located in each main region of Thailand, veterinary diagnostic laboratories associated with the universities, and some private diagnostic laboratories. These laboratories are well equipped and have full facilities for providing all basic diagnostic services. In addition, some major provinces have basic

laboratories designed to provide simple, uncomplicated techniques.

Although ADV infection is endemic in nature, the infection may be confused with others and not be recognized. In any case, laboratory diagnosis is necessary to confirm the presence of the disease (Wittmann and Rziha 1989). A combination of herd history, clinical signs, gross and microscopic findings, and serological and virological results is useful in making a presumptive diagnosis of ADV infection.

Clinically, reproductive failure may be observed when a herd first breaks with ADV (Hsu et al. 1980). Nervous signs and losses due to death from typical coagulative necrosis in liver, spleen, adrenal glands, and lung, including necrotizing placentitis, were reported among suckling pigs (Hsu et al. 1980; Sunyasootcharee et al. 1978). Concurrent bacterial infections, particularly *Streptococcus suis*, are commonly found in ADV-infected pigs (Urairong et al. 1994). In ADV-positive herds, respiratory distress and anorexia are frequently observed in nursery and/or fattening pigs, particularly after exposure to abrupt climatic changes.

The methodology to diagnose ADV infection was established in Thailand after the first outbreak in 1977. Early on, pathological and histopathologic diagnoses were the primary tools. The fluorescent antibody test on brain-impression smears and cryostat tonsil sections, histopathology on brain and tonsil of the infected pigs, and rabbit inoculation were available in most laboratories (Lou and Yang 1997; Sunyasootcharee et al. 1980). Virus isolation using a porcine kidney cell line (PK-15) was developed to help in the identification of ADV (Kongsmak et al. 1980). However, concurrent infection with hog cholera virus and ADV may pose difficulties for virus isolation (Lai et al. 1984). More recently, immunohistochemical staining on formalin-fixed tissues has been found to be a sensitive method for identifying the causative agent when concurrent infections occur (Sunsasootcharee et al. 1991).

In Thailand, only gE-deleted vaccines have been commercially available since 1995 (Damrongwatanapokin et al. 2000). In pigs, the combination of gene-deleted vaccines and compatible differential gE ELISA kits enables antibodies resulting from vaccination to be distinguished from those due to infection.

PREVENTION AND CONTROL

Control and eradication programs are variable. The most rigorous level is a *no vaccination* strategy. In some cases, vaccination with differential vaccines is used, sometimes combined with the testing and slaughter of infected pigs. And, in some cases, there is no regulation of AD. Unfortunately, only a few Asian countries, including Japan and Taiwan, currently have official control policies (Fujita 1994; Sung and Yang 1994). Initially, a

program involving the use of hyperimmune serum, vaccination, and certain management procedures for the control of ADV was established to reduce the number of fatalities and the reproductive failure caused by the ADV outbreak in Taiwan (Hsu and Lee 1984). Thailand has not yet implemented a control program, but intends to do so in the near future.

Early efforts to control the disease included work in Malaysia on the use of a formalin-inactivated vaccine in pigs and sheep that, experimentally, produced satisfactory protection (Lee et al. 1979). However, oil-adjuvanted inactivated vaccine did not work well in Singapore (Koh et al. 1979). The attenuated ADV vaccine developed for local use in China yielded satisfactory results in preventing the disease in pigs, sheep, and cattle (Li and Guo 1994). In Japan, the gC-deletion vaccine was employed since 1993 in Tohoku, and ADV was eliminated from this area in 1997 (Asai et al. 1998). Vaccination has largely changed the disease situation and tremendously reduced serious outbreaks. Due to viral latency and because vaccination and culling are done voluntarily, ADV is able to persist even in herds on a regular schedule of vaccination. When regulations and controls are based on the misconception that ADV-vaccinated pigs exposed to field virus will not become infected and will not spread infection, widespread dissemination of the disease is possible. Sporadic ADV outbreaks have been reported in China, regardless of prophylactic measures (Lou and Yang 1997; Tong and Chen 1999; Xu et al. 1997). Efficient vaccination programs rely on an understanding of the limitations of vaccine and strict controls on the movement of infected and exposed pigs, regardless of their vaccination status. Since vaccination is voluntary at the farmers' expense, and since there is no financial subsidiary in the case of the outbreak, ADV outbreaks are usually not reported. This situation makes control programs impossible in some countries.

The eradication of ADV on a nationwide basis is not yet possible for several Asian countries, including Thailand. Movement of infected animals in the absence of quarantine regulations makes eradication programs impossible. In Thailand, the placement of weanling pigs in nursery buildings in a *continuous flow* fashion is still practiced in some herds. Mixing between age groups promotes the spread of diseases, including ADV. In addition, the Danish experience suggested that the proximity of infected herds played a major role in the success of prevention and control programs (Andersen et al. 1989). It also suggested that the coincidental increased numbers of pigs within herds or within particular areas, as has occurred throughout Asia, provided an environment more conducive for ADV. Prevalence within a specific area is a major factor influencing the decision to control or eradicate the virus. Whether the policy is to control or eradicate ADV, three components are common to both programs and are usually performed simultaneously:

estimating the prevalence of infected herds, decreasing the incidence of new herd infections, and decreasing the prevalence of existing herd infections (Morrison 1994).

ACKNOWLEDGMENT

The author expresses his sincere gratitude to the staff of the Veterinary Library and Information Center, Faculty of Veterinary Science, Chulalongkorn University, for their help in the preparation of this chapter.

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7.4

Aujeszky's Disease in the Republic of Korea

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SUMMARY

Aujeszky's disease (AD) is an economically important disease of pigs and has been classified as a reportable disease in the Republic of Korea (ROK) since 1982. The first outbreak of AD was identified in June 1987 on a small farm in the Yangsan District of Kyungnam Province, the southern part of the ROK. It was believed to be due to the importation of infected breeding animals from Taiwan. Since then, AD has spread through swine operations in various regions of the ROK despite very strict movement control and the implementation of a stamping-out policy. Because of an increase in the number of outbreaks, the policy has recently been changed to vaccination using *gI* (*gE*)-deleted inactivated vaccines in conjunction with extensive serological surveillance and culling, which is the basis for the AD Eradication Campaign in the ROK.

AJUESZKY'S DISEASE OUTBREAKS

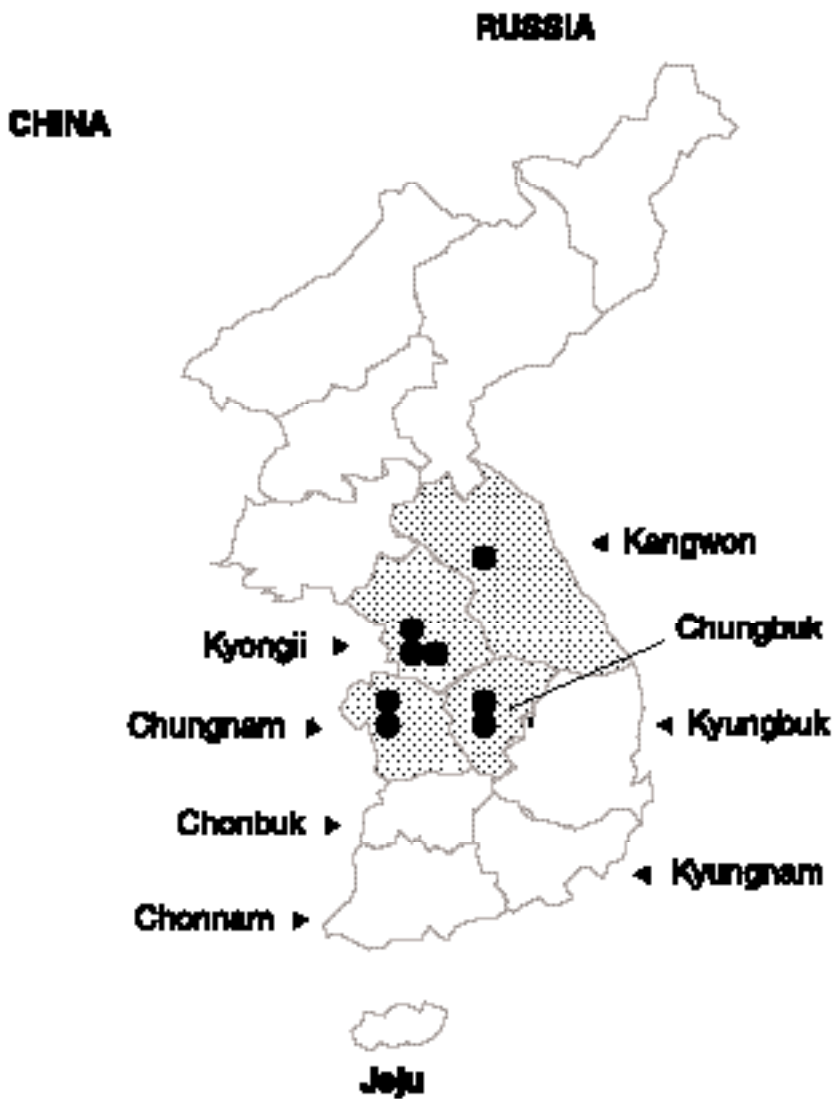
The first serological survey, conducted in 1981 and 1982, showed that the republic of Korea (ROK) was free of Aujeszky's disease virus (ADV). After AD was classified as a reportable disease in 1982, a second nationwide serological survey of domestic and imported pigs was conducted in 1983 using the virus neutralization (VN) test. However, since the VN test took a week to complete, a large-scale nationwide survey was not possible. To overcome this problem and improve turnaround of AD diagnosis, monoclonal antibodies specific to ADV were developed and applied to a radial immunodiffusion assay (modified radial immunodiffusion enzyme assay, MRIDEA) that was initially developed by Han-Soo Joo at the University of Minnesota (An et al. 1987). The modified MRIDEA kit was then distributed to Provincial Veterinary Services. From 1984 to 1987, a total of 13,000 pigs were tested by MRIDEA and VN, and none was seropositive for ADV.

In July 1987, an outbreak of neurological disease in young pigs (3 to 120 days of age) and a subsequent abortion storm among breeding stock were identified in a swine farm of approximately 954 pigs located in Yangsan District of Kyungnam Province, the southern part of the ROK (Figure 7.4.1). ADV was identified in the tonsils and

spleen of infected pigs by immunohistology and virus isolation. Farm quarantine and movement restrictions were enforced by provincial veterinarians and local police. All 954 pigs were killed and buried, and the owners were compensated. Epidemiological investigations of neighboring farms, as well as of swine operations in nearby Yangsan and Kimhae Counties, found four other AD-positive farms. All pigs on the infected farms were killed and buried, and the owners were compensated (An et al. 1988). It was therefore concluded that ADV had not been transmitted to other areas, except for the five pig farms.

Since 1987, more than 2000 pig sera have been screened for ADV antibody each year. After 1990, AD appeared to be under control. Only one AD outbreak occurred in 1992 and none in 1993 (Figure 7.4.2). In 1994, however, new outbreaks of AD were reported in swine operations in Kyonggi and Chungnam Provinces. Unfortunately, AD outbreaks in these provinces were not easily controlled because of widespread infection in large breeding farms located in intensive pig-farming areas. Aerosol transmission of ADV was also identified. By 1995, a total of 54 pig farms were infected, and 2016 pigs were culled as part of the control measures. Gradually, the incidence of AD declined, and in 1998 only 10 farms ($n = 122$ pigs) were identified as being infected with ADV. At this time, the disease had not spread to other provinces except for Kyonggi and Chungnam.

In 1999, two AD outbreaks were reported, for the first time since the initial AD outbreak in 1987, in the southern part of the ROK: one in Chonnam Province and the other in Ulsan City, Kyungnam. Epidemiological investigations of the origin of ADV conducted by the Provincial Veterinary Services showed that pigs had been purchased from farms in Kyonggi Province that had been infected with ADV in the past. All seropositive pigs were immediately culled, and no other positive animals were detected in these areas by a series of serological surveys at 3-month intervals. Although no further outbreaks of AD occurred in the southern part of the ROK, the disease gradually spread from Dangjin and Hongsong Counties in Chungnam Province to swine herds in Chungbuk Province by the movement of infected pigs in 1999 and 2000. These two counties in Chungnam are pig-dense areas with infection and reinfection occurring



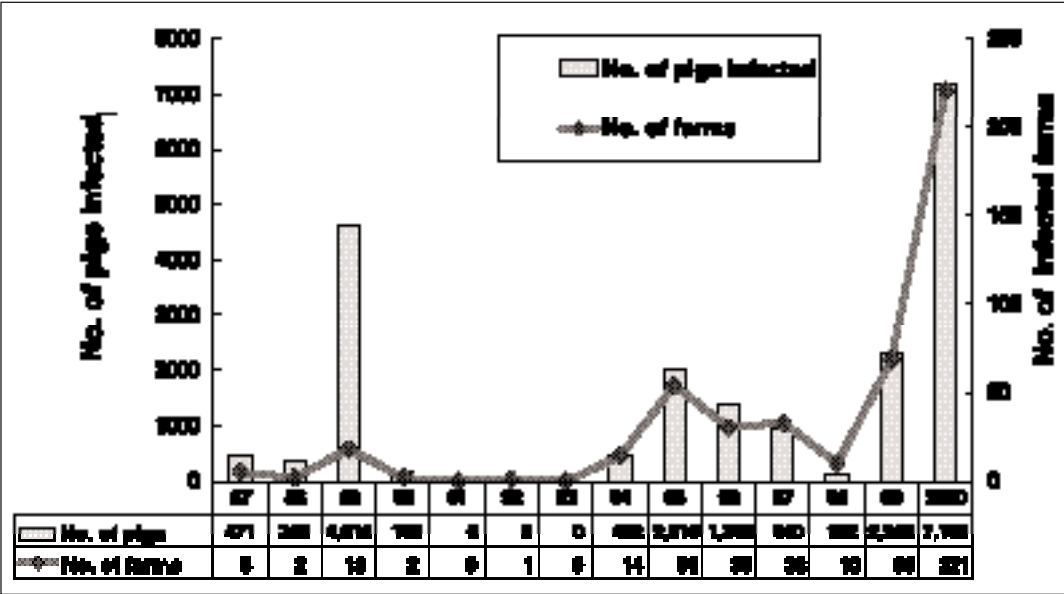
7.4.1. Map of Republic of Korea showing the nine provinces. A recent survey confirmed Aujeszky's disease virus infection in eight counties located in four provinces.

by movement of infected pigs and virus-contaminated trucks, feed, and semen.

A nationwide survey conducted in 2000 showed that 7162 sows from 221 farms in four provinces (Kyonggi, Kangwon, Chungnam, and Chungbuk) in the midregion of the ROK had serological evidence of ADV infection by the gI enzyme-linked immunosorbent assay (ELISA) (Figure 7.4.2). Sampling sizes for the serological surveys that were conducted are presented in Table 7.4.1.

DISEASE SURVEILLANCE AND CONTROL MEASURES

After the first outbreak of AD in 1987, breeding swine imported from Taiwan, as well as domestic pigs, were investigated, and three more farms infected with AD were identified. All pigs on the index farms were killed and the owners compensated. Initially, the Korean government policy was *stamping out* in order to eradicate AD



7.4.2. Aujeszky's disease in the Republic of Korea (1987 to 2000).

Table 7.4.1. Sample size used in Aujeszky's disease virus serological survey

Herd Size	Sample Size
500	≥ 20
501–1000	≥ 30
1001–5000	≥ 50
5001–10,000	≥ 70
10,000	≥ 100
Adult pigs (sows, boars)	Sample all animals

without vaccination, but this became increasingly difficult to implement because of the increased number of outbreaks and economic hardship to both the government and the producers. Since 1994, the AD eradication campaign has been based on vaccination, with progressive culling of seropositive pigs and compensation in accordance with the Directive for ADV Prevention, including the Act for the Prevention of Livestock Epidemics. An annual nationwide AD surveillance is conducted with the help of producers, the Livestock Health Control Association (LHCA), local pig producers associations, veterinary colleges, and the Provincial Veterinary Services.

For infected farms, movement restrictions are applied to all pigs, and pigs with clinical signs are immediately slaughtered, with compensation for the owners.

Seropositive finishing pigs without clinical signs are isolated and slaughtered at a designated slaughterhouse. Seropositive sows are strongly recommended for early slaughter, with partial compensation. Intensive serological examinations are conducted at 3-month intervals. The gI (gE)-deleted killed vaccines are used to prevent reinfection of infected farms.

CHARACTERIZATION OF THE KOREAN ADV ISOLATE

The ADV isolated in 1987 showed cytopathic effects similar to those of the Shope strain on porcine kidney (PK-15) cells. When piglets were intranasally or subcutaneously inoculated, nervous signs were observed about

10 days after inoculation and eventually resulted in death. Clinical signs induced by the field isolate were similar to those of the Shope strain (Lee et al. 1988).

ADV strains can be differentiated by the electrophoretic movement pattern of DNA fragments (i.e., DNA fingerprint) in agarose gels when the DNA is digested by restriction enzymes (Nishimori et al. 1987; Paul et al. 1982). To determine the origin of the Korean isolate, DNA fingerprinting was conducted and the results compared with known strains, including the Shope strain. The study demonstrated that the Korean isolate was not related to US or European ADV strains known at that time, but was close to the Taiwan strain (Kim et al. 1988), suggesting that ADV was probably introduced from Taiwan. Supporting this assumption was that three of five outbreak farms had imported breeders from Taiwan. Since 1987, many ADVs have been isolated, and DNA fingerprinting of the isolates has shown nearly the same pattern as that of the first Korean isolate, the Yangsan strain.

AUJESZKY'S DISEASE RESEARCH IN THE ROK

Monoclonal antibodies (Mabs) against ADV were produced in 1984 (Kweon et al. 1986a). These Mabs were used to detect ADV-infected animals by an indirect fluorescent antibody assay for characterization of ADV proteins and for studies on cross-reactivity with proteins of other herpesviruses, such as infectious bovine rhinotracheitis virus and Marek's disease virus (Kweon et al. 1986b). A MRIDEA was also developed using Mabs against ADV proteins with molecular masses of 22, 62, 74, 100, and 115 kD, and found to have 100% specificity and sensitivity when compared with the VN test and ELISA (Todd et al. 1981). The test kits were stable at 5°C for 2 months.

The first Korean isolate of ADV (the Yangsan strain), produced typical herpesvirus cytopathic effects, including giant cell and intracytoplasmic bridge formation when inoculated into PK-15, rabbit kidney (RK-13), and Madin-Darby bovine kidney (MDBK) cells. Subsequently, the isolate was proven to be pathogenic by experimental animal inoculation into mice, rabbits, and 10-day-old piglets (An et al. 1988). The Yangsan strain was similar to the TNU strain isolated in Taiwan by restriction fragment-length polymorphism analysis of the viral DNA (Kim et al. 1988).

A great deal of effort and many resources have been dedicated to the molecular characterization of ADV isolates and the development of marker vaccines. Hyun et al. (1996) cloned and sequenced the glycoprotein gp50 (gD) gene of the Yangsan strain. The gene was then expressed in a recombinant baculovirus-expression system. The recombinant gp50 protein had a molecular weight similar to that of the authentic gp50 and elicited

neutralizing antibody against ADV when inoculated into guinea pigs and pigs (An et al. 1993). The recombinant gp50 was mixed with inactivated gI-deleted ADV and developed as a marker vaccine; this vaccine has been extensively used in the field to control AD.

The recombinant gIII (gC) protein was expressed in a baculovirus system and used as antigen for an RIDEA (Song et al. 1992). The major capsid protein gene was also cloned and expressed in a baculovirus system (An et al. 1996). When recombinant major capsid protein was mixed with recombinant gp50 protein and then inoculated into guinea pigs, the mixed proteins elicited high serum-neutralizing antibody titers. However, the protection rate against challenge inoculation was lower in comparison with the commercial gI-deleted inactivated vaccine (Intervet) (An et al. 1996; Jun et al. 1996; Kweon et al. 1992).

Hyun et al. (1997) constructed recombinant ADV expressing β -galactosidase instead of thymidine kinase, which markedly reduced the pathogenicity of recombinant ADV. Mice survived after inoculation with $10^{6.8}$ 50% tissue culture infectious dose (TCID₅₀) of the recombinant ADV. When the gI gene was deleted from the ADV expressing β -galactosidase, pathogenicity of the gI-deleted ADV was further reduced, suggesting that thymidine kinase- and gI-deleted ADV could be used as a marker vaccine.

CONCLUSIONS

The ROK was AD free until the first outbreak was reported in 1987. Evidence suggests that the first outbreak of AD occurred through the importation of breeder pigs from Taiwan. By establishing rapid and sensitive diagnostic tests, such as the VN test, MRIDEA, and indirect fluorescent antibody assay using Mabs prior to the first outbreak, the epidemiological investigation of AD was greatly enhanced. Eradication measures—such as the restriction of movement of pigs on infected farms, the slaughter of pigs seropositive for ADV gI, and the use of gI-deleted vaccines—have been very effective in controlling the disease.

Currently, a nationwide seroepidemiological survey is being conducted. By implementing measures, such as extensive culling and compensation, along with vaccination, the ROK hopes to eradicate the AD in the near future.

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7.5

Immunity Against Aujeszky's Disease Virus

Federico A. Zuckermann

SUMMARY

An understanding of the role that different types of immune effector mechanisms have in mediating protective immunity against virus diseases of swine is key to the development of effective vaccines for use against this species. The abundant literature available on the efficacy of vaccines against Aujeszky's disease virus (ADV) has provided evidence that inactivated ADV vaccines are not as effective as modified-live virus (MLV) vaccines at stimulating protective immunity. The fact that a similar titer of virus-neutralizing antibodies is present in pigs receiving either type of vaccine has fueled the speculation that cell-mediated immunity must therefore be responsible for mediating protection from disease. Certainly, the lesser ability of inactivated ADV vaccines to provide protective immunity must reflect the inability of this type of vaccine to induce sufficient levels of immune effector mechanism(s) important for protection. However, the exact nature of this deficit is unknown. We have examined this issue by measuring the serum titer of virus-neutralizing antibodies and the frequency of ADV-specific interferon-gamma (IFN- γ)-producing cells in the peripheral blood mononuclear cells of pigs after immunization with either inactivated or MLV ADV vaccines. We found that while both MLV and inactivated vaccines can induce similar levels of neutralizing antibodies, an inactivated vaccine is not as effective as an MLV vaccine at stimulating ADV-specific IFN- γ -producing cells. Indeed, we found a correlation between the intensity of this response and the level of protective immunity. This correlation was further confirmed by the observation that pigs immunized with inactivated virus in combination with either human recombinant interleukin 12 or an oil-in-water adjuvant developed an enhanced IFN- γ response and level of protective immunity, as compared with pigs receiving the inactivated virus alone. In contrast, the titer of virus-neutralizing antibodies produced in response to the inactivated vaccine was minimally affected by either of these adjuvants. Our studies clearly demonstrated that an inactivated ADV vaccine is less efficient than an MLV vaccine at inducing cell-mediated and protective immunity in pigs. The data also provided evidence for the existence of a dichotomy in the regulation of porcine humoral and cellular immune responses. The positive

correlation between a strong cellular, but not humoral, immune response, with a high level of protective immunity, suggested that cell-mediated immunity mediates protection against this viral disease.

INTRODUCTION

Aujeszky's disease (AD) is characterized by fatal encephalitis in newborn pigs and a milder disease in older swine. Infection with Aujeszky's disease virus (ADV), an alphaherpesvirus, is primarily manifested as severe depression, anorexia, pyrexia, ataxia, and respiratory distress (Baskerville et al. 1973). The clinical response to virus challenge depends on the immune status and age of the animal (Kluge et al. 1992). Challenge of vaccinated animals with virulent ADV results in a reduction in the growth rate (weight gain), and even weight loss, depending on the severity of the challenge and level of protective immunity conferred by the vaccine (Vannier 1985; Van Oirschot and De Leeuw 1985; Wardley et al. 1991; Zuckermann et al. 1998a). Weight change observed within 7 days after ADV challenge has been shown to be a sensitive, reproducible, and statistically sound parameter that allows for the quantification of the level of protective immunity conferred by different types of ADV vaccines (Stellman et al. 1989). Although protective immunity can be readily induced by immunization with either live or inactivated ADV vaccines (Donaldson et al. 1987; Wardley et al. 1991; Zuckermann et al. 1998a,b), there are measurable differences in clinical response. Use of this measurement has demonstrated that, indeed, inactivated vaccines are less effective than live vaccines at inducing protective immunity (De Leeuw and Van Oirschot 1985; Stellman et al. 1989; Vannier 1985; Zuckermann et al. 1998a,b). We have analyzed in detail the humoral and cellular response to vaccination with either inactivated or modified-live virus (MLV) vaccines. We present data indicating that a strong cellular immune response is associated with a high level of protective immunity.

MATERIALS AND METHODS

Groups of pigs were immunized either with commercially available inactivated or MLV vaccines. The development of

humoral and cellular immunity was monitored by a standard virus-neutralizing (VN) test and an interferon- γ (IFN- γ) ELISPOT assay, respectively (Zuckermann et al. 1998a). Protection was measured based on the weight change from the day of challenge to day 7 after challenge, as described by Stellman et al. (1989). Animals were challenged with 10 LD₅₀ of the wild-type ADV Becker strain.

RESULTS AND DISCUSSION

Although the nature of the immune response responsible for mediating protection from AD is unknown, some inferences can be made by correlating the intensity of a given type of immune response and the level of protection from virus challenge. Immunization with MLV vaccines generates a robust cytotoxic T-lymphocyte (Zuckermann et al. 1990) and lymphoproliferative responses (Kimman et al. 1992, 1995; Van Oirschot 1978/79; Zuckermann and Husmann 1996; Zuckermann et al. 1998a). In our studies, we found that, although the intensity of the lymphoproliferative response to either inactivated or MLV vaccines may or may not differ, the MLV vaccine induces a three- to fivefold higher frequency of ADV-specific IFN- γ -producing cells than does an inactivated vaccine (Zuckermann et al. 1998a,b). Remarkably, there is a dichotomy between the humoral and cellular immune responses to these two types of vaccines. While in some instances the inactivated vaccine is capable of inducing an equal or even higher titer of VN antibodies than the MLV vaccine, the inactivated vaccine is less capable of stimulating the generation of virus-specific IFN- γ -producing cells (Zuckermann et al. 1998a). Since the MLV vaccine induced a higher level of protection from challenge than the inactivated virus vaccine, a high level of protective immunity correlates with the presence of a strong cell-mediated immune response. Further evidence of this correlation was obtained by the observation that while immunization with an unadjuvanted, inactivated ADV vaccine is capable of inducing a significant humoral immune response, it only promotes a minimal cell-mediated immunity (CMI). The protection afforded by unadjuvanted, inactivated vaccine was also minimal. The addition of an oil-in-water adjuvant to the inactivated virus enhanced significantly both the intensity of CMI and the level of protective immunity. However, it had only a minimal effect on the titer of VN antibodies. A similar correlation was obtained by utilizing interleukin 12 as an adjuvant for the inactivated vaccine. Using this cytokine, we found that as little as 2 μ g of interleukin 12 administered to pigs in combination with an inactivated ADV vaccine enhanced the strength of the frequency of virus-specific IFN- γ -secreting cells as well as the level of protective immunity induced by the inactivated virus (Zuckermann et al. 1998b). In contrast, the titer of VN antibodies was either not affected or even slightly decreased.

The data clearly showed differences in the quality and quantity of the immunity induced by a live versus inactivated ADV vaccine. Although inactivated commercial vaccines are equally efficient as an MLV vaccine at inducing humoral immunity, they induce only a weak and transient virus-specific IFN- γ response. In contrast, an MLV vaccine induces a robust virus-specific IFN- γ response. In all of the different ADV vaccine formulations tested, a high level of protective immunity correlated with the presence of a strong IFN- γ response, whereas the titer of VN antibodies did not. Although the results from this study do not rule out a role for humoral immunity in protection, they do suggest that CMI participates in providing a high level of protective immunity. We would argue that the level of CMI is indeed the major factor in determining the level of protection from ADV-induced disease. At the very least, a strong IFN- γ response in a pig is a good predictor that the animal has developed a strong protective immune response against this virus.

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7.6

The Porcine Lymphotropic Herpesviruses: Emerging Pathogens in Xenotransplantation?

Bernhard Ehlers and Michael Goltz

SUMMARY

Xenotransplantation—the transplantation of animal organs into humans—offers one of the possible solutions for the continuously growing shortage of human organs in allotransplantation. The favored donor species is the pig. However, concerns have been raised about the virological safety of xenotransplantation. There are continuous efforts to assess the potential risk of known porcine viruses for humans who undergo immunosuppressive treatment during xenotransplantation, and to identify as-yet-unknown porcine viruses.

A recent search for new herpesviruses in pigs resulted in the identification of novel porcine gammaherpesviruses. They were named porcine lymphotropic herpesviruses 1 and 2 (PLHV-1 and PLHV-2). In this chapter, we present evidence that these viruses are involved in a novel lymphoproliferative disease in swine, summarize what is presently known about their genetic and biological properties, and discuss their pathogenic potential for animals, as well as human xenotransplant recipients.

INTRODUCTION

Research on a porcine virus usually starts with the occurrence of a new disease caused by a previously unrecognized viral entity. Examples of such novel viruses are described in this book. Another cause for a new disease might be a known microorganism previously thought to be nonpathogenic or reported to be involved in other diseases. Alternatively, an old disease formerly not known to have an infectious etiology may come into the focus of viral research. The first detection of the porcine lymphotropic herpesviruses 1 and 2 (PLHV-1 and PLHV-2) represents another alternative, i.e., the discovery of novel viruses in healthy pigs. However, in this chapter, their association with a novel porcine lymphoproliferative disease syndrome is also described.

The reason for an intense search for novel porcine viruses is the current interest in xenotransplantation (XT), the transplantation of animal organs into humans (Auchincloss and Sachs 1998). XT is considered a solu-

tion for the present shortage of organs available for allotransplantation (human-to-human transplantation). However, there are scientific as well as regulatory and public concerns about virological safety in XT because the possibility cannot be excluded a priori that animal donors might harbor microorganisms with pathogenic potential for the human recipient and the population at large. In the past, several pathogenic viruses have been transmitted from nonhuman primates to humans (Gao et al. 1992; Holmes et al. 1995), which is one of the reasons why nonhuman primates are considered unsuitable organ donors for XT (Chapman et al. 1995). With pigs serving as donors, the risk of transmitting diseases to humans is supposed to be markedly lower. For many centuries, pigs have been raised by humans and, until recently, no serious infectious disease transmission had been observed, with the exception of some strains of influenza virus. For these, as well as for physiological, ethical, and economic reasons, pig organs are currently favored as transplants (Fishman 1994). However, new porcine pathogens with zoonotic potential have emerged in recent years. An example is the novel porcine paramyxovirus (Nipah virus) that caused the death of more than 100 people in Malaysia (Farrar 1999). This and other emerging porcine viruses are described in this book. In addition, recipients of xenogeneic organs receive immunosuppressive treatment to prevent organ rejection (Auchincloss and Sachs 1998). With an impaired immune system, not only pathogens but also even nonpathogenic microorganisms might adapt to the human recipient and cause disease. Therefore, recipients of xenotransplants have to be monitored carefully for the transmission of animal viruses. However, this is obviously only possible for known viruses. By default, unknown viruses are excluded from monitoring because of the lack of detection methods and are therefore even more difficult to control (Günzburg and Salmons 2000).

In 1998, we started a search for unknown herpesviruses in domestic pigs for several reasons. Herpesviruses, in particular Epstein-Barr virus (EBV) and human cytomegalovirus, are frequently transmitted or reactivated in allotransplantation, often with severe clin-

ical complications (Ferry and Harris 1994; Van Zanten et al. 1998). Herpesviruses, in particular many species of the Gammaherpesvirinae subfamily like EBV and human herpesvirus 8 (HHV-8), are associated with oncogenesis and lymphoproliferative diseases (Knecht et al. 1997; Whitby and Boshoff 1998). Moreover, several herpesvirus species are well adapted to their natural host, but cause severe and often fatal disease after transmission to other hosts. This is exemplified by some ruminant malignant catarrhal fever viruses like alcelaphine herpesvirus 1 (AIHV-1) (Reid and Buxton 1989) and others (see below), several monkey herpesviruses like herpesvirus simiae (B virus) (Brown 1997), herpesvirus saimiri, and herpesvirus ateles (Fleckenstein and Desrosiers 1982), as well as a recently discovered elephant herpesvirus (Ehlers et al. 2001; Richman et al. 1999). In addition, before the start of our search, only one alphaherpesvirus [Aujeszky's disease virus (Mettenleiter 1991; Wittmann and Rziha 1989)] and one betaherpesvirus [porcine cytomegalovirus (Goltz et al. 2000; Ohlinger 1989)], but no gammaherpesviruses, were known. Therefore, we examined pigs for the presence of gammaherpesviruses by a polymerase chain reaction (PCR) assay that targeted highly conserved motifs of the herpesvirus DNA polymerase (DPOL) with degenerate and deoxyinosine-substituted primers (Ehlers et al. 1999a). We analyzed porcine blood and tissue samples and identified short sequences of two novel porcine herpesviruses with high similarity to gammaherpesviruses. Derived from their presence in blood and lymphatic tissues, they were named porcine lymphotropic herpesvirus 1 (PLHV-1) and 2 (PLHV-2) (Ehlers et al. 1999b).

DETECTION OF PORCINE GAMMAHERPESVIRUSES

PLHV-1 and/or PLHV-2 were detected in more than 80% of blood and spleen samples from domestic pigs by a PLHV-specific PCR. PLHV-1 was found to be more prevalent (Ehlers et al. 1999b). In feral pigs, the situation was reversed, i.e., a high prevalence of PLHV-2 and a very low prevalence of PLHV-1 were found (Ulrich et al. 1999). These data indicated that domestic pigs might be the predominant host for PLHV-1 and feral pigs for PLHV-2. However, apparently both viruses can cross-infect. Evolutionarily, it is tempting to speculate that PLHV-1 diverged from PLHV-2 with the derivation of the domestic pig from the feral pig as animal husbandry developed.

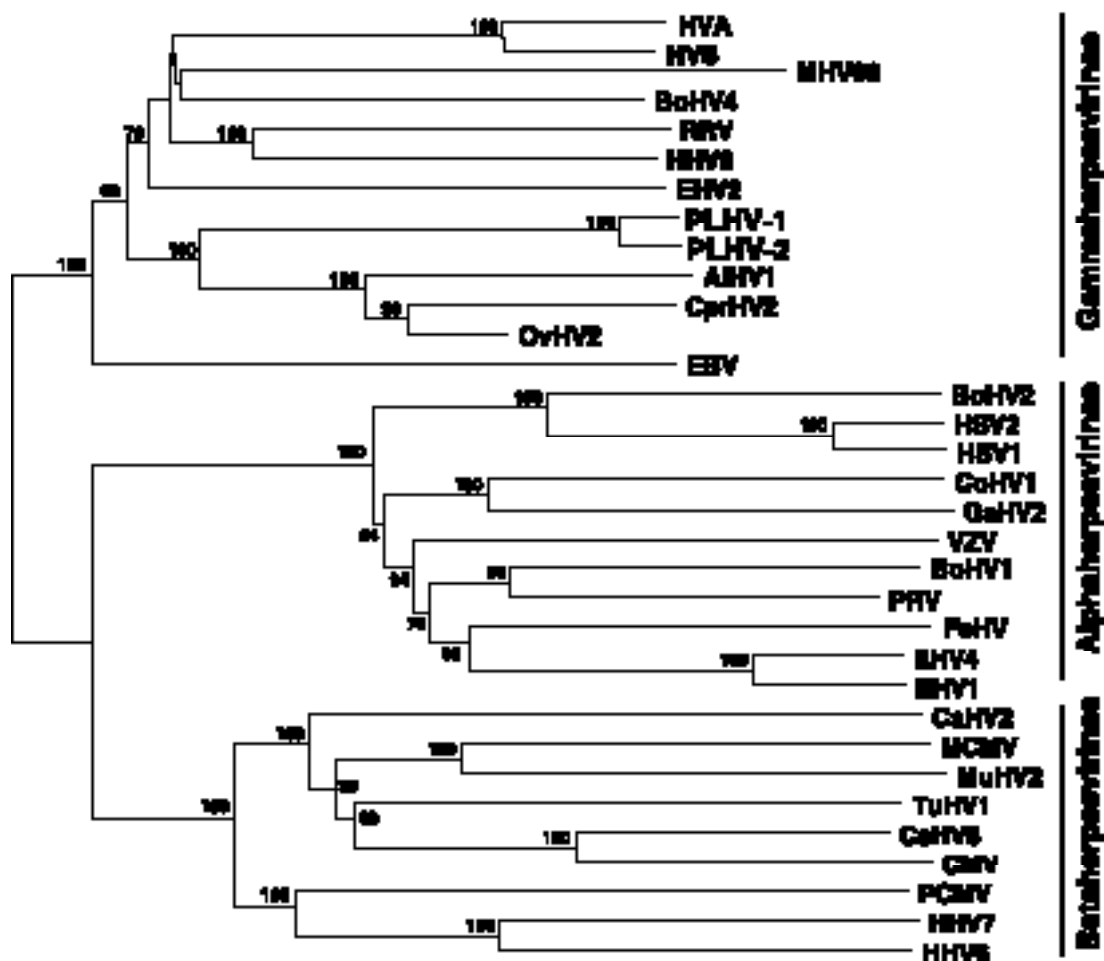
PLHVs were found in pig samples from several parts of Germany and from the United Kingdom, Spain, Holland, France, and the United States. Therefore, they are probably globally prevalent. Only minimal sequence variation was found among PLHV-1 and PLHV-2 isolates, not exceeding an average of 1% in protein-coding regions (Michael Goltz, Sabine Beckmann, and Bernhard Ehlers, unpublished data). The sequence stability might

indicate that the worldwide distribution of these viruses happened only very recently in evolution. It ensures the universal applicability of PCR-based detection systems.

Approaches to characterize the genomes of PLHV-1 and PLHV-2 are hampered by the fact that, so far, culture of the viruses has not been successful. Therefore, the genomes were amplified from PLHV-positive pig samples by a PCR technique called genome walking (Siebert et al. 1995). Up to now, about 65 kilobase pairs (kbp) of PLHV-1 and 30 kbp of PLHV-2 have been sequenced. These sequences contain approximately 50 open reading frames (ORFs) that encode viral proteins, including (1) those that are essential for viral growth, like DPOL and the glycoprotein B, and (2) possible virulence-associated proteins like a G-protein-coupled receptor (Ulrich et al. 1999; Michael Goltz and Bernhard Ehlers, unpublished data). Phylogenetic analysis of PLHVs based on DPOL showed a close relationship to the herpesviral subfamily Gammaherpesvirinae, in particular the ruminant herpesviruses AIHV-1, ovine herpesvirus 2 (OvHV-2), and caprine herpesvirus 2 (CprHV-2) (Figure 7.6.1) (Chmielewicz et al. 2001; Ehlers et al. 1999b; Ulrich et al. 1999).

Since PLHVs were unknown until recently and were detected in healthy pigs, it might be assumed that these viral entities are well adapted to their porcine host and nonpathogenic for pigs and other vertebrates. However, there are several reasons to doubt this assumption.

First, as already mentioned, the PLHVs encode putative virulence-associated proteins. Counterparts of these proteins have already been found in other gammaherpesviruses, including the ORF74-encoded protein of HHV-8. This virus causes Kaposi sarcoma and other tumors in humans (Whitby and Boshoff 1998). The ORF74-encoded protein of HHV-8 probably plays a functional role in the genesis of Kaposi sarcoma (Kirshner et al. 1999). Second, the closest related viruses, AIHV-1 and OvHV-2, are nonpathogenic in their natural hosts, the African wildebeest and the sheep, respectively, but they cause a highly lethal lymphoproliferative disease, i.e., malignant catarrhal fever, in domestic cattle (Reid and Buxton 1989). More importantly, OvHV-2 was also reported to cause malignant catarrhal fever in pigs (Løken et al. 1998). CprHV-2, which is also closely related to the PLHVs, was only recently detected in domestic goats. It was found to be endemic and probably nonpathogenic in its natural host. However, it seems to cause a disease syndrome in Sika deer (Chmielewicz et al. 2001; Li et al. 2001). From these data, it can be summarized that the PLHVs belong to a group of herpesvirus species that are nonpathogenic in their natural host but highly pathogenic in related hosts or even, as in the case of OvHV-2, in more distantly related hosts. In turn, PLHV-1 and PLHV-2 may have the same potential, i.e., they may be nonpathogenic in pigs, but pathogenic in other animal species or in humans who undergo immunosuppressive treatment in XT.



7.6.1. *Phylogenetic analysis of porcine lymphotropic herpesviruses 1 and 2 (PLHV-1 and PLHV-2). Phylogenetic trees were constructed with the neighbor/joining method using the tree-building module of MacVector™ based on a multiple amino acid sequence alignment of conserved regions of DNA polymerase (DPOL) proteins. All DPOL amino acid sequences, including PLHV-1 and PLHV-2 DPOL, were deduced from DPOL genes of alpha-, beta- and gammaherpesviruses available in the GenBank® database. The herpesviral subfamilies are indicated. The trees were statistically evaluated by bootstrap analysis (100-fold resampling). The bootstrap values are indicated at the branches of the tree.*

The first direct evidence of the possible pathogenic potential of the PLHVs came from very recent studies at the laboratories of David Sachs and Clive Patience. Miniature swine were subjected to a protocol of allogeneic hematopoietic stem cell transplantation in which the animals were preconditioned by thymic irradiation, T-cell depletion, and immunosuppressive treatment with cyclosporine. Under these conditions, a high incidence of posttransplant lymphoproliferative disease (PTLD) was observed, resembling PTLD in humans, which represents a spectrum of EBV-driven lymphoid proliferations ranging from a reactive polymorphic expansion of EBV-infected lymphocytes to

monoclonal B-cell lymphomas. The disease was observed in more than 40% of treated animals; most of these animals died whereas a minority recovered from the disease after cessation of immunosuppressive drug treatment. In the blood and lymph nodes of diseased animals, a novel porcine gammaherpesvirus was detected by PCR with degenerate primers targeting the gammaherpesviral glycoprotein B gene. Subsequent PCR with specific primers revealed that the novel virus was present in high amounts. Both before the onset of treatment and after cessation of treatment in animals surviving the disease, only very low amounts of this virus were found. The rise in copy number in the course of the

disease was about 10^5 , probably indicating the onset of lytic viral infection. Like the PLHVs, the novel virus was found to be closely related to ALHV-1 (Huang et al. 2001). Subsequently, it was demonstrated by sequence comparison that PLHV-1 and the PTLT-associated gammaherpesvirus were probably identical (Michael Goltz, Bernhard Ehlers, Clive Patience, and Tom Ericsson, unpublished data).

These data show that PLHV-1 (and possibly also PLHV-2, because of its close genetic relatedness) is associated with, and possibly etiologically involved in, the genesis of PTLT in pigs. The experimental conditions that induced the onset of porcine PTLT included severe experimental immunosuppression and an allogeneic stimulus. Presently, the question of whether any natural disease is caused by, or associated with, activation of the PLHVs remains open. Studies undertaken to shed light on this question are difficult to perform because it has not been possible to culture the PLHV viruses up to now, thus preventing experimental infection of PLHV-negative pigs. Likewise, it is difficult to observe tumor development in commercial pigs because they are usually slaughtered at around 6 months of age. However, in sows and boars kept for commercial breeding or in hobby breeds, petting zoos, etc., there may be a rare chance to observe lymphoproliferative diseases and other tumorigenic processes. It will be important to determine whether PLHV lytic growth can be observed in such neoplastic diseases. To achieve this goal and for the evaluation of the PLHV status of pig breeds intended to be used as donor animals in XT, the availability of nucleic acid-based and antibody-based detection methods is of crucial importance. PCR protocols have been published (Ehlers et al. 1999b; Ulrich et al. 1999), and both a quantitative PCR assay and serological test have been developed (Michael Goltz and Bernhard Ehlers, unpublished data). These methods will also aid in monitoring the possible transmission of PLHV from pigs to human xenotransplant recipients. If transmission were to occur, then the question is whether PLHV, once adapted to the recipient, would be able to facilitate the onset of human PTLT. Further analyses of PLHV gene content and function and their comparison with those of EBV will help to answer this question.

Finally, the question of whether PLHV-1 and PLHV-2 will be the last porcine herpesviruses to be detected should be considered. Knowledge will be gained from further systematic analysis of pigs with pan-herpes-PCR methods and other universal detection systems. Continuing efforts of this kind are needed to produce pig breeds of maximum quality with respect to virological safety in order to render them suitable as donors in XT, and to monitor the viral infection status of human XT patients reliably.

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8 ARBOVIRAL INFECTIONS

8.1 Eastern Equine Encephalomyelitis

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SUMMARY

Eastern equine encephalomyelitis (EEE), a viral disease that is endemic in the eastern United States, is transmitted by mosquitoes and has an endemic cycle in wild birds with sporadic outbreaks among horses, humans, and a variety of domestic and wild animals. EEE infrequently causes deaths among very young pigs, usually in nursing piglets that have central nervous system signs. Histological lesions are primarily confined to gray-matter areas of the brain and are characterized by neuronal necrosis with neutrophil infiltration in acute cases. Diagnosis is based on isolation and identification of the virus or demonstration of viral RNA or antigen in tissues. Risk of infection is related to abundance of mosquito vectors, proximity to wetlands, facility design, and age of pigs.

INTRODUCTION

Isolation of distinctly different viruses from outbreaks of encephalitis in horses on the West and East Coasts of the United States and elucidation of the route of transmission in the 1930s were crucial to the study of western and eastern equine encephalomyelitis (Giltner and Shahan 1933; Merrill et al. 1934). Investigations of subsequent epidemics and research have expanded our knowledge of the geographical distribution, life cycle, host range, and epidemic behavior of eastern equine encephalomyelitis (EEE). Application of new methodologies in virology, epidemiology, entomology, and medicine will hopefully provide the necessary information to diagnose, prevent, predict, and treat EEE rapidly.

Serological surveys conducted in the 1950s detected antibody titers in pigs in Georgia, New Jersey, and Wisconsin (Feemster et al. 1958; Karstad and Hanson 1958, 1959). An outbreak of EEE resulting in the death of 160 piglets in the coastal plain of Georgia confirmed that swine are at risk (Pursell et al. 1972). Death losses in three subsequent outbreaks ranged from a few to 280 piglets (Anonymous 1995; Elvinger et al. 1994; Pursell et al. 1983). A serosurvey revealed serum antibody titers in domestic and feral swine in Georgia (Elvinger et al.

1996a). Economic losses attributed to decreased production, reduced return on investment, and veterinary fees were significant for individual producers. Actual financial losses to producers and costs for mosquito control have not been calculated. Based on the infrequent diagnosis of EEE in swine and the limited geographical distribution of cases, this disease has had minimal impact on pork production in the United States.

EEE is endemic in the Atlantic and Gulf Coast states. The number of hogs and pigs located in the endemic area is dynamic. Based on comparison of the most recent Census of Agriculture data, there was a significant net increase in total number of hogs and pigs in this region of the United States from 1992 to 1997 (USDA-NASS 1999). Much of the increase occurred in North Carolina, where swine numbers have quadrupled in 10 years and now approach 10 million head. Much of the population is located in coastal and near coastal areas that are prone to flooding. The two counties with the highest inventory of hogs and pigs in the United States are in this area. Other states in the area have maintained stable numbers, and a few such as Florida, Georgia, and Louisiana have encountered net losses. Expansion of the swine industry into endemic areas will increase the potential for the reemergence of EEE in swine.

ETIOLOGY

Eastern equine encephalomyelitis virus (EEEV) is a member of the genus *Alphavirus* in the family *Togaviridae*. Eastern encephalitis is an accepted synonymous name for the disease and virus. Alphaviruses are enveloped, spherical, single-stranded RNA viruses that are 60 to 65 nm in diameter. The ability to replicate in, and be transmitted by, mosquitoes is a characteristic feature of alphaviruses. EEEV, western equine encephalomyelitis virus (WEEV), and Venezuelan equine encephalomyelitis virus (VEEV) are important members of the genus that produce neurological disease in horses and other domestic animals and in humans. Only EEEV produces natural infection in swine. Experimental disease can be produced in pigs with all three viruses.

EPIDEMIOLOGY

Although initially diagnosed in horses, EEE is primarily an enzootic disease of wild birds. In endemic areas, EEEV cycles annually in passerine birds via *Culiseta melanura*, a mosquito that feeds exclusively on birds and breeds in freshwater wetlands. Red maple and loblolly bay are among a few trees that provide the required habitat for development of the vector mosquito. Passerine birds such as sparrows, blackbirds, and cardinals develop viremia with inapparent infection and serve as a source of virus for other genera and species of mosquitoes that transmit the disease to pigs and other vertebrates. Standing water from excessive rainfall or flooding provides an environment necessary for proliferation of mosquitoes that have been associated with transmission of EEEV in inland areas. Salt-marsh mosquitoes play a major role in coastal areas. Introduction and proliferation of foreign mosquitoes, such as *Aedes albopictus* from Asia, that feed on both birds and mammals potentially could increase the risk of EEE in swine and other species (Mitchell et al. 1992). Infection of swine and other domestic animals is incidental, and cases occur within close proximity to wetlands. Among pigs and other vertebrates, EEE is an epidemic disease with only occasional outbreaks and high mortality.

Epidemiological data reveal that EEE is endemic in Atlantic and Gulf Coast areas. The geographical range of EEE extends from the coastal area to the Mississippi River valley, southeastern Canada, the Caribbean, and northeast South America. Reported cases of EEE in swine have occurred in the coastal plain of Georgia in 1971, 1982, and 1991 and Florida in 1994. Epidemics of EEE among people have been seen at approximately 10-year intervals. Epidemics of EEE among horses occur at approximately 5-year intervals. This pattern of past epidemics is not predictive of future epidemics.

A limited epidemiological study of the 1991 outbreak in Georgia revealed an understated finding of no shelter for the piglets. Typical pork producers in the region managed farrow-to-finish operations utilizing fenced enclosures and hutches on pasture for farrowing facilities. These structures afforded little protection from the elements and no protection from mosquitoes. Swarms of mosquitoes and mosquito-covered piglets were consistently reported by producers. A shift toward total confinement operations may reduce exposure of pigs to mosquitoes and environmental stress, thereby lowering the incidence of EEE in pigs.

Reported cases of EEE in swine involved nursing piglets from 1 to 4 weeks of age and a 2-month-old piglet, suggesting that only neonates and very young animals develop fatal disease with neurological lesions. Prolonged viremia and higher virus titers in experimentally infected young animals may explain this age-related susceptibility for development of neurological disease

(Hurst 1950). Hypoglobulinemia in a nursing piglet suggests that failure of passive transfer also may be a predisposing factor in natural infections (Elvinger et al. 1994).

The swine cases occurred in May, July, November, and December. This seasonal distribution is similar to the year-round infections that occur among horses and people in Florida. In temperate areas, most cases occur between July and October. Killing frosts and cold temperatures result in migration of passerine birds and reduction or absence of vector mosquitoes.

Evaluation of climatic data associated with outbreaks of EEE in horses has shown a correlation between higher than normal levels of precipitation in June and July (Francy and Wagner 1992). A similar association was seen with October precipitation in the year preceding an outbreak. No significant association was seen for warmer than normal temperatures. Excessive precipitation and swarming of mosquitoes did precede the 1991 outbreak of EEE in swine (Elvinger et al. 1994). Higher than normal precipitation is predictive of increased risk for EEE outbreaks. Adequate or increased water levels in wetlands may facilitate breeding of endemic and epidemic vector mosquitoes; however, this is only one of several requisite elements for an epidemic of EEE.

The minimal essential elements for an outbreak of EEE are viremic birds, a suitable mosquito vector, and susceptible pigs. Factors that limit the frequency, distribution, and severity of epidemics are complex and partially explain the low incidence of disease reported in swine. The first element requires the simultaneous presence of susceptible passerine birds, *Culiseta melanura*, and EEEV. Abundant rainfall and warm temperatures favor mosquito breeding. Passerine birds have a transient viremia limiting the time during which they can serve as a reservoir. Mosquitoes may be infected for life. Other species of mosquitoes that feed on both birds and mammals are required to transmit EEEV to pigs. Ultimately, infected mosquitoes must feed on susceptible pigs.

PATHOGENESIS

The primary route of natural infection is by hematophagous arthropod bite into the skin. Experimental studies have partially elucidated the pathogenesis of EEE (Baldwin et al. 1997; Karstad and Hanson 1959; Pursell et al. 1972). Piglets have been infected by intracranial, intradermal, intravenous, and oral injections of virus. All routes of administration resulted in detectable viremia as early as 6 hours post injection (PI), and viremia persisted up to 168 hours PI. A transient elevation of rectal temperature of less than 12 hours' duration occurred at 24 hours PI. Only a few pigs exhibited neurological signs at 1 to 3 days PI. Virus was isolated from oropharyngeal and rectal swabs from 6 to 96 hours PI. Persistence of the virus in tonsil for up to 20 days was

confirmed by isolation or oligonucleotide probes. Virus was recovered only from the central nervous system and tonsils of pigs without viremia. The virus has no specific cell receptors. Trophism for neural tissue is evident, but other tissues, such as the heart, may be affected. In one study, transient liver necrosis was detected at 12 hours PI, and lesions were resolved at 48 hours PI (Baldwin et al. 1997). Virus-neutralizing antibodies are detectable at about 5 days PI.

Viremic pigs, horses, pheasants, and other infected animals may play a minor role as a source of virus for vectors. Pecking in caged pheasants and cannibalism are uncommon routes of bird-to-bird mechanical and oral transmission, respectively (Satriano et al. 1957). Pigs occasionally exhibit similar biting behavior toward pen mates, which is another potential route for transmission in swine. Commingling turkey poults with orally inoculated poults resulted in spontaneous horizontal transmission of EEEV with fatal enteritis and reisolation of the virus from inoculated and in-contact poults (Brown and Roberts 1992). Some of the pigs commingled with experimentally infected pigs developed serum-neutralizing antibody against EEEV (Baldwin et al. 1997; Karstad and Hanson 1958). Absence of clinical signs of disease suggests that the amount of virus shed by experimentally infected pigs is insufficient to produce clinical disease and that lateral transmission is probably not a major factor in natural disease in swine.

CLINICAL SIGNS

With the exception of one 2-month-old piglet, clinical disease has been described only in nursing piglets (Elvinger et al. 1994; Pursell et al. 1972, 1983). Neurological signs included depression, loss of coordination, circling, paddling, seizures, and somnolence. Weight loss, anorexia, and vomiting have also been reported. No clinical signs have been observed in exposed older pigs and sows that developed antibody titers.

PATHOLOGY

Antemortem and postmortem examinations have revealed no diagnostic gross lesions for EEE. Histopathology is not pathognomonic, but diagnostically significant lesions are present in the brain in acute lesions. Diffuse and localized lesions predominate in the gray-matter areas of the cerebral cortices, thalamus, and hypothalamus. Neuronal necrosis with neutrophil infiltration is strongly suggestive of EEE. Removal of necrotic neurons by phagocytic cells is a common finding. Vasculitis and thrombosis occur in severe lesions. Viral inclusions were not detected. Microglial proliferation and lymphocytic perivascular cuffing are features of late lesions.

Myocarditis was described in one EEE outbreak (Elvinger et al. 1994), and similar lesions were produced

in experimental infections (Baldwin et al. 1997). Random foci of myocardial necrosis were infiltrated by macrophages, lymphocytes, and neutrophils. Myocardial lesions are variable and of no diagnostic significance.

DIAGNOSIS

A live pig with recent onset of neurological signs is the specimen of choice. If fresh tissues are submitted from a field necropsy, brain should be included in addition to routine specimens. Neurological disease in nursing and recently weaned piglets coupled with compatible histopathologic lesions is sufficient to justify a presumptive diagnosis of EEE.

Identification of the virus is required to make a definitive diagnosis. Isolation of the virus from brain or blood specimens is routinely performed using Vero or BHK-21 (baby hamster kidney) cells, with identification of the isolated virus by indirect immunofluorescence. Viral antigen in fresh tissues may also be detected by enzyme-linked immunosorbent assay (ELISA) (Scott et al. 1988). A variety of methods are available for ongoing and retrospective diagnosis of EEE in formalin-fixed tissues. Immunohistochemistry and ELISA have been developed to detect EEEV antigen in tissues (Monath et al. 1981; Patterson et al. 1996). DNA in situ hybridization and polymerase chain reaction techniques are available to detect EEEV RNA in tissues (Armstrong et al. 1995; Gregory et al. 1996; Vodkin et al. 1993). Detection of antibody is a useful method for surveillance, but it is of limited diagnostic value due to the rapid progression of the clinical disease in piglets. Standard tests are serum neutralization, hemagglutination inhibition, and complement fixation. Immunoglobulin M (IgM) and G (IgG) ELISAs have been used to diagnose EEE (Sahu et al. 1994). The presence of IgM indicates recent infection or vaccination. IgG antibody appears later and persists, making it useful for surveillance.

EEE in pigs is probably underdiagnosed, because death of a few piglets does not always result in submission of specimens to a diagnostic laboratory. Significant economic losses often precede requests for veterinary service. Factors such as cost, availability, and quality of services often influence whether samples will be submitted to a diagnostic laboratory. Special procedures required to diagnose EEE may not be included in the routine laboratory protocol for evaluating central nervous system disease in piglets. Awareness of the disease is necessary for early and correct diagnosis of EEE.

THERAPY, PROPHYLAXIS, AND PREVENTION

Antiviral drugs for the treatment of EEE are not currently available and would likely be cost prohibitive and not

approved for use in swine. Palliative treatment consisting of artificial alimentation, fluid therapy, and seizure control are also not feasible and pose a risk of exposure to caregivers. The isolation of pigs exhibiting clinical signs and the killing of animals in extremis may be justified. Infected carcasses should be rapidly removed and disposed of by an approved method to prevent cannibalism and access to scavengers.

In the early stage of an outbreak of EEE in swine, extra-label use of an EEE vaccine licensed for equine use may reduce death losses. Experimental studies have shown that vaccination of pregnant sows provides protection to piglets against viral challenge (Elvinger et al. 1996b). Routine use of the vaccine is not justifiable unless there is a high prevalence of EEE on the premises or immediate area.

The endemic life cycle of EEE involving wild birds coupled with its wide geographical range makes eradication of EEE an untenable goal. A strategy to prevent EEE could have many facets. Killing passerine birds that are the amplifier host is unconscionable. Draining or filling of wetlands to eliminate mosquito-breeding areas has been used to reduce the incidence of EEE. Current trends in environmental protection are directed at maintaining and reestablishing wetlands and may result in expansion of endemic areas. Widespread application of insecticides is costly and has potential for an adverse impact on the environment. Control of mosquitoes with insecticides in farrowing and nursery facilities and the surrounding area is economically feasible. Geographical information system technology coupled with abundance data on endemic and epidemic mosquito vectors from the area surrounding epidemics in Massachusetts has been used to identify deciduous wetlands as areas at risk for EEE transmission and can be used to schedule pesticide applications efficiently (Moncayo et al. 2000). This methodology could be adapted to other areas. Use of window screens and location of facilities on well-drained sites distant to wetlands also may reduce exposure of pigs to mosquitoes. Swine producers, veterinarians, and laboratory diagnosticians should monitor surveillance data on sentinel animals and epidemiological reports of EEE to determine the prevalence of EEE in their region.

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8.2

Japanese Encephalitis Virus

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SUMMARY

Japanese encephalitis (JE) virus is regarded as an important emerging virus because it is clearly spreading into new areas, increasing its geographical distribution (Mackenzie et al. 2001). Its progress through Asia has been attributed to changes in land use for agriculture and particularly the huge increase in rice growing, but movement eastward into the islands north of Australia suggests that this is not the only factor involved. Pigs are the principal amplification hosts of Japanese encephalitis virus, especially in epidemic areas, and, together with certain ardeid birds, are maintenance hosts in endemic areas.

JE activity tends to be epidemic in more temperate climates, with discrete and obvious epidemics and a relatively low seroprevalence in human and animal populations. This pattern of activity probably reflects susceptibility of the vectors to unfavorable climatic fluctuations. In tropical areas with monsoonal rainfall, the virus tends to be endemic, with occasional cases in young children and a high seroprevalence in susceptible populations, including pigs.

JAPANESE ENCEPHALITIS VIRUS

Japanese encephalitis (JE) is a member of the family *Flaviviridae*, genus *Flavivirus*, in a serological complex known as the JE serogroup, to which are assigned 10 antigenically related members. These are JE, Murray Valley encephalitis (MVE), Kunjin (KUN), West Nile (WN), Alfuy (ALF), St. Louis encephalitis (SLE), Koutango (KOU), Usutu (USU), Yaounde (YAO), and Cacipacore (CPC) viruses (Heinz et al. 2000; Mackenzie et al. 2002). KOU, USU, and YAO viruses are found only in Africa (Karabatsos 1985). SLE virus, an important pathogen, is found only in parts of North, Central, and South America (Monath and Heinz 1996). CPC virus has been isolated only in South America. MVE, KUN, and ALF viruses are all Australasian members of the serological complex. MVE virus is the cause of Australian encephalitis of people and is the most important Australasian member of the complex. KUN virus can also cause encephalitis, although the disease is generally milder than that caused by MVE virus, but it can also cause a febrile illness, some-

times with polyarthralgia and polyarthritis. WN virus is found principally in Africa, the Middle East, western Asia, and southern Europe (with a very large epidemic recently in Romania), and most recently in the United States. It also is a major human pathogen. The geographical range of WN overlaps that of JE virus in India and Pakistan.

JE virus, the most important member of the group, is found in eastern and southern Asia (Burke and Leake 1988; Endy and Nisalak 2002; Umenai et al. 1985; Vaughn and Hoke 1992), and is most closely related to MVE virus by genetic (Poidinger et al. 1996) and antigenic criteria.

PUBLIC HEALTH AND VETERINARY SIGNIFICANCE OF JAPANESE ENCEPHALITIS VIRUS

JE virus is responsible for over 50,000 cases of encephalitis annually, with about 15,000 deaths [reviewed by Umenai et al. (1985), Burke and Leake (1988), Vaughn and Hoke (1992), Monath and Heinz (1996), Tsai (1997), Mackenzie et al. (1998), and Solomon and Vaughn (2002)]. About 1 in 25 to 1 in 300 infections with JE virus results in clinical disease (Benenson et al. 1975; Grossman et al. 1974; Halstead and Grasz 1962; Tsai 1997; Vaughn and Hoke 1992); thus, most infections are asymptomatic. Approximately 25% of clinical cases of JE are fatal, 50% have some form of neurological sequelae such as quadriplegia or mental retardation, and 25% recover fully. The World Health Organization recognizes JE as an important emerging pathogen.

In many countries where JE virus is known to be present, animal health authorities place little significance on the disease (Daniels 2002). Although veterinary data are even less readily available than are public health data, it is accepted that most infections of domestic animals are also asymptomatic. However, JE virus is an animal pathogen causing fetal encephalitis, abortion, and stillbirth in pigs, hypospermia and aspermia in boars (Joo and Chu 1999), and encephalitis in horses. Horse-racing authorities in countries with a well-developed industry, such as Singapore, Malaysia, Hong Kong, and Japan, require vaccination of horses (Ellis et al. 2000). However, although vaccines are available for use in the pig industry

in Japan (Hammon et al. 1971; Igarashi 2002), it seems that porcine disease attributable to JE is not widely recognized outside of Japan, and if it occurs, it is mostly undiagnosed (Daniels 2002). Takashima et al. (1988) confirmed that reproductive disease in pigs still occurs where JE infections are seasonally epidemic and are diagnosed where there is a laboratory capability to support investigations of cases.

THE NATURAL HISTORY OF JAPANESE ENCEPHALITIS VIRUS

Viruses in the JE serogroup are arboviruses that require a mosquito vector for transmission of infection among vertebrate hosts. The life cycle of JE virus is primarily between rice-field breeding mosquitoes and domestic pigs and/or waterbirds. Two classical studies have contributed much to the knowledge of JE ecology. The first was in Japan by Scherer and Buescher and their colleagues (Buescher and Scherer 1959; Buescher et al. 1959a,b; Scherer and Buescher 1959; Scherer et al. 1959a–d), and the other by Grossman et al. in Thailand (Gould et al. 1974; Grossman et al. 1973a,b, 1974; Johnsen et al. 1974). The main mosquito vector throughout Asia is *Culex tritaeniorhynchus*. The major sylvatic hosts are ardeid birds, especially the black-crowned night heron, *Nycticorax nycticorax*, which is considered to be the most important maintenance host, although egrets and other herons may also be involved.

The domestic pig is considered an amplifier host, although it can function as a maintenance host. Other domestic and wild animals are not believed to play a role in the zoonotic cycles, with the possible exceptions of domestic dogs and bats (Burke and Leake 1988; Monath and Heinz 1996; Vaughn and Hoke 1992).

Swine

Pigs are the principal amplification hosts of JE virus, especially in epidemic areas, and are maintenance hosts in endemic areas. The importance of pigs in the maintenance and amplification of JE virus was demonstrated by the studies of Scherer and Buescher and their colleagues in Japan (Scherer et al. 1959c–e). There was a high JE seroprevalence among pigs, and pigs developed a significant viremia following natural infection with JE virus that lasted 2 to 4 days and was capable of infecting *Cx. tritaeniorhynchus* mosquitoes. *Culex tritaeniorhynchus* transmitted JE between pigs in a laboratory setting, and the large numbers of susceptible young pigs on commercial farms provided an immunologically naïve population for amplification of JE virus each year.

Numerous investigations have confirmed the importance of pigs in the natural history of JE (Burke et al. 1985a,b; Carey et al. 1968; Fukumi et al. 1975; Gingrich et al. 1992; Grossman et al. 1974; Hurlbut 1964; Johnsen et al. 1974; Konno et al. 1966; Lee et al. 1962; Oda et al.

1996; Okuno et al. 1973; Peiris et al. 1992; Sazawa 1968; Simpson et al. 1970; Thein et al. 1988; Van Peenen et al. 1974b, 1975; Yamada et al. 1971). In serological surveys, pigs consistently display higher geometric mean antibody titers than other domestic or wild animals.

Despite their obvious importance in amplifying JE virus activity, pigs are not essential for epidemic transmission (Rosen 1986). Pigs are not present on certain islands where epidemics of JE virus have occurred, such as Langkawi (Fang et al. 1980), and only a few pigs were found on Lombok (Olson et al. 1983). The reported low incidence of JE in Indonesia was suggested to be due to the relatively low numbers of pigs in this predominantly Muslim country (Wuryadi and Soroso 1989). Similarly, there were substantially fewer pigs than other domestic animals in parts of India where JE outbreaks have been recorded (Banerjee et al. 1979; Rajagopalan and Panicker 1978; Reuben et al. 1992).

Birds

Serological studies in Japan in 1950 and 1951 showed that avian infection with JE was widespread (Hammon et al. 1958a). Significant numbers of black-crowned night herons (*Nycticorax nycticorax*) and plumed egrets (*Egretta intermedia*) nesting near Tokyo were shown to have antibodies to JE virus. Ardeid birds are now accepted as the maintenance hosts of JE virus in Japan (Buescher et al. 1959b,c; Scherer et al. 1959b) and India (Rodrigues 1988). Their susceptibility has been confirmed in experimental infections (Boyle et al. 1983; Hammon et al. 1951).

In India, early studies showed that pond herons (*Ardeola grayii*) and, to a lesser extent, little cormorants (*Phalacrocorax niger*) had antibodies to JE [cited by Soman et al. (1977)]. JE virus was also isolated from gray herons (*Ardea cinerea*), paddy birds (*Ardeola grayii*), and a black-crowned night heron (Carey et al. 1968). JE viremia was demonstrated in cattle egrets and pond herons after experimental infection (Soman et al. 1977), as was experimental bird-mosquito-bird transmission.

Sparrows, ducks, and pigeons may have a minor involvement in JE transmission cycles. Among wild-caught Japanese tree sparrows (*Passer montanus saturatus*), 20% to 37% had neutralizing antibodies to JE, and experimental infections resulted in viremia and induced neutralizing antibodies (Hasegawa et al. 1975). Occasional infections of tree sparrows (*Passer montanus*) or English sparrows (*Passer domesticus*) have been reported from Thailand (Johnsen et al. 1974) and elsewhere in India (Bhattacharya et al. 1986; Loach et al. 1983). Although a moderate viremia was again observed in experimentally infected sparrows (Banerjee et al. 1979), they have not been considered significant hosts.

Ducks have been suggested as an alternative vertebrate host in areas where pigs were less frequently bred or the incidence of seroconversion to JE was lower than

expected. Experimental transmission of JE had been reported between ducks and from ducks to chickens by *Cx. tritaeniorhynchus*, and infected *Cx. bitaeniorhynchus* were able to transmit the virus to ducklings (Dhanda et al. 1977). A significant number of ducks were found to have antibodies to JE virus in studies in India (Bhattacharya et al. 1986; Khan and Banerjee 1980; Loach et al. 1983), China (Huang 1982), Malaysia (Simpson et al. 1970), Indonesia (Olson et al. 1983), and Thailand (Johnsen et al. 1974). Nonetheless, ducks are believed to be relatively unimportant. Similarly, although pigeons developed a viremia after experimental infection (Banerjee et al. 1979), serological evidence is inconsistent. One study found positive serology (Bhattacharya et al. 1986) and another negative (Huang 1982).

Chickens appear to be only rarely infected with JE and probably have no role in transmission. Most studies have found either a low level of seroconversion (Bhattacharya et al. 1986; Huang 1982; Johnsen et al. 1974; Loach et al. 1983) or none at all (Khan and Banerjee 1980; Olson et al. 1983; Simpson et al. 1970).

OTHER VERTEBRATE SPECIES

The two species susceptible to encephalitis, humans and horses, are not believed to contribute to transmission cycles since the levels of measurable viremia are comparatively low. Mosquito vectors have a feeding preference for large ruminants (Rodrigues 1988), which in turn show a high prevalence of serological evidence of infection (Carey et al. 1968; Huang 1982; Johnsen et al. 1974; Lee et al. 1962; Oda et al. 1996; Olson et al. 1983; Peiris et al. 1993; Reuben et al. 1992; Thein et al. 1988). However, most studies have found that cattle and buffalo do not develop a high-enough viremia to be a significant source of infection for mosquitoes (Carey et al. 1969; Johnsen et al. 1974; Ilkal et al. 1988). One animal that could possibly participate in natural cycles of transmission is the bat (Mackenzie et al. 1998). Bats show serological evidence of natural infection, and experimental studies show detectable viremias (Sulkin and Allen 1974). The potential involvement of these animals in maintenance and spread of JE infections should be further investigated.

Insect Vectors

Various types of evidence contribute to an understanding of vector-borne infections. The natural hosts may be identified by evidence of infection in free-living members of the species concerned, but transmission studies are preferred as proof of vector potential of any insects from which virus has been isolated. Other aspects of vector and host biology are also considered when assessing the likely relative importance of any species to the cycle of transmission.

JE virus has been isolated from a wide range of field-

caught mosquitoes. While *Culex tritaeniorhynchus* predominates in Asia, other species such as *Cx. gelidus* and *Cx. vishnui* may be important in some areas (Burke and Leake 1988; Huang 1982; Vaughn and Hoke 1992). Similarly, the vectors of JE in southern India (*Cx. tritaeniorhynchus*, *Cx. pseudovishnui*, and *Cx. vishnui*) are different from the possible vectors in northern India (*An. hyrcanus*, *An. barbirostris*, *Cx. bitaeniorhynchus*, and *Cx. epidesmus*). JE has been isolated from *Cx. annulirostris* and *Cx. gelidus* in the Torres Strait north of Australia.

Experimental studies of vector competence have confirmed that a number of species of mosquito are competent to act as vectors of JE virus (Burke and Leake 1988; Vaughn and Hoke 1992). Experimental transmission involves either mosquitoes being fed an infected blood meal or fed on an infected host followed by feeding on a susceptible host after a period of extrinsic incubation in the insect. The second host is examined for evidence of infection. Although *Cx. tritaeniorhynchus* can be infected with quite low doses of JE virus (Hill 1970; Soman et al. 1977), only pigs and certain birds develop levels of viremia sufficient to infect significant numbers of mosquitoes.

Much work has been conducted to determine which species of mosquito are likely to feed on potential vertebrate hosts of the virus and the times when risk of transmission is highest. The major *Culex* vectors of JE bite during the night, particularly in the period shortly after sunset and in the early morning between midnight and about 4 a.m. (Gould et al. 1974; Wada et al. 1970), and prefer animals to humans for obtaining blood meals. *Culex tritaeniorhynchus* feed preferentially on bovines [reviewed by Rodrigues (1988), Colless (1959), Mitchell et al. (1973), Gould et al. (1974), Thein et al. (1988), and Reuben et al. (1992)], even when pigs are equally available. However, in a study carried out in Assam, northern India, a high percentage of *Cx. tritaeniorhynchus* (40%) and *Cx. vishnui* (35%) was found to have fed on pigs rather than other species prevalent in the area, whereas *Cx. pseudovishnui* was not attracted to pigs (0.4%) (Bhattacharyya et al. 1994). Traps baited with black-crowned night herons were 3 to 15 times more attractive to *Cx. tritaeniorhynchus* than traps containing egrets or chickens (Scherer et al. 1959a), supporting the observation that these birds are an important host of JE.

The distance that potential vector species can fly is an important aspect of JE virus ecology, particularly for the movement between rice-field breeding sites and villages where pigs and humans are often in close proximity. Studies vary in details but indicate a range of normal activity. In Japan, *Cx. tritaeniorhynchus* mosquitoes flew up to 1.5 km and to at least 50 feet (15 meters) above ground (Scherer et al. 1959a). Unfed mosquitoes showed a maximum dispersal 1 day after release of 5.1 km, and a maximum recorded dispersal of 8.4 km 3 days after release with a mean dispersal of 1.0 km over a 7-day period

(Wada et al. 1969). Dispersal of mosquitoes is predominantly random, although it was observed that *Cx. tritaeniorhynchus* avoided flying over hills. However, as recent experience has indicated, winds can result in long-distance dispersal of potentially infected mosquitoes (Ritchie and Rochester 2001). This is the hypothesized mode of spread in the most recent extension of the range of JE into the islands to the north of Australia.

The mechanisms by which JE survives periods of cold and drought, during which there is no observed mosquito activity, are not known with certainty. Local survival rather than reintroduction from endemic areas is the favored hypothesis, for instance, in Japan at the northern extremity of the range of distribution of the virus (Takashima et al. 1988). Both mosquito- and vertebrate-mediated mechanisms have been proposed, such as survival in overwintering mosquitoes (usually adult females), and vertical transmission of virus (parent to offspring), such as transovarial transmission. The alternative vector-mediated strategy would be migratory movements of virus-infected mosquitoes. Vertebrate-mediated mechanisms include immigration of infected vertebrates, such as birds, and the sequestering of virus in host tissue, with subsequent recrudescence of virus in the blood in spring (Burke and Leake 1988; Sulkin and Allen 1974).

Although vertical transmission has been described for many arboviruses (Leake 1984), it is seldom the dominant mode of transmission. However, it is considered a mechanism by which a virus could survive inclement conditions.

EMERGING RECOGNITION OF THE GEOGRAPHICAL DISTRIBUTION OF JAPANESE ENCEPHALITIS VIRUS

The geographical range of JE virus extends from Japan, Korea, and maritime Siberia southward through China to the Philippines, and throughout southeastern Asia, from Indonesia to Thailand, Vietnam, Laos, and Myanmar. More recently, the known range has extended westward into India, Bangladesh, Nepal, and Sri Lanka (Tsai 1997; Umenai et al. 1985; Vaughn and Hoke 1992). The virus was detected in southeastern Pakistan in 1993 (Igarashi et al. 1994). Since JE is not widely recognized as a veterinary problem, information on occurrence and spread is more readily obtained from reports of human infection.

In Japan, minor epidemics of "summer encephalitis" have been recorded since the early 1870s. The disease gained prominence with a larger epidemic in 1924, which resulted in 6125 cases and 3797 deaths [W. C. Rappleye, cited by Burke and Leake (1988)]. JE was initially termed "Type B" epidemic encephalitis to distinguish it from epidemic encephalitis lethargica, "type A" but the "B" is no longer used. Severe epidemics occurred in 1935,

when JE virus was first isolated, and in 1948 and 1966. Since 1966, the incidence of human infection in Japan has decreased, due both to a decline in the vector populations following increasing use of pesticides on rice crops and to widespread vaccination of people. There are now fewer than 50 human cases per year (Igarashi 1992, 2002).

JE is prevalent in all provinces of China except Xinjiang (Sinkiang) and Xizang (Tibet). Clinical cases were recognized in 1940, and the virus was first isolated in 1941 (Huang 1982). Despite massive vaccination programs, 10,000 to 20,000 cases are still reported annually, with a case-fatality rate of around 10% (Igarashi 1992; Umenai et al. 1985).

Large epidemics of JE were first reported in Korea in 1949 (5548 cases and 2429 deaths) (Vaughn and Hoke 1992). The incidence has been relatively low since 1969, probably due again to increased use of pesticides in rice paddies and to mass vaccination (Igarashi 1992; Vaughn and Hoke 1992).

An outbreak of JE was reported in Guam in 1947 (Hammon et al. 1958b). A second outbreak in the Pacific occurred in 1990 on the island of Saipan that resulted in 10 cases (Paul et al. 1993). The antibody prevalence in residents after the outbreak was 4.2%, but none of 288 sera collected prior to the outbreak were positive, suggesting that JE was a recent introduction. The seroprevalence in pigs was 96%, and it was suggested that the outbreak ended due to the exhaustion of a supply of susceptible amplifier hosts.

Hence, the early observations of JE were predominantly in East Asia. Farther south, limited information is available for Vietnam. The first record of JE in northern Vietnam was in 1965. The epidemic pattern of JE in Vietnam varies, with an epidemic season in the subtropical north between June and August, while the disease has been reported all year round in the tropical south, with a slight peak in July (Igarashi 1992).

The first outbreak of JE in Thailand was reported in 1969 from the Chiangmai Valley (Yamada et al. 1971). Subsequently, two distinct patterns of disease have been recognized in Thailand: annual epidemics in the subtropical north (Grossman et al. 1973a) and endemic activity clearly based on porcine infections, but with only occasional human cases, in the south (Burke et al. 1985a).

To the west of Thailand, JE was first recognized in Myanmar in 1974 in Shan State, which borders Chiangmai Province of Thailand, and cases have been reported each year thereafter (Umenai et al. 1985). Over 50% of pigs were found to have antibodies to JE virus during a prospective serological study in 1982 in Rangoon. Known mosquito vectors were shown to be prevalent, but no cases of human infection were observed during the study period (Thein et al. 1988), suggesting an endemic situation.

Little is known about JE virus activity in Bangladesh. In the first reported outbreak, virus could not be isolated and no new cases were recorded over the next 2 years, suggesting that the disease had been introduced and later died out (Khan et al. 1981). However, seroepidemiology has shown a high prevalence of JE antibody in the northeastern (Sylhet) and southwestern (Kushtia) parts of the country (Islam et al. 1982).

India is another of the countries where JE is considered an emerging problem. Cases of JE were first recognized in 1955 in North Arcot and the neighboring districts of Tamil Nadu and in adjoining areas of Andhra Pradesh (Work and Shah 1956). Occasional cases were reported subsequently in south India, with about 63 cases between 1955 and 1966, and virus was isolated from mosquitoes (Carey et al. 1968, 1969). However, JE did not emerge as a major public health problem in India until 1973. JE has subsequently spread to many parts of the country (Tsai 1997).

The first direct evidence of JE virus in neighboring Pakistan was obtained in 1992 when JE was diagnosed by polymerase chain reaction (PCR) in a patient with encephalitis (Igarashi et al. 1994). A serological study in 1983 indicated that JE might have been present previously (Sugamata et al. 1988).

To the north of India, in Nepal, epidemic encephalitis due to JE virus was first reported in 1978 in the southern lowland region bordering India, a rice-growing area (Joshi 1986; Parajuli 1989). The first proven outbreak of JE in the Kathmandu Valley (altitude, 1300 meters) occurred in 1995, with 15 cases in one hospital and a mortality rate of 53% (Zimmerman et al. 1997). Serological testing of sera from free-roaming pigs in the Kathmandu Valley found that 23 of 44 were positive for JE antibodies.

JE virus was first isolated in Sri Lanka in 1968 (Hermion and Anandarajah 1974). In 1985, the first major outbreak was documented. A prospective study was carried out during 1987 and 1988, in which epidemic activity, human infections, seroconversion in swine, and virus isolation from mosquitoes were investigated. Seroconversion in sentinel pigs preceded human cases by 2 to 3 weeks. A number of virus isolations was made from various mosquito species (Peiris et al. 1992).

To the southeast of Thailand, in Malaysia, JE was first suggested in a report of illness among British prisoners of war during the Second World War (Cruikshank 1951). It was subsequently confirmed in the Malaysian Peninsula in the early 1950s, with virus isolations, serological evidence of human and equine infections, and cases of encephalitis (Paterson et al. 1952; Pond et al. 1954). Epidemiological studies in Sarawak demonstrated that JE was the principal cause of human arboviral encephalitis in that area (Smith et al. 1974). Cases continue to occur in the Malaysian Peninsula (Cardosa et al. 1995).

Only limited information is available on the incidence of JE in the Philippines. Serological surveys have

indicated the presence of JE (Basaca-Sevilla and Halstead 1966; Cross et al. 1977), but JE virus was not found until a number of isolates were obtained from mosquitoes trapped on Luzon in 1977 (Ksiazek et al. 1980).

JE used to be endemic in Singapore, with domestic pigs the host (Hale et al. 1957), but JE virus was eradicated there by vector control and phasing out of pig farming. The last fatal case was in 1984. No cases were observed in 1993 (Anonymous 1994).

Seroepidemiological surveys of human and animal populations indicated that JE was present in various parts of Indonesia in the 1960s (Hotta et al. 1970), but the first report of any clinical illness attributed to JE was in 1971 from Jakarta [L. K. Kho, cited by Van Peenen et al. (1975) and by Wuryadi and Soroso (1989)]. Pigs were incriminated as the amplifying hosts (Van Peenen et al. 1974a,b). Ten sentinel pigs developed antibodies to JE, with virus being isolated from the blood of three (Van Peenen et al. 1975), with similar results being obtained in the same area 15 years later (Daniels et al. 1995). Serological studies of pigs and other village livestock have since shown evidence for JE widespread throughout Indonesia (Daniels et al. 1995; Olson et al. 1983, 1985).

Until recently, there was an opinion that the southeastern limit of JE virus was defined by the Wallace Line, an imaginary line separating the Oriental zoogeographical region from the Australasian zoogeographical region (Kanamitsu et al. 1979; Mackenzie et al. 1997) that passes between Bali and Lombok islands. However, JE virus was isolated from mosquitoes on Lombok (Olson et al. 1985). In addition, JE has been isolated from *Cx. tritaeniorhynchus* trapped on Flores Island (J. G. Olson, unpublished results), and serological evidence for JE found in sentinel pigs in Irian Jaya in 1989–1990 (P. W. Daniels and I. Sendow, unpublished results). Wallace (1869) noted that, although some species of fauna were separated by the strait between Bali and Lombok, more mobile species of insects were common among the islands to both east and west. The arrival of other viruses such as bluetongue and bovine ephemeral fever into northern Australia after the introduction of susceptible ruminant hosts suggests that there would be no significant barrier to the spread of arboviruses in the region (Daniels et al. 1995).

It was against this background that another significant extension to the range of JE occurred to the east. In March and April 1995, there were three cases of encephalitis, two of which were fatal (Hanna et al. 1996a), in an outbreak in the Torres Strait Islands of northern Australia. The nearest-known focus of JE human infection prior to this event was in Bali, approximately 3000 km to the west, and the nearest place from which JE virus had previously been isolated was on Flores Island, approximately 2200 km to the west.

An extensive epidemiological study in both the Torres Strait Islands and Papua New Guinea investigated the

extent of virus activity in the region, the possible source of the virus causing the outbreak, and the risk of JE virus spreading into mainland Australia. Serological studies in Papua, New Guinea clearly indicated that JE had been present in the southwest in Western Province since 1989 and that most pigs had been exposed to the virus. Human disease was demonstrated, and isolates of JE virus were obtained from *Cx. sitiens*-group mosquitoes (Johansen et al. 2000; Mackenzie et al. 2002). A further outbreak of JE on Badu Island in the Torres Strait occurred in 1998, with one human case and seroconversions of pigs on six islands (Hanna et al. 1999). Infection also emerged on mainland Australia, with the first indigenous human case and with seroconversions of pigs (Hanna et al. 1999). JE activity recurred in the Torres Strait in 2000, and virus was isolated from sentinel pigs and *Cx. gelidus* mosquitoes (Pyke et al. 2001; Van den Hurk et al. 2001). However, JE has not been observed since 1998 on mainland Australia until the time of writing, despite active surveillance using sentinel pigs.

MOLECULAR EPIDEMIOLOGY

Early molecular studies based on nucleotide sequences of the prM gene of a wide range of JE virus isolates revealed variation among isolates from different regions or from different time periods within regions. Four genotypic groupings were identified. Genotype 1 contained isolates from Cambodia and northern Thailand; genotype 2 included isolates from southern Thailand, Malaysia, and Indonesia; genotype 3 contained isolates from Japan, Korea, China, the Philippines, Indonesia, India, and Sri Lanka; and genotype 4, identified subsequently, was restricted to Indonesia (Chen et al. 1990, 1992).

The isolates from the subtropical and temperate areas of the more northern countries, where epidemic patterns of infection were observed, tended to group together. This was interpreted as evidence for an overwintering mechanism, rather than annual reintroductions of virus by migratory wildlife from tropical regions (Chen et al. 1990).

Molecular epidemiological studies of 10 JE virus isolates in the Torres Strait Islands of Australia, based on nucleotide sequence analyses of the prM, E, NS1, and NS5-3'UTR areas of the genomes, showed greater than 99% homology. This may be interpreted as indicating that the outbreak originated from a single focus of virus activity (Hanna et al. 1996a; Mackenzie et al. 1997; Ritchie et al. 1997).

Further extensive comparisons were conducted of one of the human isolates in the Torres Strait Island outbreak, the FU strain, based on E and cognate prM gene sequences from 64 isolates from across the geographical range of JE (Williams et al. 2000). In each case, the FU strain grouped with isolates from Indonesia and Malaysia, conforming with the second of the genotypes

listed above, as identified by Chen et al. (1990). These included a 1970 isolate from Kuala Lumpur and a 1981 isolate from Bali (Mackenzie et al. 1998).

Williams et al. (2000) sequenced the full genome of the FU strain and compared it with whole JE virus genome sequences from 15 other isolates. In this much smaller comparison, FU was most closely aligned with an isolate from Korea. This Korean isolate had grouped with isolates from the first of Chen's (1990) genotypes in the prM and E gene analyses, that is, with isolates from Cambodia and northern Thailand. The other isolates in the full genome analysis were from Japan, China, Taiwan, and India—that on prM and E gene analysis grouped in the largest of Chen's (1990) genotypes. The full genome analysis showed FU as being quite distant from these. Full-length sequences from any of the isolates in the second Chen genotype (Chen et al. 1990) were not available, and the close relationship observed with each of the prM and E genes could not be confirmed.

Hence, the isolates from the Torres Strait, at the southeastern extremity of the known distribution of JE, were more closely related genotypically to JE isolates from the adjacent tropical areas of Southeast Asia than to the more geographically distant isolates of mainland and East Asia to the north. This observation would be consistent with a gradual extension of the range of JE to the east through the islands of Indonesia, under local influences, rather than a large jump from elsewhere in Asia as a result of wild bird migration or human-assisted movement.

Chen et al. (1990) noted that the isolates from two of their groupings—from the Indian subcontinent and East Asia and from the grouping involving northern Thailand and Cambodia—were from areas where JE activity was epidemic. The isolates in their other genetic groupings were mainly from areas where JE was endemic. Williams et al. (2000) cautioned against interpreting such data as suggesting that the propensity for epidemic or endemic activity can be deduced from the genetic analyses. The strong association with geographical place of isolation would be a strong confounding factor to any such hypothesis. An epidemic or endemic pattern of virus activity may be as much or more influenced by, firstly, the effect of climate on the vector and, secondly, by the immune status of the vertebrate host populations rather than by any genetic potential of the virus detectable in the simple analyses attempted to date.

In a most recent development, the virus causing the 2000 incursion into the Torres Strait was found to be of a different genotype from the FU isolate, indicating that a second strain of JE virus has emerged in the Australasian zoogeographical region (Pyke et al. 2001).

DIAGNOSIS

Since the distribution of JE is increasing and new countries and new areas are becoming infected, it is pertinent

to review diagnostic capability, especially for porcine infections. The Office International des Epizooties (OIE) lists JE as an equine disease, B (Takashima 2001). Definitive diagnosis in that species is by isolation of virus from samples from the central nervous system. Suckling mouse brain inoculation is still listed as the primary isolation method, with inoculation of cell cultures an option. Specific immunoglobulin M (IgM) or IgG antibodies may be detected in cerebrospinal fluid. For serology, the plaque reduction neutralization test is preferred, and hemagglutination inhibition and complement fixation tests are described (Takashima 2001). Improvement of diagnostic tests for JE has been an active area of research in recent years.

An important consideration in JE diagnosis is that other flaviviruses have various shared antigenic determinants that, with frequent exposure to heterologous viruses, can lead to a complex range of anamnestic responses. The eminent American virologist, Karl Johnson, referred to this as the flavivirus antigenic hall of mirrors [cited by Mackenzie et al. (1998)].

VIRUS DETECTION IN MOSQUITOES

The conventional virus isolation procedures listed by the OIE (Takashima 2001) do not lend themselves to rapid diagnosis or the screening of large numbers of samples. The potential for detection of virus in pools of wild-caught mosquitoes as a surveillance strategy are reviewed below. To become practical for JE surveillance, the concept must be supported by diagnostic test development. Such tests may include antigen detection enzyme-linked immunosorbent assays (ELISAs) (Gajanana et al. 1995; Peiris et al. 1992; Sithiprasana et al. 1994) and genomic amplification by PCR (Mackenzie et al. 1998). The major problem with the latter procedure is the presence of various inhibitors of the reverse transcriptase-polymerase chain reaction (RT-PCR) in mosquito suspensions, and the requirement of a nucleic acid isolation step to circumvent these inhibitors. However, one of its major advantages is that PCR can be reliably carried out on desiccated mosquitoes, so mosquito surveillance can be undertaken without the recourse to cold chain requirements. Williams et al. (2001) and others [reviewed by Mackenzie et al. (1998)] have developed and used RT-PCR for the detection of JE virus under various circumstances. Real-time PCR may offer even more advantages in specificity and speed of test procedure and is undergoing evaluation.

Serology

Serological diagnosis of flaviviruses, and of members of the Japanese encephalitis serological group in particular, is complicated by the need to contend with antigenic cross-reactions from prior exposure to heterologous flaviviruses (Beaty et al. 1995; Calisher 1994). Although

ELISAs (Chang et al. 1984; Konishi and Yamaoka 1982; Ohkubo et al. 1984) and IgM-capture ELISAs (Burke et al. 1985c; Cardoso et al. 1993) have been described for porcine sera, these problems have not been overcome in all situations.

Williams et al. (2001) investigated antibody responses in pigs after primary, secondary, and heterologous challenges with JE, MVE, and KUN viruses. They used blocking ELISAs incorporating monoclonal antibodies to each virus and compared these ELISAs with microtiter serum neutralization tests. Plaque reduction neutralization tests were also included in the investigation (R. Lunt, D. Boyle, K. Newberry, et al., unpublished results). The blocking ELISAs were performed using a modification of the methods of Hall et al. (1995) and Johansen et al. (1997). Hall et al. (1995) differentiated between MVE and KUN virus infections of sentinel chickens with these ELISAs.

To greater or lesser degrees, the JE ELISA detected antibodies even in pigs that had not been exposed to JE, and the ELISAs for other flaviviruses detected cross-reacting antibodies in pigs inoculated with JE (Williams et al. 2001). Hence, the currently available ELISA is not specific for detection of infections with JE in an environment where related flaviviruses are circulating.

Similar problems were encountered with interpretation of results in virus neutralization assays following both primary and secondary inoculations with each of the viruses. Following secondary virus challenge, identification of the infecting agent became virtually impossible. Factors contributing to equivocal diagnosis included variation in immune responses between individual animals, broadening of the immune response following secondary exposure, and anamnestic antibody response to the original infecting virus subsequent to exposure to heterologous virus (Williams et al. 2001).

Broadened and anamnestic immune responses following secondary flavivirus infection have been well documented (Beaty et al. 1995; Calisher 1994; Westaway et al. 1974) and are characteristic of sequential infections of this type. Although the plaque reduction neutralization test has demonstrated better resolution and sensitivity than the microtiter serum neutralization test (Kanamitsu et al. 1979; Ksiazek and Liu 1980), it is also affected by broadly neutralizing antibodies following sequential flavivirus infections (Jirakanjanakit et al. 1997; Kanamitsu et al. 1979; Spicer et al. 1999; Westaway 1965). The evaluation of plaque reduction neutralization using the pig sera generated in this study was consistent with these findings (R. Lunt, D. Boyle, K. Newberry, et al., unpublished results).

Research to develop improved diagnostic tests must be continued. A reliable serological test to assist in surveillance and rapid and specific diagnosis is needed, particularly in countries where JE occurs in epidemic patterns or where it is emerging in new areas.

MANAGEMENT AND CONTROL

Effective management of JE is based on an ability to predict and detect infection in an area and to respond with measures to prevent or reduce the impact of infection on susceptible species. Detection and the development of local knowledge is most effectively based on the long-term use of a surveillance system supported by an effective laboratory capability.

Prevention of JE virus infection may be attempted through immunization of clinically susceptible hosts, immunization of amplifying hosts, vector control, and vector avoidance.

Surveillance

Arboviral surveillance methodologies are usually directed toward four different approaches: surveillance of domestic animal or human hosts, especially clinically; surveillance of wild vertebrate hosts; surveillance of vector abundance and distribution, and, where possible, virus isolation from field-caught mosquitoes; and the serological monitoring of sentinel animals (Daniels et al. 1996; Mackenzie et al. 1994, 1998).

Since the ratio of infections to clinical cases is low in susceptible species, especially people and horses, clinical surveillance approach is not an efficient way of detecting or monitoring JE infections. Nonetheless, detection of disease in people has been the means whereby the incursions of JE into the Torres Strait Islands and the Australian mainland were detected (Hanna et al. 1996a, 1999). On ethical considerations, it is obviously not the ideal approach and does not provide early warning to susceptible populations.

Wild vertebrate host surveillance, such as the monitoring of crow deaths from WN infection in the United States (Eidson et al. 2001), has not been reported to be efficacious for predicting JE outbreaks (Burke and Leake 1988). JE does not cause clinical disease in currently known wildlife hosts. In northern Australia, if JE became endemic, serological sampling of young feral pigs could be potentially useful in monitoring the extent of virus spread.

Vector abundance and distribution can be a useful guide as an epidemic predictor, especially in temperate and subtropical climates where extreme weather events can have a significant impact on mosquito populations due to flooding, rises in sea level, or drought. Virus isolation procedures from insect collections, however, are too time-consuming to be used in routine surveillance (Burke and Leake 1988), and frequently would be too expensive and too difficult logistically. Rapid detection methods, such as PCR, to monitor large pools of mosquitoes for the presence of virus might enable a virus-specific surveillance system based on collections of vectors to be developed. This approach has been used successfully for the monitoring of WN infections (White

et al. 2001), has shown promise for bluetongue surveillance in Australia (Melville et al. 1996), and is currently being investigated for use in northern Australia for JE (C. A. Johansen, S. A. Ritchie, Van den A. F. Hurk, and J. S. Mackenzie, unpublished observations).

Sentinel animals currently remain the most appropriate surveillance method for predicting JE activity. Success depends on selection of a manageable host species that is susceptible to infection. Sentinel chickens have been used successfully for surveillance for related flaviviruses (Mackenzie et al. 1992, 1994; Spencer et al. 2001), but, although meeting the first requirement, do not appear to be suitable for JE surveillance. Chickens showed a high incidence of seroconversion in some studies (Loach et al. 1983), but the proportion of birds found to seroconvert was low in most (Gould et al. 1974; Johnsen et al. 1974; Lee et al. 1962; Simpson et al. 1970).

Sentinel pigs have been used successfully to monitor JE activity in Thailand (Burke et al. 1985b; Gingrich et al. 1987; Johnsen et al. 1974), Japan (Maeda et al. 1978), India (Geevarghese et al. 1987a,b, 1991), Indonesia (Daniels et al. 1995; Van Peenen et al. 1974b) and, more recently, in the Torres Strait Islands and northern Australia. The use of domestic pigs as sentinel animals should be considered only in areas away from human habitation, unless all residents have been vaccinated. Alternative sentinel hosts should be explored.

In Sri Lanka, porcine JE infection occurred in synchronous bursts associated with monsoonal rains and was correlated with significant bovine, ovine, and human seroprevalence in areas of both epidemic (dry zone) and endemic (wet zone) disease. However, the JE seroprevalence in cattle and goats was a better predictor of human infection risk than was porcine seroprevalence (Peiris et al. 1993). One interpretation could be that infection in humans and ruminants occurred only when circumstances favored a "spilling over" of infection from the porcine-mosquito ecosystem.

That observation may suggest that cattle may not give as good an early warning of the risk of human infection as do sentinel pigs. However, cattle are still considered potentially useful for JE surveillance (Mackenzie et al. 1998). They do not develop a significant viremia following infection with JE virus and are, therefore, not believed to participate in natural JE transmission cycles. They appear to show a high proportion of animals in a group that seroconvert and are attractive to most mosquito vector species.

VACCINATION

Vaccination programs have reduced the incidence of human JE in Japan, South Korea, Thailand, China, and elsewhere in Asia (Anonymous 1998; Burke and Leake 1988; Tsai and Yu 1994; Vaughn and Hoke 1992). The efficacy of various JE vaccines and the future prospects

for new and improved vaccines have recently been reviewed by Monath (2002). JE vaccines for human use are manufactured in Japan, South Korea, Taiwan, India, and Thailand as formalin-inactivated virus propagated in mouse brain and in China from formalin-inactivated virus grown in primary hamster kidney cell cultures, using either the Nakayama or Beijing-1 (Ja-GAr-01) JE virus strains, or together as a bivalent vaccine. An attenuated live human vaccine (SA 14-14-2 virus) has been used successfully in China. A number of new approaches are under investigation, the most interesting being a chimeric vaccine using the 17D yellow fever virus (Monath 2002).

Following the JE outbreak in the Torres Strait, inactivated JE vaccine was offered to all of the residents of the outer Torres Strait islands in December 1995 to January 1996. Nearly 9000 doses were administered, with about 88% of the residents receiving at least two doses (Hanna et al. 1996b). In addition, a surveillance system was established to monitor for further incursions using young seronegative sentinel pigs (Shield et al. 1996). The success of both programs was evident when pigs on Saibai Island seroconverted to JE virus in 1996 (Shield et al. 1996) and 1997 without any further human cases.

Attenuated live JE vaccines produced by various manufacturers have been used in pigs in Japan since 1972 to prevent reproductive disease in pigs or to suppress amplification of the virus (Fujisaki et al. 1975; Sasaki et al. 1982; Ueba et al. 1978). The attenuated live vaccine was shown to be more efficacious against natural challenge than a killed vaccine (Ueba et al. 1978). Similar studies in Australia have shown that attenuated vaccine prevents detectable viremia in experimentally challenged, vaccinated pigs, whereas killed vaccines do not (P. W. Daniels, R. Lunt, and D. Middleton, unpublished results, 2001). Vaccination of pigs to interrupt transmission cycles is likely to be ineffective or prohibitively expensive in most circumstances due to the high turnover of the population. Special circumstances for such use of vaccines may be found where the pig population is small and the risk of amplification of virus is high, such as in the Torres Strait Islands of Australia.

There has been conjecture whether a natural equivalent to vaccination may occur where susceptible hosts are exposed to other flaviviruses in the JE serogroup. In parts of India, JE and WN viruses cocirculate. In pigs, infection with WN virus did not prevent the occurrence of a low viremia on subsequent infection with JE virus, although the viremia was probably too low to infect mosquitoes. There was a boosting effect on the already existing WN antibodies (Ilkal et al. 1994). Preliminary studies with Australian members of the JE serogroup have indicated that prior infection with MVE, but not KUN, suppresses development of a JE viremia on experimental challenge (Daniels et al. 2001; Williams et al. 2001).

Vector Management

Control of JE, other than through immunization, is by a combination of vector control and vector avoidance. Although adulticides and larvacides have been employed for vector control in some countries in Asia [reviewed in Burke and Leake (1988) and Vaughn and Hoke (1992)], they have not been very effective, requiring frequent applications and often resulting in insect resistance. In addition, their wide-scale use invokes environmental concerns and potential human health (toxicological) problems. The use of insecticides will probably be largely restricted to outbreak suppression in semiurban areas.

Environmental modifications can be useful. These include reducing vector-breeding sites around pig farms and removing pig farms and pig pens from near human habitation.

JAPANESE ENCEPHALITIS AS AN EMERGING DISEASE

The geographical distribution of JE is increasing and, where new areas become involved, cases of human encephalitis are reported. It is likely that in regions not supported by diagnostic laboratories with a high level of expertise, both human and animal infections and disease may be underreported. Childhood vaccination has reduced the incidence of neurological disease in the human population in countries that have adopted this practice (Tsai 1997).

Human factors are likely to be involved in the spread of JE. The increase in rice growing throughout much of Asia has been noted. This increases the habitat for the mosquito vectors. There has also been a major expansion in the pig industry in many countries throughout Asia, possibly also a contributing factor by increasing the population of amplifying hosts (Ellis et al. 2000). In addition, the substantial increase in the frequency and speed of human travel and the associated aircraft and vessels has created an exponential increase in the opportunities for distribution of infectious agents and their vectors. However, the preliminary molecular studies already reviewed seem to indicate processes more akin to encroachment of range rather than long-distance dispersal. In some situations, wind can be a factor in driving such encroachment (Ritchie and Rochester 2001), but local movements of infected vectors and vertebrate hosts on various types of transport could also be involved.

Although porcine disease due to JE is not reported as a major production issue, in Asia as elsewhere there is increasing concern regarding zoonotic disease. It can be expected that, where intensive pig production is conducted close to human populations, there will be increasing community concern to ensure that public health issues are adequately addressed. Management of the mosquito vector may be the most cost-effective strategy in most

situations. Vaccination of the human population working on farms and living in close proximity should be encouraged.

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8.3

West Nile Virus

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SUMMARY

West Nile virus (WNV), which is a member of the Flaviviridae and closely related to Japanese encephalitis virus, infects and causes disease in birds, which are a primary reservoir, humans, and several species of mammals, including horses. It is transmitted primarily by *Culex* mosquitoes, but species of *Aedes* and several other genera of mosquitoes are also capable of transmitting the virus. Experimental studies have shown that pigs are susceptible to WNV infection, but there are no reports indicating that WNV infection affects swine productivity. The low level of viremia observed in experimentally infected pigs implies that pigs are not an epidemic reservoir for WNV. However, the genomic and biological variability of WNV suggests the possible existence of strains capable of producing high levels of viremia and production loss among pigs.

INTRODUCTION

West Nile virus (WNV) was first isolated in 1937 by Smithburn et al. (1940) from the blood of a woman in Uganda who presented with a mild febrile illness. The virus is a member of family Flaviviridae; the type species of which is the yellow fever virus. Flaviviruses are characterized by single-stranded positive-sense RNA genomes that are protected by enveloped icosahedral capsids. The principal envelope protein of flaviviruses is glycosylated and is designated the E protein, which is the primary target for both neutralizing antibodies and hemagglutination-inhibiting antibodies. The family has been grouped into eight antigenic complexes based on cross-neutralization mediated by polyclonal hyperimmune serum (Burke and Monath 2001). Extensive cross-reactivity between complexes does occur with the hemagglutination inhibition test. These complexes are the Modoc, Rio Bravo, tick-borne encephalitis, Tyuleniy, Uganda S, Dengue, Ntaya, and Japanese encephalitis (JE) groups. Some flaviviruses are not sufficiently related to each other to enable categorization into a specific antigenic complex. WNV belongs to the JE antigenic complex. Other flaviviruses in this complex include Alfuy, Kokobera, Kunjin, Murray Valley encephalitis, and the Stratford virus in Australia; JE virus in Asia; Koutango

and Usutu viruses in Africa; St. Louis encephalitis virus throughout the Western Hemisphere; and WNV established in Africa, Europe, the Middle East, Asia and, most recently (1999), North America.

EPIDEMIOLOGY

With few exceptions, most flaviviruses are transmitted by arthropods, including mosquitoes and ticks. The principal vectors of flaviviruses vary depending on the specific viruses. The primary vectors of WNV are mosquitoes belonging to the genus *Culex*, but the virus has also been isolated from species of *Aedes*, *Ochlerotatus*, *Culiseta*, *Psorophora*, *Anopheles*, *Mimomyia*, and *Mansonia* (Burke and Monath 2001; Turell et al. 2001). There is concern that ticks may be involved in the natural history of WNV.

Birds are considered the amplifying host. The virus has been isolated from naturally infected chickens, crows, herons, and pigeons (Taylor et al. 1956; Work et al. 1955). Experimental infections and serological surveys of birds also indicate that sparrows, doves, various raptor species, geese, blue jays, starlings, coots, grackles, house finches, American robins, red-winged blackbirds, and ducks are also susceptible to WNV infection. Virus titers in viremic birds, including crows and house sparrows, can persist for as long as 6 days and reach levels that can exceed $10^{7.7}$ median mouse lethal dose 50%/ml (LD_{50} /ml) (McIntosh et al. 1969; Taylor et al. 1956).

WNV has been isolated from a wide variety of mammals, including dogs, donkeys, horses, mules (Taylor et al. 1956), camels (Kemp et al. 1973), the Nile grass rats (*Arvicanthis niloticus*) (Kemp et al. 1974), and cats (Komar 2000). Experimental infections and serological surveys of mammalian species indicate that pigs (Geevarghese et al. 1987; Ilkal et al. 1994), water buffalo, goats, sheep (Taylor et al. 1956), hamsters, rabbits, non-human primates, and bats (Burke and Monath 2001) are also susceptible to WNV infection.

THE DISEASE

WNV infection of humans can be inapparent, present as a febrile illness, and on occasion cause encephalitis and death. It is most severe in elderly individuals. In

addition, hepatitis has been associated with WNV infections of people in the Central African Republic. Epidemiological data prepared by the Iowa State Department of Public Health (Currier 2001) suggests that 20% of WNV-infected people will develop recognizable clinical signs, 1% of these individuals will develop neurological manifestations, and fewer than 0.2% will die. The clinical presentation of WNV and its virulence may be related to genomic differences between geographical isolates. Burke and Monath (2001) suggested that the human disease pattern in the Central African Republic might be due to the uniqueness of a local strain of WNV that is genomically distinct from WNV strains in other geographical regions. The virulence of WNV also appears to be strain dependent. This relationship was demonstrated by Umrigar and Pavri (1977), who found that WNV viremia in mice varied by the geographical origin of the isolate; and by Odelola and Fabiyi (1977), who compared Nigerian WNV isolates and reported differences in their biological characteristics.

In animals, WNV infection can be inapparent or it can cause encephalitis, as has been reported in naturally infected birds, such as crows (Steele et al. 2000), and in horses (Cantile et al. 2001). One report describes experimental infection in three dogs, two of which developed mild myopathy (Blackburn et al. 1989). Although pigs can be infected with the virus, there is no indication in the literature that WNV causes disease in pigs, unlike the closely related JE virus. Ilkal et al. (1994) reported viremia in three of four weanling pigs infected with an Asian strain of WNV that persisted for 1 to 4 days and reached maximum titers ranging from $10^{2.2}$ to $10^{2.7}$ mouse LD₅₀/ml. However, the widespread differences in genomic and biological characteristics among WNV strains within and between geographical regions suggest the possible existence of WNV strains that could generate higher viremia of longer duration and/or cause disease and production losses in different age classes of pigs. There is also the possibility that WNV-infected pig populations could indirectly contribute to WNV in nearby poultry and horses by serving to amplify the WNV infection rate in specific mosquito populations. Whether these possibilities exist and to what extent need to be assessed. For this reason, there is a need to determine the effect of different strains of WNV on different age classes of pigs.

PUBLIC HEALTH

The public health significance of WNV-infected pigs is not considered high because of the relatively low levels and short duration of viremia reported in pigs to date (Ilkal et al. 1994). Nevertheless, abattoir workers and veterinarians are at risk if they injure themselves with virus-contaminated equipment, such as knives and needles. In addition, there are no studies reporting the

risk of human infection from virus-infected aerosols generated during slaughter or necropsy procedures. As a precaution, individuals are encouraged to wear gloves, eye protection, and a facemask to minimize the risk of infection resulting from contact with infected blood and tissue, particularly brain. Studies are also needed to determine the time that WNV remains infectious in fresh or frozen meat to properly assess the risk to the consumer.

The public health significance of WNV-infected pigs will need to be reassessed if WNV strains are identified that induce higher levels of viremia of longer duration in pigs. Part of this assessment should be an evaluation of the WNV-vectoring capacity of the different mosquito species that are associated with farms in different regions that will blood-feed on pigs and serve as bridge vectors to human populations.

DIAGNOSIS AND PREVENTION OF WNV

WNV is not currently considered a health problem for pigs. If necessary, however, it can be diagnosed by standard virus isolation and identification techniques using Vero cells. The brain would appear to be the organ of choice for virus isolation based on collective experience with other WNV-infected animals. The virus is considered a biosafety level 3 agent and will require BLS-3 facilities for its isolation. The reverse transcriptase–polymerase chain reaction can also be used to identify the presence of virus in brain or other tissue (Lanciotti et al. 2000). Serological diagnosis can be made by antibody-capture enzyme-linked immunosorbent assays, the serum-virus neutralization test, the hemagglutination inhibition assay, and complement fixation. Care must be taken in the interpretation of test results because cross-seroreactivity among flaviviruses is extensive. The plaque reduction serum-virus neutralization assay using a 90% endpoint is highly specific.

WNV outbreaks can be controlled effectively by mosquito abatement. Efforts to develop vaccines for people and animals are in progress (Davis et al. 2001).

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8.4 Arthropod Vector and Vertebrate Host Associations of West Nile Virus

Carl J. Mitchell

SUMMARY

West Nile virus (WNV) is widely distributed throughout the world and, in 1999, it was introduced into North America, where it has since become endemic. The virus is infectious for a variety of vertebrates, including humans and a wide variety of avian and mammalian species. The information available at present indicates that swine are poor hosts for WNV. However, there is uncertainty concerning the potential for WNV to cause abortion in sows and stillbirth of fetuses infected in utero, as does the closely related Japanese encephalitis virus. A mosquito-borne disease, the ecology of WNV is complex and, in the absence of vaccines, prevention is based on the control of vector populations.

INTRODUCTION

West Nile virus (WNV) was first isolated in 1937 from the blood of a febrile patient in the West Nile district of Uganda (Smithburn et al. 1940). WNV is an RNA virus in the family Flaviviridae, genus *Flavivirus*. Early studies by Smithburn (1942) demonstrated that WNV could be differentiated by serological tests from the antigenically related St. Louis encephalitis (SLE) and Japanese encephalitis (JE) viruses. These flaviviruses are among those currently assigned to the JE antigenic complex (Calisher 1988). WNV is widely distributed in Africa, the Middle East, parts of Europe, and South Asia. Kunjin virus (KUN), a subtype of WNV, is found in Australia and Indomalaysia. In 1999, WNV was recognized for the first time in the Western Hemisphere, where it was associated with outbreaks of encephalomyelitis among people and horses, and fatalities among several species of birds in New York [Centers for Disease Control and Prevention (CDC) 1999a,c; Steele et al. 2000]. Further WNV activity in New York and in several other states during 2000 (CDC 2000b,g) suggests the virus is now firmly established and likely to extend its geographical range in the United States, and perhaps to other countries in the Western Hemisphere.

The basic transmission cycle of WNV, involving mosquitoes and birds, was elucidated quite early. Philip and Smadel (1943) demonstrated the experimental transmis-

sion of WNV by *Aedes albopictus* mosquitoes, and Kitaoaka (1950) showed that *Culex pipiens* and *Cx. tritaeniorhynchus* were competent experimental vectors. The latter study was especially prescient because subsequent studies implicated these species as vectors of WNV in nature. The pioneering investigations by Taylor et al. (1953, 1956) and Work et al. (1953, 1955) provided a basic understanding of the ecology of WNV in Egypt, and a rationale and impetus for pursuing similar studies elsewhere. This found expression in several areas, including Israel, South Africa, France, the former Czechoslovakia, the former USSR, India, and Pakistan, and more recently in Romania, Russia, and the United States as WNV came to be more widely recognized as the cause of illness, and occasionally of more severe neurological disease in people, equines, and some avian species. Also, studies on KUN virus were conducted in Australia and Indomalaysia (Hall 2000). The significant accomplishments made almost half a century ago by small groups of broadly trained scientists working under less than ideal conditions and with limited budgets are sometimes overlooked. However, they form the basis for current advances and continue to offer insights into how such investigations should be conducted. Review articles (Halouzka 1999; Hayes 1989; Hubálek and Komar 2000; McLean and Komar 2002; Peiris and Amerasinghe 1994; Rappole et al. 2000) should be consulted, along with a series of articles published in *Viral Immunology* (Calisher 2000; Hall 2000; Hubálek 2000; Hubálek et al. 2000; Jordan et al. 2000; Lustig et al. 2000). A series of reports (CDC 1999a–c, 2000a–g, 2001) summarizes information concerning the recent outbreaks of WNV in the United States.

EPIDEMIOLOGY

In many areas, most WNV infections in humans occur during childhood and are subclinical or produce only mild febrile disease (Taylor et al. 1956). However, severe WNV infections have been described in adolescents from Israel (Flatau et al. 1981; Pruzanski and Altman 1962) and young children in India (George et al. 1984). Severe disease is usually observed in the elderly. This has also been the pattern during recent outbreaks in the United

States in which clinical cases have occurred mainly among adults (CDC 2000e). Clinical disease usually is characterized by rapid onset of fever lasting 3 to 6 days and accompanied by flu-like symptoms and signs common to a number of other viral fevers. These may include headache, laryngitis, myalgia, conjunctivitis, retrobulbar pain, nausea, vomiting, insomnia, maculopapular rash, and lymphadenopathy (Goldblum et al. 1954; Taylor et al. 1956). Patients with more severe cases may develop hepatitis, pancreatitis and, especially in the elderly, meningitis and encephalitis (Hubálek and Halouzka 1996; Peiris and Amerasinghe 1994). Death is rare, and permanent sequelae have not been documented.

Early outbreaks of WNV infection in the Middle East, sub-Saharan Africa, southern Europe, and southern Asia often were referred to in the literature as West Nile fever, reflecting the relatively benign nature of the disease. More severe disease has been reported recently in Romania, Russia, and the United States (CDC 2000a; Lvov et al. 2000; Platonov et al. 2001; Tsai et al. 1998). Historically, the largest outbreaks have occurred in Israel (Klingberg et al. 1959) and in South Africa, where a widespread epidemic in 1974 resulted in thousands of human infections (McIntosh et al. 1976). Apparent-to-inapparent infection ratios may vary widely. In Israel, a clinical attack rate above 60% was reported in a population of approximately 1000 in a military camp (Klingberg et al. 1959). During an epidemic in southeastern Romania in 1996, 393 human cases were documented, with the ratio of apparent-to-inapparent infections estimated to lie between 1:140 and 1:320 (Tsai et al. 1998). In contrast, in areas of Egypt where WNV is endemic and exposure usually occurs during childhood, up to 90% of the adult population may have antibodies to WNV (Darwish and Ibrahim 1975; Taylor et al. 1956).

In 1999, the localized outbreak in the borough of Queens, New York City, resulted in 62 patients with severe central nervous system disease, 7 of whom died (CDC 2000a, 2001). A subsequent serosurvey in a north Queens neighborhood estimated the incidence of recent WNV infection at 2.6% (CDC 2001). In 2000, although only 21 persons were reported with acute illness attributed to WNV, human infections were widely distributed among four counties in New York State, five in New Jersey, and one in Connecticut (CDC 2001). However, 10 of the 21 cases resided in the borough of Staten Island, New York City. A subsequent serosurvey of Staten Island residents indicated that 4 (0.46%) of 871 showed evidence of recent WNV infection. In addition, 0.12% of residents sampled in Suffolk County, NY, and none of 731 individuals surveyed in Fairfield County, CT, had demonstrable WNV antibodies (CDC 2001). However, despite the widely expanding epizootic in 2000, only about one-third as many human cases were confirmed in 2000 as in 1999.

In humans, the incubation period, i.e., the time from the bite of an infected mosquito to the onset of clinical

disease, can be as brief as 3 days (Hannoun et al. 1964). The upper limit for incubation is less clearly defined. WNV can be isolated from whole blood or serum, with virus titers as high as $10^{3.3}$ ID₅₀/ml (mouse infectious dose 50% per milliliter) in naturally acquired infections (Goldblum et al. 1957). Such virus titers are adequate to infect some species and strains of vector mosquitoes (Taylor et al. 1953 and *vide infra*). Duration of viremia in natural infections may be 4 to 6 days (Goldblum et al. 1957) in many patients, and sometimes up to 10 days (Hubálek and Halouzka 1999). Hubálek and Halouzka (1996) cited a report of WNV being isolated from a patient's blood 22 days after the individual left the area where the infection presumably was acquired, but later suggested (Hubálek and Halouzka 1999) that viremias of such long duration may only occur in immunocompromised patients.

WNV can be isolated from up to 77% of serum specimens collected on the day of onset of fever (Goldblum et al. 1957). High clinical attack rates, e.g., 636 cases of West Nile fever in a population of about 1000 in a military camp in Israel (Klingberg et al. 1959), coupled with information about the frequency, magnitude, and duration of viremia in humans, suggest the possibility of human-mosquito-human transmission of WNV. If such transmission occurs, the virus could be introduced into new areas by viremic, and possibly asymptomatic, humans. The outbreak in New York City during 1999 may have resulted from such an introduction. This possible mode of transmission should be investigated further, especially in epidemic situations.

Generally, human WNV infections occur in midsummer to late summer in temperate regions, a pattern consistent with the peaks in incidence during the 1999 and 2000 outbreaks in the northeastern United States (CDC 1999c, 2000g). In contrast to the temporal pattern displayed by equine and human cases of eastern equine and western equine encephalitis virus infection in the United States, human cases of WNV infection occurred well in advance of equine epidemics in 1999 and 2000 (CDC 2000g). Therefore, clinical WNV disease in horses is unlikely to be a useful surveillance tool for predicting risk to humans.

WEST NILE VIRUS GENOTYPES

Two genotype lineages of WNV have been proposed (Berthet et al. 1997; Savage et al. 1999). One lineage includes WNV from Europe, the Middle East, and north, central, and west Africa, and subtype Kunjin from Australia and Indomalaysia. The other lineage includes isolates of WNV from west, central, and east Africa, including Madagascar. Strains of WNV isolated during the 1999 outbreak in New York have been characterized (Briese et al. 1999, 2000; Jia et al. 1999; Jordan et al. 2000; Lanciotti et al. 1999). A high degree of similarity

between WNV RNA from a 1999 New York isolate and a 1998 isolate from Israel led to speculation about the possibility of an Israeli origin for the former (Lanciotti et al. 1999).

ARBOVIRUS TRANSMISSION CYCLES

Horizontal transmission of arboviruses, i.e., arthropod-vertebrate-arthropod, is accomplished in two ways. Biological transmission of mosquito-borne viruses consists of an infected mosquito feeding on a susceptible vertebrate host, usually a bird or mammal, but sometimes either, depending on the virus and vector involved. If infected by the bite, the vertebrate host becomes viremic for a variable period. More mosquitoes feed and ingest the virus. Following an extrinsic incubation period, during which time the virus replicates and reaches the mosquito's salivary glands, infected mosquitoes feed again and continue the transmission cycle. Infected mosquitoes remain infected for life and have the potential to transmit the virus each time they feed, which generally precedes each gonotrophic cycle and can range from none to a few or many times. Mechanical transmission can occur in some cases, usually following an interrupted feeding on a viremic host, by transfer of virus from contaminated mouthparts of mosquitoes in the absence of virus replication.

Vertical transmission of some arboviruses may occur between mother and offspring via infected eggs. This has been demonstrated for WNV (*vide infra*). Venereal transmission of some arboviruses can take place between adult male and female mosquitoes, but this probably is of minor importance for WNV maintenance or epidemiology.

Different mosquito species and geographical strains of the same species may vary in their vector competence, i.e., in their susceptibility to infection and ability to transmit different arboviruses (Mitchell 1983). Similarly, vertebrate species may vary in their susceptibility and capacity to serve as virus-amplifying hosts by circulating viruses in the blood at concentrations sufficient to infect vector mosquitoes (vertebrate host competence). Most virus-vector relationships are fairly specific within each ecological setting.

ARTHROPOD VECTOR ASSOCIATIONS OF WEST NILE VIRUS

Hubálek and Halouzka (1999) listed 43 mosquito species in seven genera from which WNV has been isolated in Africa, Europe, and Asia. Among these, only two species, *Culex pipiens* and *Aedes vexans*, are common to the list of 14 species associated with WNV in the United States during 1999–2000 (CDC 2000f,g). Other species from which WNV has been isolated in the United States include *Cx. restuans*, *Cx. salinarius*, *Ochlerotatus canadensis*, *Oc. can-*

tator, *Oc. japonicus*, *Oc. triseriatus*, and *Oc. trivittatus*. Five additional species—*Ae. albopictus*, *An. punctipennis*, *Cs. melanura*, *Oc. atropalpus*, and *Psorophora ferox*—were positive for WNV by reverse transcriptase-polymerase chain reaction (RT-PCR) assays.

Hubálek and Halouzka (1999) indicated that WNV has been isolated most frequently from mosquito species in the genus *Culex*. The main vector in Africa and the Middle East is *Cx. univittatus*; however, this species is currently recognized as part of a species complex, and *Cx. univittatus* (*sensu strictu*) may not occur north of sub-Saharan Africa. Harbach (1988) indicated that the member of the complex found in Egypt and Israel, and hence the species from which WNV has frequently been isolated in that region, is *Cx. perexiguus*. *Culex univittatus* appears to be restricted to the temperate highlands of southern and eastern Africa and the southwestern corner of the Arabian Peninsula. *Culex univittatus* is also an important WNV vector in South Africa and Madagascar, although WNV also has been isolated frequently from *Ae. albocephalus* in Madagascar. In Europe the species yielding WNV isolates most often include *Cx. pipiens*, *Cx. modestus*, and *Coquilletidia richiardii*, and in Asia *Cx. quinquefasciatus*, *Cx. tritaeniorhynchus*, and *Cx. vishnui* have been implicated as vectors. The molestus biotype of *Cx. pipiens*, which is autogenous (i.e., not dependent on a blood meal for egg development), stenogamous [i.e., able to breed (mate) in small, confined spaces], and anthropophilic, is prevalent in the Middle East (Harbach et al. 1984) and parts of Russia (Lvov et al. 2000), where it is suspected of being an important vector. Taylor et al. (1956) stated that the molestus biotype of *Cx. pipiens* found in their study areas in Egypt rarely fed on birds. Taylor et al. (1953) demonstrated that the molestus biotype could be infected by feeding on WNV-infected mice with virus titers within the range of titers reported in human infections (Southam and Moore 1954; Goldblum et al. 1957). Tahori et al. (1955) showed that a strain of the molestus biotype from Israel also was a competent vector of WNV.

Hayes (1989) listed 11 mosquito species, including *Cx. pipiens* plus subspecies *pallens* and biotype molestus, that had been shown to be competent vectors of WNV in experimental transmission experiments. These studies were done mainly with African, European, and Asian strains of mosquitoes. Kay et al. (1982) found that six strains of *Cx. quinquefasciatus* from Australia were poorly susceptible or refractory to per os infection with KUN virus and none of four strains tested transmitted the virus by bite.

Jupp (1976) reported that 41% of a South African strain of *Cx. univittatus* became infected after feeding on a viremic chick circulating $10^{2.9}$ SMIC LD₅₀/ml (suckling-mouse intracerebral lethal dose 50% per milliliter) of WNV. Estimates of 10% infection thresholds, expressed as titers found in viremic chicks, for local strains of *Cx. univittatus*, *Cx. pipiens*, *Cx. quinquefasciatus*, and

Cx. theileri were $<10^{2.7}$, $10^{2.7}$, $10^{2.7}$, and $<10^{4.1}$ adult mouse LD₅₀/ml, respectively. Information on WNV infection thresholds of other mosquito species and strains is scant or lacking. This is a fertile area for future research.

Akhter et al. (1982) fed *Cx. tritaeniorhynchus* and *Cx. quinquefasciatus* on five chicks that were circulating $10^{4.9}$ to $10^{5.3}$ SMIC LD₅₀/ml of WNV in their blood. All *Cx. tritaeniorhynchus* ($n = 100$) became infected, and 59% ($n = 17$) to 90% ($n = 19$) of the *Cx. quinquefasciatus* did so, depending on the dose of WNV ingested. A dose-response relationship exists between the virus titer of the infective meal and the proportion of mosquitoes that will become infected and, among those that do become infected, that subsequently can transmit certain arboviruses (Mitchell 1983). An Israeli strain of the molestus biotype of *Cx. pipiens* transmitted WNV to infant mice after feeding on high-titered blood/virus mixtures, but not when the titer was $10^{4.3}$ /ml (Tahori et al. 1955). Jupp (1974) noted that a reduction in the infecting WNV titer from $10^{6.5}$ to $10^{4.3}$ SMIC LD₅₀/ml caused a decrease in the transmission rate from 89% to 33% in *Cx. univittatus*. Therefore, mosquitoes that become infected on very low doses of WNV generally are less likely to transmit the virus.

Turell et al. (2001) fed a variety of mosquito species from the eastern United States on viremic chicks circulating WNV at titers of about $10^{5.2}$ Vero cell plaque-forming units per milliliter (PFU/ml) and $10^{7.0(\pm 0.3)}$ PFU/ml. They found that *Ae. albopictus*, *Oc. japonicus*, and *Oc. atropalpus* were highly susceptible to infection and almost all mosquitoes with disseminated infections transmitted WNV by bite. *Culex pipiens* and *Oc. sollicitans* were moderately susceptible to infection, and *Ae. vexans*, *Ae. aegypti*, and *Oc. taeniorhynchus* were less susceptible.

Four criteria must be met to incriminate a mosquito species as a vector: (1) isolation of the disease-producing agent from wild-caught specimens, (2) demonstration of the ability of the species to become infected by feeding on a viremic host, (3) demonstration of its ability to transmit by bite, and (4) field evidence confirming association of the infected arthropod with the vertebrate population in which the infection is occurring. Perhaps the most difficult criterion to fulfill is the latter. This is usually approached indirectly by collecting blood-fed mosquitoes in the field and identifying the sources of the blood. With the exception of *Cx. pipiens*, which meets the criteria for an endemic/epidemic vector of WNV, one or more of the four criteria have yet to be satisfactorily fulfilled for the remaining 13 mosquito species found positive for WNV or WNV RNA in the United States.

Culex pipiens has yielded several WNV isolates during recent outbreaks (CDC 2000f), and a New York strain of this species has been shown to be a competent vector of WNV under experimental conditions (Turell et al. 2001), but controversy exists about its feeding habits in the epidemic zone. In the northern parts of its range in the United States, *Cx. pipiens* is mainly ornithophilic

(Tempelis 1975) and, with the exception of the autogenous molestus biotype, may feed only rarely on people. It is an endemic vector and almost certainly contributes to WNV amplification and epidemics among susceptible bird species. However, evidence is lacking to show that anautogenous (i.e., dependent on a blood meal for egg development) *Cx. pipiens* is an important vector of WNV to people and horses. By the same token, evidence associating the molestus biotype with WNV transmission to humans in the United States is lacking, although it is suspected of being a vector in the Middle East and Russia (vide supra). Therefore, one or more mosquito species other than *Cx. pipiens* may be serving as bridge vectors between bird populations and humans and horses.

Following experimental infection of adult female mosquitoes by intrathoracic inoculation, low levels of vertical transmission of WNV to progeny have been reported for *Ae. aegypti*, *Ae. albopictus*, and *Cx. tritaeniorhynchus* (Baqar et al. 1993) and *Cx. pipiens* (Turell et al. 2001). Miller et al. (2000) reported the isolation of WNV from a small sample of male *Cx. univittatus*-complex mosquitoes collected in Kenya, suggesting vertical transmission or venereal transmission as the mechanism of infection. Two pools of female *Culex pipiens* and one pool of female *Culex* species collected at Fort Totten (Queens, NY) during January and February 2000 were positive for WNV RNA by a TaqMan RT-PCR assay, and WNV was isolated from one of the *Cx. pipiens* pools in cell culture (CDC 2000c; Nasci et al. 2001). WNV also has been isolated from the tick *Argas hermanni*, collected during the winter in Egypt (Schmidt and Said 1964).

One possible mechanism for arbovirus persistence through the winter in temperate regions is vertical transmission of virus in late summer and fall to cohorts of mosquitoes that are programmed for overwinter diapause (Mitchell 1988). Previously unfed, vertically infected female mosquitoes could emerge from hibernaculae in late winter or spring and initiate new transmission cycles by feeding on susceptible birds or other animals. Unless the *Cx. pipiens* from the winter collections in New York were of the molestus biotype, which may breed year-round in underground habitats, it seems likely that they would have become infected by vertical transmission of WNV. Current evidence indicates that anautogenous populations of *Cx. pipiens* in temperate regions undergo facultative reproductive diapause during the fall and winter, and blood-fed nondiapausing individuals are unlikely to survive the winter (Mitchell and Briegel 1989a,b). Environmental temperatures also influence the rate of replication, and hence the length of the extrinsic incubation period, of arboviruses in arthropod vectors; WNV is no exception (Jupp 1974; Cornel et al. 1993; Dohm and Turell 2002).

WNV has been isolated from three species of ticks (*A. hermanni*, *Ornithodoros capensis*, and *Dermacentor*

marginatus) that also are capable of transmitting the virus under experimental conditions (Hurlbut 1956; Hubálek and Halouzka 1999). In addition, *Hyalomma marginatum*, *H. detritum*, *Rhipicephalus turanicus*, *R. muhsamae*, and *Amblyomma variegatum* have yielded isolates of WNV (Hayes 1989; Hubálek and Halouzka 1999). Species of ornithophilic hard ticks that are competent vectors would be admirably suited to seeding new areas with WNV and extending its range, and soft ticks might be involved in overwinter maintenance of the virus (Schmidt and Said 1964). However, supporting data are difficult to obtain, and the importance of ticks in WNV transmission cycles has not been determined.

Studies suggest the possible involvement of nidicolous hemipteran bugs, mites, and ticks in WNV transmission in rookeries of colonial birds such as swallows, martins, and rooks (Iakimenko et al. 1991; Sixl et al. 1988). This possibility should be investigated further in the United States, where crows are infected frequently and have high mortality rates (CDC 2000a,e,g).

VERTEBRATE HOSTS

Hayes (1989), Hubálek and Halouzka (1999), Komar (2000), and McLean and Komar (2002) cite a number of studies demonstrating experimental WNV infection or evidence of naturally occurring infections in a variety of vertebrate animals, including crows, falcons, chickens, ducks, geese, herons, doves, pigeons, sparrows, pigs, horses, donkeys, mules, sheep, water buffalo, cattle, camels, dogs, lemurs and other primates, wild rodents, laboratory mice, rats, hamsters, guinea pigs, hedgehogs, rabbits, frogs, and humans. Recent reports (CDC 2000g; Komar 2000) indicate that bats (vide infra) and other mammals (gray squirrel, striped skunk, eastern chipmunk, domestic rabbit, and cat) may become infected with WNV in the northeastern United States. However, only birds have been implicated as significant amplification hosts of WNV activity during epidemics.

Birds

In Egypt, high-titered viremia levels of long duration, sufficient to infect vector mosquitoes, were demonstrated in several species of birds by Work et al. (1955) and Taylor et al. (1956). Five species of wild birds infected by mosquito bites usually were viremic for 3 to 4 days. All species had virus titers sufficient to infect some mosquitoes that fed on them, but the hooded crow (*Corvus corone sardonius*) and the house sparrow (*Passer domesticus*) developed viremias with the highest titers. Experimentally infected hooded crows had a 100% fatality rate; however, a high WNV antibody prevalence rate for crows captured in the wild indicated that such a uniformly high mortality rate was unusual in this species (Work et al. 1955). Nonetheless, these observations on the lethality of WNV infections in crows are significant. They should

have provided clues to the etiology of infections in related species of corvids that were dying in significant numbers in New York City during the summer of 1999. In South Africa, 13 avian species were shown to be susceptible to WNV, and only one of 66 inoculated birds failed to develop a detectable viremia (McIntosh et al. 1969). Viremia usually persisted for at least 3 days and was of sufficient titer to potentially infect the principal local vector, *Cx. univittatus*, based on the known per os susceptibility of this species. The susceptibility of a North American strain of *P. domesticus* to WNV infection and its ability to circulate virus at titers sufficient to infect vector mosquitoes for up to 5 days have been demonstrated (N. Komar, personal communication).

A variety of avian species are infected in nature and have yielded WNV isolates or been shown to have antibody against WNV (Hayes 1989; Hubálek and Halouzka 1996, 1999; Komar 2000; Rappole et al. 2000). The latest published data on WNV surveillance in birds in the United States indicate that, during the year 2000, more than 4000 WNV-infected dead birds were reported from 12 states and the District of Columbia, and since 1999 WNV infection has been identified in dead specimens of 76 avian species in the United States (CDC 2000g). Species reported to be infected most frequently were the American crow, blue jay (CDC 2000g), and exotic zoo birds (Steele et al. 2000). Crows and blue jays may have high death rates (CDC 2000a,e). Other infected species included the following: fish crow, black-crowned night heron, great blue heron, mute swan, sandhill crane, Canada goose, mallard, ring-billed gull, laughing gull, bald eagle, merlin, red-tailed hawk, broad-winged hawk, rock dove, American robin, northern mockingbird, eastern bluebird, house sparrow, song sparrow, and yellow-rumped warbler (CDC 1999c, 2000e).

The susceptibility of crows to WNV infection and the lethal nature of such infections in large numbers of individuals make the mortality rate in wild crows a useful surveillance tool, at least in the United States. Available data indicate that crows with WNV infections are likely to have high-titered viremias and may become more sedentary a few days before death (CDC 2000d). Bird-to-bird transmission of WNV is potentially an important mechanism for virus transfer in nature and could have a dramatic impact on WNV ecology and epidemiology. McLean et al. (R. G. McLean, personal communication) found no bird-to-bird transmission of WNV among crows held in separate cages in the same cage rack. However, in an aviary where unrestrained crows infected by needle inoculation were intermixed with uninfected crows, all nine inoculated crows died within 4 to 7 days. Among uninoculated unrestrained control crows, five of seven died after the last crow death in the needle-inoculated cohort.

Langevin et al. (2002) detected brief (1 day), low-level viremia in a chicken (hen) 3 days after its cage mate

was needle inoculated with WNV-NY99. The onset of viremia was delayed by 1 day in comparison to the inoculated chicken. Senne et al. (2000) did not find evidence of direct transmission of WNV to cage mates of chickens that had been infected by needle inoculation. In studies involving seven other species of birds (house sparrow, house finch, European starling, American robin, rock dove, American coot, and mallard) infected by mosquito bites, no WNV infection was detected among cage-mate controls (N. Komar, personal communication). Infection was detected in one of two 3-week-old domestic geese that had been in contact with WNV-NY99 needle-inoculated goslings [see McLean and Komar (2002)], but not in uninfected controls that had been in contact with needle-inoculated turkey poults (Swayne et al. 2000).

Young chickens develop viremia after peripheral inoculation of WNV (Taylor et al. 1956), and such infections may be lethal in individuals less than 3 days old (Turell et al. 2001). Viremia levels are highest in young chicks and consistently reach titers of about 10^7 PFU/ml of blood 2 days after infection (Turell et al. 2001). Titers of $\geq 10^3$ SMIC LD₅₀/ml are sufficient to infect some species of highly susceptible vector mosquitoes (Jupp 1976). When Swayne et al. (2000) inoculated 7-week-old chickens and 2-week-old turkeys with WNV, it was recovered from the blood of chickens up to 8 days after infection, and virus titers in one bird reached a peak of 10^5 TCID₅₀/ml from day 3 to day 4. Most turkeys had titers of $<10^2$ TCID₅₀/ml. Fecal shedding of virus was detected in cloacal swabs from chickens on days 4 and 5 after infection and from turkeys on days 4 and 7.

Equids

Before 1999, WNV had been reported to cause equine encephalomyelitis in Egypt (Schmidt and El Mansoury 1963), the Rhone delta of France (Panthier et al. 1966), Morocco (Tber 1996), Israel (Malkinson et al. 1999), and Italy (Cantile et al. 2000). Most equine infections were thought to result in mild clinical disease or inapparent infections, with only occasional cases of severe disease. However, unusually high morbidity and mortality rates were reported for a cluster of equine cases on Long Island, Suffolk County, NY, during the 1999 epizootic (Bunning et al. 2002). Of 83 horses, 36 (43%) sampled from a circumscribed area within a radius of 6 miles were seropositive, and the clinical attack rate was 42% among the seropositive animals. The mortality rate for the 22 clinical cases from Suffolk County was 35%. These findings raised the question of whether horses might be serving as amplifying hosts for WNV. During 2000, a total of 60 equine cases of WNV were reported from the United States and 24 equine cases during August-September in the Rhone delta of southern France.

Studies documenting the experimental infection of equids with WNV are few, and early attempts to induce and study clinical disease met with equivocal results

(Schmidt and El Mansoury 1963; Taylor et al. 1956). Joubert et al. (1971) and Oudar et al. (1971) produced fever in four of nine equids (jenny, horse, and seven foals) following simultaneous needle inoculation of WNV by the subcutaneous and intravenous routes. Three of the four foals that became febrile developed meningoencephalomyelitis and specific histopathologic lesions in central nervous system tissue (Guillon et al. 1968).

Schmidt and El Mansoury (1963) reported transient (1 day) trace amounts of WNV in the blood of two of six donkeys infected by needle inoculation, but three horses included in the study did not develop detectable viremia. Viremia titers and duration were not reported in a quantitative manner in the studies by Joubert et al. (1971) and Oudar et al. (1971). A study conducted by US Department of Agriculture (USDA) staff [see Bunning et al. (2002)] also reported low levels of viremia ($\leq 10^{2.5}$ TCID₅₀/ml) in four horses infected by needle inoculation of a strain of WNV isolated from a horse during the 1999 epizootic.

Previous studies failed to settle the question of whether equids infected with WNV produce viremia levels of sufficient magnitude and duration to infect vector mosquitoes. Bunning et al. (2002) fed WNV-infected mosquitoes on 12 horses. All horses became infected, as determined by virus isolation or seroconversion, and one horse developed clinical encephalomyelitis with symptoms becoming evident on day 8 after infection. Among the 10 horses that became viremic, titers were generally highest ($\leq 10^3$ PFU/ml) from days 3 to 5, during which times groups of uninfected *Ae. albopictus* were fed daily on eight of the horses. A total of 652 fed mosquitoes, including three lots that fed on the clinically ill horse on days 8 and 9 after infection, survived 7 to 10 days of incubation before being tested individually for the presence of virus. None of these mosquitoes became infected. These data and previously published results suggest that horses infected with WNV develop viremia of low magnitude and short duration and are unlikely to serve as important amplifying hosts.

Swine

WNV was isolated from the serum of a naturally infected pig in Karnataka, India (Ilkal et al. 1994). Serum-neutralizing antibodies were reported from domestic pigs from the same area (Geevarghese et al. 1987), and a low prevalence of hemagglutination inhibition (HI) and complement-fixing antibodies was found in pigs in and around Chandigarh, India (Ratho et al. 1999).

Experimental studies on pigs inoculated sequentially with WNV and Japanese encephalitis virus (JEV), or in reverse order, demonstrated cross-protection in one direction (Ilkal et al. 1994). Pigs with JEV antibodies did not develop viremia of WNV when challenged with WNV, but pigs with WNV antibodies developed low-level JE viremias when challenged with JEV.

Among four pigs inoculated intramuscularly with a high-titered dose ($10^{6.7}$ SMIC LD_{50}) of a WNV strain that had been passed eight or nine times in mouse brain, only three developed detectable viremia, and all developed HI antibodies with titers of 20 to 320 (Ilkal et al. 1994). Viremia levels in two pigs ranged from $10^{2.0}$ to $10^{2.7}$ LD_{50} /ml and were detectable on days 1 and 2 after infection. Titers ($\leq 10^{2.3}$ LD_{50} /ml) in the third pig were detectable on days 1 to 4. An additional three pigs, infected by the bites of 7 to 21 WNV-infected *Cx. vishnui* mosquitoes, failed to develop detectable viremias although each developed HI antibodies against WNV 7 to 10 days after being bitten. The authors concluded that pigs were poor hosts for WNV, but further studies are warranted, especially concerning the question of whether WNV might cause abortion in sows and stillbirth of fetuses infected in utero, as does the closely related JEV.

Dogs and Cats

A serosurvey of dogs in South Africa found WNV antibodies in 37% of 377 sera tested, and WNV was isolated from one of 110 dog sera that were negative for HI antibodies (Blackburn et al. 1989). Also, WNV was isolated from the blood of two of three dogs infected experimentally and viremia levels, although low ($\leq 10^{2.8}$ /ml), were deemed sufficient to infect a small proportion of the local vector, *Cx. univittatus*, based on the known per os susceptibility of this species to WNV (Jupp 1976). Two of the dogs had a mild recurrent myopathy, but no other abnormalities were noted. Four dogs bitten by mosquitoes infected with the New York WNV strain had low-level viremia of short duration (R. A. Bowen, personal communication). Evidence to date indicates that dogs do not play an important part in the ecology and amplification of WNV activity.

Komar (2000) reported a WNV isolate from a cat in New Jersey. Currently, pathogenesis studies in cats infected with WNV are under way. Preliminary data show that some cats infected experimentally by mosquito bite may circulate virus at levels sufficient to infect vector mosquitoes and that some cats can become infected by eating WNV-infected mice (R. A. Bowen, personal communication). However, because of their domesticated habits, it seems unlikely that cats will play a significant role in WNV amplification.

Bats

WNV has been isolated from bats, and serological evidence suggests that different species become infected in many parts of the world (Taylor et al. 1956; Akov and Goldwasser 1966; Paul et al. 1970; Fontenille et al. 1989). Recently, WNV infection was confirmed in two species of bats, *Eptesicus fuscus* and *Myotis lucifugus*, in New York (CDC 2000f). There is an extensive literature on flavivirus infections in bats (Sulkin and Allen 1974), including infections with SLE and JE viruses, which are

closely related to WNV. Some species, including *E. fuscus* and *M. lucifugus*, are susceptible to flavivirus infection by the oral route as well as by mosquito bite (La Motte 1958). These and other bat species could play a role in flavivirus transmission cycles and as overwintering hosts (Cross et al. 1971).

PREVENTION, TREATMENT, AND CONTROL

Commercial WNV vaccines are currently unavailable for human or veterinary use, but studies are under way in several laboratories to correct these deficiencies. Mice and young geese vaccinated with an attenuated live strain of WNV were 100% protected from intracranial challenge with wild-type WNV isolated from a naturally infected moribund goose (Lustig et al. 2000). Also, a recombinant plasmid DNA vaccine induced protective immunity in mice and horses challenged with WNV by needle inoculation (mice) and infectious mosquito bites (mice and horses) (Davis et al. 2001).

There is no specific treatment for WNV infection. Supportive care and palliative measures may reduce mortality rates and the severity of clinical disease. The current search for inhibitors of hepatitis C virus may result in the discovery of compounds that inhibit the replication of flaviviruses in general (Leyssen et al. 2000).

Avoidance of mosquito bites by the use of repellents or other measures can significantly reduce the risk of being infected with mosquito-borne viruses. Vector control also may effectively reduce the risk of being bitten by infected mosquitoes. The greatest successes in consistently suppressing pest and vector mosquito populations have occurred in areas with tax-supported, organized mosquito control agencies, e.g., California, Florida, and New Jersey, in the United States. Source reduction and treatment of aquatic habitats with biological control agents and/or chemicals, accompanied by surveillance of adult mosquito populations and appropriately timed adulticide treatments, yield the best results. Commonly used biological control agents include *Bacillus thuringiensis israelensis*, *Bacillus sphaericus*, and larvivorous fish such as *Gambusia affinis*. A variety of chemicals, including organophosphates, carbamates, pyrethroids, and insect growth regulators, are used for mosquito control in the United States (Rose 2001). In the event of a mosquito-borne arbovirus outbreak, emergency measures often are implemented to reduce adult mosquito populations quickly. These measures include application of ultra-low-volume space sprays by ground-operated or aerial equipment. In some cases, such treatments may rapidly reduce the number of vector mosquitoes (Mitchell et al. 1969). However, beginning such treatments early enough during an epidemic to reduce virus transmission significantly is difficult, and emergency measures often are unknowingly implemented when epidemics are

waning. Preemptive adult mosquito control was undertaken in some communities in the northeastern United States after WNV activity was detected during 2001 (CDC 2000d,e). It is unclear how successful such measures were in reducing the risk of infection and the number of cases in treated areas (CDC 2001). Carefully designed and executed control demonstrations will be required to answer these questions.

ACKNOWLEDGMENTS

I am grateful to Michael Bunning (Armed Forces Medical Intelligence Center, Ft. Detrick, MD), Richard Bowen [Colorado State University (CSU), Ft. Collins, CO], and Kathlene Julian [Centers for Disease Control (CDC), Ft. Collins] for providing literature; to Michael Turell (US Army Medical Research Institute of Infectious Diseases, Ft. Detrick, MD), Robert McLean (National Wildlife Health Center, US Geological Survey, Madison, WI), and Nicholas Komar and Roger Nasci (both CDC, Ft. Collins) for sharing unpublished results and manuscripts; and to Charles Calisher (CSU, Ft. Collins), Nicholas Komar, and Richard Bowen for reviewing the manuscript and making corrections and helpful suggestions.

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9 PORCINE CIRCOVIRUS

9.1

Animal Circoviruses

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SUMMARY

Circoviruses, the smallest viruses of animals, have been recognized for decades. Although the pathogenic mechanism is unclear, these viruses are capable of causing characteristic histopathologic changes (lymphoid depletion) and clinical manifestations (growth retardation, dermatitis, and jaundice) in animals. In swine, porcine circovirus is being evaluated for its role in new diseases such as postweaning multisystemic wasting syndrome and porcine dermatitis-nephropathy syndrome.

PORCINE CIRCOVIRUS

History and Taxonomy

Porcine circovirus (PCV) was first recognized as a noncytopathic viral contaminant of a continuous pig kidney cell line PK-15 (ATCC-CCL31) (Tischer et al. 1974, 1982). Based on its unique morphological and genomic characteristics, the virus has been classified in the genus *Circovirus* in the family Circoviridae along with psittacine beak and feather disease virus (PBFDV) (Lukert and Jeal 1995; Meehan et al. 1997). Chicken anemia virus, another member of family Circoviridae, was recently assigned to the new genus *Gyrovirus* (Pringle 1999). Although human TT virus was initially assigned to the family Circoviridae (Miyata et al. 1999), it has been proposed to place the virus into a new family named Circonoviridae (Mushahwar et al. 1999).

In 1998, a DNA viral agent morphologically similar to PCV was isolated from pigs with clinical problems of progressive weight loss and respiratory distress in herds in western Canada. Although the new virus shared morphological similarities with PCV, it was antigenically and genetically distinct (Allan et al. 1998b; Meehan et al. 1998; Morozov et al. 1998). To reflect these differences, circoviruses isolated from pigs clinically affected with wasting syndrome were designated PCV type 2 (PCV2) and the PK-15 cell contaminant was denoted PCV type 1 (PCV1) (Allan et al. 1998a; Meehan et al. 1998). Although PCV2 was recognized only recently, a retrospective serological survey found that PCV2 may have been present in the domestic swine population since at least 1972 (Walker et al. 2000).

Physicochemical Properties

PCV is a small, nonenveloped virus approximately 17 nm in diameter with icosahedral symmetry. The virus contains a negative-sense, single-stranded circular DNA genome (Tischer et al. 1982). The buoyant density of PCV1 is 1.33 to 1.37 g/cm³ on a CsCl gradient (Allan et al. 1994; Tischer et al. 1974). PCV1 does not hemagglutinate erythrocytes of various animal species, including pig, sheep, cattle, chicken, turkey, and guinea pig (Allan et al. 1994). Whether PCV2 can hemagglutinate erythrocytes of any species is not known.

PCV is highly resistant to inactivation in the environmental, which is a concern for effective cleaning and disinfection of swine production buildings and facilities. Allan et al. (1994) found that PCV1 remained infectious even after exposure to pH 3 and high temperatures, 56°C and 70°C for 15 minutes (Allan et al. 1994). Quaternary ammonium (Roccal-D) and phenol (One-Stroke Environ) disinfectants were shown to be effective in inactivating PCV2 in vitro (Royer et al. 2000).

Genomic Organization and Gene Expression

The genomes of PCV1 and PCV2 are 1759 and 1768 nucleotides long, respectively (Hamel et al. 1998; Meehan et al. 1997, 1998; Morozov et al. 1998). Overall, PCV1 and PCV2 share less than 80% nucleotide sequence homology and approximately 75% homology at the amino acid level (Morozov et al. 1998). Computer-aided analyses of PCV1 and PCV2 DNA identified 11 potential open reading frames (ORFs) for both viruses (Hamel et al. 1998). However, ORFs 1 and 2 comprise more than 90% of the genome. ORF1 is more conserved between PCV1 and PCV2 than other ORFs, and showed 83% and 86% homology at the nucleotide and amino acid levels, respectively (Morozov et al. 1998). In contrast, 67% nucleotide and 65% amino acid homology was observed in ORF2 between PCV1 and PCV2. For this reason, it was suggested that ORF2 or its product could be used to differentiate the two types of PCV (Mahe et al. 2000).

Circoviruses are postulated to replicate via a double-stranded replicated form using a rolling circle mechanism (Mankertz et al. 1997; Meehan et al. 1997). Viral mRNAs are believed to be transcribed in the nucleus from both DNA strands (i.e., ambisense) (Meehan et al.

1998). Early protein(s) and nonstructural protein(s) of PCV are postulated to be involved in the induction and regulation of viral DNA replication (Tischer et al. 1995c). Accumulation of structural proteins, a process independent from DNA synthesis, takes place in the cytoplasm of infected cells and results in cell destruction (Tischer et al. 1995c).

The estimated molecular size of the deduced polypeptide products encoded by individual potential ORF ranges from 2 to 36 kD (Hamel et al. 1998). ORF1 is believed to encode for the putative replicase protein, which is required for the replication of viral genome (Mankertz et al. 1998). The predicted molecular mass of the ORF1 product is 35.7 kD for PCV1 and 35.8 kD for PCV2. The ORF2 is postulated to encode for capsid protein, the major structure protein (Mankertz et al. 1998). The molecular mass of the deduced ORF2 product is predicted to be 27.8 kD for PCV1 and 27.8 kD for PCV2 (Hamel et al. 1998). The predicted sizes of proteins encoded by individual ORFs 3 to 11 of PCV1 are approximately 23, 13, 10, 6.7, 6, 4, 3.4, 3.7, and 3 kD, respectively. Molecular masses of PCV2 ORF 3 to 11 products are predicted to be approximately 12, 7, 6, 3, 2, 2, 5, 4, and 2 kD, respectively (Hamel et al. 1998). Although computer-aided sequence analyses identified 11 potential ORFs, only one protein, with molecular mass of 36 kD, was immunologically and chemically identified in the PCV1 virion (Tischer et al. 1982). Another protein with a molecular mass of 31 to 33 kD was also identified later in PCV1, but its role has not been determined (Tischer et al. 1995b). Although the actual composition of PCV2 structural protein(s) has not been well characterized, a longitudinal study revealed that pigs experimentally infected with PCV2 developed antibodies to three virus-specific putative polypeptides with approximate molecular mass of 28, 28.5, and 35 kD (Pogranichnyy et al. 2000). Investigators speculated that 28- and 35-kD proteins are products of ORF2 and ORF1, respectively.

Viral Replication

PCV has been found to infect many different types of cells in a variety of tissues. The virus or viral antigens were isolated or detected in liver, spleen, Peyer's patches of the intestine, lung, tonsil, kidney, and other tissues, with the exception of the central nervous system (Allan et al. 1995; Rosell et al. 1999). In these tissues, the main target cells are lymphocytes, macrophages, hepatocytes, and renal tubular and ileac epithelial cells (Rosell et al. 1999). Intensive basophilic inclusion bodies of 5 to 25 nm in size can be seen within cells of tissues from infected pigs (Harding and Clark 1997; Kiupel et al. 1998). However, it is not known how PCV establishes infection and replicates in pigs.

In vitro, PCV can replicate in PK-15 cells, African green monkey kidney cell line Vero, semicontinuous pig lung cells, semicontinuous swine testicle cells, primary

bovine kidney cells, semicontinuous bovine lung cells, semicontinuous bovine testicle cells, primary lamb kidney cells, semicontinuous lamb testicle cells, and other primary and permanent cell cultures (Allan et al. 1994). In cell culture in vitro, the virus does not cause visible lysis of affected cells (Allan et al. 1994; Tischer et al. 1982), and the presence of the virus can be demonstrated by only indirect methods, such as immunofluorescence assay (Allan et al. 1998a; Ellis et al. 1998; McNeilly et al. 1999; Sorden et al. 1999; Tischer and Buhk 1988), in situ hybridization (Allan et al. 1998a; Choi and Chae 1999; Ellis et al. 1999; McNeilly et al. 1999), and polymerase chain reaction-based assays (Larochelle et al. 1999; Morozov et al. 1998). Using these techniques, PCV can be found in only a small number of cells. Heterogeneous cytoplasmic inclusion bodies consisting of electron-dense paracrystalline arrays of small nonenveloped viral particles are also found in infected cells (Kiupel et al. 1998; Stevenson et al. 1999).

In vitro circovirus replication depends on the availability of cellular enzymes that are expressed during the S phase of cell growth (Tischer et al. 1987). The yield of progeny virus and the number of infected cells were found to be increased by pretreatment of cells with D-glucosamine, but care must be taken since the reagent has a toxic effect on the cell culture (Allan et al. 1998a; Tischer and Buhk 1988). D-Glucosamine synchronizes the cell cycle and initiates virus replication by enabling the PCV genome to enter the nucleus of cells. If the cell is not treated, the virus will enter the nucleus of the daughter cell at the end of mitosis, resulting in very few cells becoming infected (Tischer et al. 1987).

Epidemiology

The presence of PCV has been reported virologically and/or serologically in Canada, France, Germany, Great Britain, Japan, Korea, the Netherlands, Northern Ireland, Spain, Taiwan, and the United States (Allan et al. 1998a; Choi and Chae 1999; Dulac and Afshar 1989; Edwards and Sands 1994; Kiupel et al. 1998; LeCann et al. 1997; Segales et al. 1997; Tischer et al. 1995a; Wellenberg et al. 2000). The limited numbers of serological surveys have demonstrated that PCV infection is ubiquitous in swine populations (Allan et al. 1994; Dulac and Afshar 1989; Tischer et al. 1986, 1995a; Walker et al. 2000). In one study, the prevalence of PCV infection was estimated to be 95% in the German swine population (Tischer et al. 1987). However, early serological surveys were conducted using PCV1 as antigen. Recent studies revealed cross-reactivity between PCV1 and PCV2 antibodies (Allan et al. 1998a; Mahe et al. 2000; Pogranichnyy et al. 2000). Consequently, estimates of the prevalence of PCV1 or PCV2 infection need to be reassessed. Recently, using a multiplex polymerase chain reaction assay that enables typing of PCV, it was demonstrated that PCV2 is the main type of PCV circulating in

the swine population (Ellis et al. 2000; Larochelle et al. 1999). Only 4% to 8% of the viruses identified in the field were PCV1 (Larochelle et al. 1999).

Field observations suggest that PCV can cross the placenta and infect fetuses, i.e., congenital infection (Allan et al. 1995; West et al. 1999). PCV2 has been detected in hearts or fetal thoracic fluids of aborted or stillborn fetuses (West et al. 1999). A field-based longitudinal study demonstrated vertical transmission of PCV1 in sows exposed to the virus at some point during pregnancy (Hines and Lukert 1994).

Horizontal transmission of PCV has also been documented in pigs commingled with inoculated pigs (Tischer et al. 1986). PCV1 and PCV2 were found in nasal secretions and feces from experimentally inoculated pigs, although the animals did not show any clinical signs or pathological changes (Allan et al. 1995; Krakowka et al. 2000; Tischer et al. 1986). These observations suggest that PCV can spread through nose-to-nose contact and/or fecal-oral exposure.

Mice were reported to be susceptible to PCV2 under experimental condition (Kiupel et al. 2001). Nevertheless, involvement of nonporcine species in the transmission of PCV to pigs remains to be determined.

Early seroepidemiological surveys demonstrated that PCV1 was prevalent in swine. The virus has been detected by polymerase chain reaction assays in 4% to 6% of pigs, regardless of their health status (Larochelle et al. 1999; Ouardani et al. 1999), but has not been associated with any particular disease. Under experimental conditions, pigs inoculated with PCV1 seroconverted to the virus by day 7 after inoculation, and antibody titers continued to raise for 5 weeks after inoculation (Tischer et al. 1986). The virus was detected in various tissues and shed in nasal secretions and feces (Tischer et al. 1986), suggesting that naïve pigs were susceptible to PCV1 (Allan et al. 1995). However, experimental inoculation of PCV1 failed to induce pathological changes or clinical disease in pigs, suggesting that PCV1 was not pathogenic for swine (Krakowka et al. 2000; Tischer et al. 1986). Although tremors were reproduced in young pigs infected with circovirus, and circovirus was isolated from newborn piglets with *congenital tremors* (i.e., chronic contractions of the skeletal muscles), the virus isolate was not characterized genetically or antigenically (Hines and Lukert 1994).

PCV2 is also prevalent in domestic swine populations (Tischer et al. 1995a; Walker et al. 2000) and has been implicated in numerous disease syndromes, such as wasting syndrome (Ellis et al. 1998; Harding and Clark 1997) and dermatitis/nephropathy syndrome (Rosell et al. 1999, 2000). However, a causal role of PCV2 in these diseases has not been conclusively demonstrated under experimental conditions.

Both PCV1 and PCV2 have been found in aborted fetuses collected from the field in conjunction with repro-

ductive failure and in fetuses obtained from pregnant sows inoculated with the virus, suggesting that the virus can cross the placental barrier (Allan et al. 1995; West et al. 1999). However, the significance of these findings related to reproductive failure is unknown to date.

OTHER CIRCOVIRUSES OF ANIMALS

Chicken Anemia Virus

Chicken anemia virus (CAV) was first identified in Japan in association with a disease resulting in 50% mortality (Yuasa et al. 1979). Clinically, CAV infection is characterized by growth retardation, depression, ruffled feathers, anemia, and marked pallor that extends to internal organs (Yuasa and Imai 1986). Serological surveys have indicated that the virus is ubiquitous in commercial chicken flocks (McNulty et al. 1989).

CAV is an icosahedron and contains a 2.3-kb single-stranded circular DNA (McNulty et al. 1989; Todd et al. 1990). The virion is 18 to 26 nm in diameter, with a density of 1.35 to 1.36 g/cm³ (McNulty et al. 1990; Todd et al. 1990). The virus is antigenically and genetically distinct from PCV and PBFDV (Bassami et al. 1998; Todd et al. 1991). Three ORFs have been identified and encode for three putative proteins with molecular weights of 51.7, 24.1, and 13.3 kD, respectively (Noteborn et al. 1991). Only one protein with a molecular mass of 50 kD was detected by gel electrophoresis (Todd et al. 1990).

CAV has been reported to be associated with several diseases or syndromes in chicken, i.e., “blue wing” disease, hemorrhagic anemia syndrome, gangrenous dermatitis, and aplastic anemia (Yuasa 1993). In severe cases, naïve hens infected with CAV show anemia, subcutaneous hemorrhages, and gangrenous dermatitis, which lead to enhanced susceptibility to secondary viral or bacterial infections (Rosenberger and Cloud 1989, 1998). The virus can be detected in all tissues, but thymus and bone marrow are reported to be the most severely damaged (Taniguchi et al. 1983). Usually, infected birds die, but some may recover after 3 to 4 weeks (Rosenberger and Cloud 1998).

Experimentally, the disease was demonstrated in chicks inoculated with CAV at 1 day of age, but was more difficult to reproduce in chicks at 3 weeks of age or older or in chickens with neutralizing antibody (Yuasa et al. 1983). The pathogenicity of CAV is reportedly enhanced when chickens are concurrently infected with infectious bursa disease virus or Marek’s disease virus (Yuasa et al. 1980). Dual infection results in much higher mortality and morbidity and more severe lesions than does CAV infection alone (McNulty 1997).

Histopathologic lesions in infected chickens are characterized by lymphocyte depletion in the cortex and medulla of thymus and bone marrow (Rosenberger and Cloud 1998). Infected chickens are also depleted of

erythrocytes, thrombocytes, and granulocytes, and precursors of these cells are replaced by adipose tissue. The bursa of Fabricius and spleen are also depleted of lymphoid cells, but less severely than the thymus (Rosenberger and Cloud 1998), suggesting that T cells are the main targets of virus. There is some degree of swelling in the liver and bursa of Fabricius. Muscular atrophy and hemorrhage have been observed (Taniguchi et al. 1983; Yuasa et al. 1979). Severe lymphoid depletion in lymphoid tissue and the hematopoietic system leads to the speculation that CAV may induce immunosuppression. In one study, the immune response of infected chickens to vaccines against different viruses, such as turkey herpesvirus and Marek's disease virus, was depressed after inoculation with CAV (Otaki et al. 1988).

Exposing breeder flocks to the virus before chickens go to egg production prevents transovarian transmission. Acquired immunity in the flock is known to prevent vertical transmission and horizontal transmission of the virus for 1 day to 2 weeks (Rosenberger and Cloud 1998). In the United States, the current control strategy for CAV is to expose serologically negative birds at 12 to 15 weeks of age to known positive flocks (Fussell 1998). In Europe, vaccination with autogenous live virus has been proven to prevent vertical transmission of CAV by mimicking natural infection (Fussell 1998). Hens that are hyperimmunized with an inactivated CAV vaccine are reported to perform better than unvaccinated hens and have better body weight gain, viability, and feed conversion rates. Furthermore, vaccination was reported to be efficacious in protecting chicks from developing the disease on a farm with ongoing disease problems (Fussell 1998).

Psittacine Beak and Feather Disease Virus

PBFDV is another member of family Circoviridae and a circular single-stranded DNA virus with icosahedral symmetry (Ritchie et al. 1989). The virus infects a wide variety of species of wild and captive birds and causes anorexia, vomiting, weakness, dystrophic feathers, and deformities of the beak and bones. In some cases, severe leukopenia or anemia and pancytopenia have been observed (Schoemaker et al. 2000).

Histopathologically, hepatic necrosis and atrophy of lymphoid follicles with occasional necrotic foci and polymorphic basophilic polymorphic inclusion bodies typical of circovirus are frequently observed (Schoemaker et al. 2000). Many of the feather abnormalities are due to retention of a hyperkeratotic feather sheath. Necrosis of epidermal cell and epidermal hyperplasia and hyperkeratosis were reported in naturally exposed bird (Pass and Perry 1984). Necrosis and degeneration of epithelial cells lining the developing feather follicles are common because the virus infects and replicates in these cells (Jacobson et al. 1986; Latimer et al. 1991; Ritchie et al. 1990). The virus can also be detected in epithelial cells and macrophages within thymus and bursa. Necrosis of

epithelial cells of the tongue and mouth has also been reported (Jacobson et al. 1986; Ritchie et al. 1990).

Once exposed, birds can harbor the virus for 10 to 15 years, and most develop cryptosporidial infections that generally occur in birds with immunodeficiency. The majority of infected adult birds develop viremia, but birds with well-established humoral and cell-mediated immunity clear the virus and undergo asymptomatic infection (Ritchie et al. 1992). Several factors are considered important as to whether birds will have a protective immune response or develop fatal disease. Some of these factors include the presence or absence of maternal antibodies, route of viral exposure, exposure dose, and presence or absence of conditions that promote immunotolerance (Ritchie 1995).

Pigeon Circovirus

Pigeon circovirus first was recognized in Canada in 1986 and, in 1989, was reported in Australia (Woods et al. 1993). Clinically, infected birds develop anorexia, lethargy, and rapid weight loss, and die. Histologically, the most common lesions are lymphofollicular hyperplasia and discrete lymphofollicular necrosis in the bursa of Fabricius, cytoplasmic inclusions in macrophages and epithelial cells, lymphoid depletion, atrophy, and cystic changes with lymphoid hyperplasia (Woods et al. 1994). Infections are most common in animals at 7 to 8 weeks of age. Mortality rates range from 1% to 100%. Although it is not clear whether death is due to secondary infections, birds are also commonly infected with *Pasteurella* sp., *Chlamydia psittaci*, *Escherichia coli*, mycoplasmosis, and ascaridiasis (Ritchie 1995). Pigeon circovirus is antigenically different from the PBFDV, but causes a similar clinical problem (Woods et al. 1994).

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9.2 Postweaning Multisystemic Wasting Syndrome and Porcine Circovirus: A United States Perspective

Perry A. Harms

SUMMARY

Although porcine circovirus (PCV) has been recognized since 1974 (Tischer et al. 1982), it was only recently associated with clinical disease in swine, including postweaning multisystemic wasting syndrome, congenital tremors, abortion, and porcine dermatitis and nephropathy syndrome. PCV infection is common in swine, but disease is seen in a relatively small proportion of herds and in only some of the pigs within an affected herd. Given the limited tools currently available for the prevention and control of PCV, understanding the pathogenesis of PCV and the roles of postulated cofactors will be important in learning to deal with PCV in affected herds.

INTRODUCTION

Porcine circovirus (PCV) has recently been associated with a new disease syndrome termed postweaning multisystemic wasting syndrome (PMWS). PCV has been recognized since 1974, when it was identified as a contaminant of a continuous cell line derived from porcine kidney cells (Tischer et al. 1982). The cell contaminant virus is now referred to as porcine circovirus type 1 (PCV1) and appears to be nonpathogenic for swine (Allan et al. 1995; Tischer et al. 1986). A genetically and antigenically different strain, porcine circovirus type 2 (PCV2), has been associated with PMWS (Ellis et al. 1998; Meehan et al. 1998; Morozov et al. 1998). PMWS was first described in Canada as a wasting syndrome in growing pigs (Clark 1997; Harding and Clark 1997).

One of the intellectual challenges to understanding PMWS is reconciling the fact that antibodies to circovirus are widespread throughout the pig population (Walker et al. 2000), yet the disease seems to affect a relatively limited number of herds and only some of the pigs within a herd. While the presence of PCV2 is necessary for the development of PMWS, other factors appear to be needed for expression of the disease.

The definition of PMWS or PCV-2-associated disease continues to evolve. For clarity, a diagnosis of PMWS requires the fulfillment of three criteria (Sorden 2000):

1. There must be clinical disease. This most often includes wasting, pneumonia, or diarrhea.
2. Characteristic microscopic lesions must be present, including lymphoid depletion, intracytoplasmic inclusions, or granulomatous inflammation, in one or multiple organs.
3. There must be demonstration of PCV2 in the tissues.

All three of these criteria need to be met for a diagnosis of PMWS.

The clinical signs are not specific and do not allow for a definitive diagnosis. The identification of PCV2 in the tissues is an important aspect of diagnosis, but is not sufficient in itself. Most pigs develop antibodies to PCV2 at some time during the finishing period, and identifying PCV2 DNA or antigen in clinically normal pigs is not uncommon. Therefore, microscopic examination of the tissues and identification of the characteristic lesions, along with viral identification and observation of clinical disease, are needed to confirm a diagnosis of PMWS.

The range of disease that falls under the umbrella of PMWS has also expanded over the past several years as new diagnostic tools have improved diagnostic capabilities. Lymphoid depletion was the defining microscopic lesion when intracytoplasmic inclusions were the primary means of confirming a diagnosis. Wider use of immunohistochemistry has helped define other microscopic lesions, such as peribronchiolar fibrosis, as important landmarks by showing spatial relationship between the virus and the lesions.

In addition to PMWS, PCV2 has been associated with several other conditions, including congenital tremors, abortion, and porcine dermatitis and nephropathy syndrome (PDNS). PCV was reported as a cause of congenital tremors in 1984 (Hines and Lukert 1994). More recently, Stevenson et al. (2001) showed several congenital tremor cases to be associated with PCV2. Canadian researchers have associated PCV2 with abortions (West et al. 1999), and it was identified in fetal tissues and myocardial lesions were a distinguishing feature. A similar case has been reported in a herd in Iowa (Janke 2000).

PCV2 has also been shown to be a common finding in pigs with PDNS (Rosell et al. 2000), which was first described in 1993 by Smith et al. Affected pigs have necrotizing skin lesions that are most common in the perineal area and on the hind limbs. The renal lesions include vasculitis and glomerulitis. Most cases of PDNS involve a low percentage (1% or 2%) of the animals in a group (Hicks and Sorden 2000). PDNS has not been reproduced experimentally, and the implication of PCV2 in the pathogenesis of the disease is by association only.

CLINICAL SIGNS

Dyspnea and wasting are the two most common signs reported in cases of PMWS. Pneumonia, dyspnea, or other respiratory signs were reported in over 70% of the cases diagnosed at the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL) from 1996 to 1998 (Harms 1999). Similar findings have been reported in Canada, with 71% of the herds experiencing pneumonia (Harding et al. 1998). The second most common sign reported was wasting or ill thrift, which was present in approximately 50% of cases. Other clinical signs included diarrhea, pallor, and occasional icterus. These clinical signs are similar to those reported from other countries (Allan et al. 1998, 1999; Harding 1997; Segales et al. 1997).

PMWS or PCV2-associated disease primarily affects pigs in the late nursery to early finishing period. Harding et al. (1998) reported that most pigs were 6 to 8 weeks of age (average, 7 weeks) at the time they first developed the disease. The disease in the United States tends to occur later in a pig's life, with a peak at 12 to 14 weeks of age, but there have been cases in pigs ranging from 3 weeks of age to mature replacement gilts.

MORBIDITY AND MORTALITY

Morbidity and mortality associated with PMWS vary considerably from farm to farm. In general, the morbidity rate is low within an affected group. A review of cases at ISU-VDL showed that 64% of the herds reported a morbidity rate of less than 10%, with many herds in the 1% to 5% range (Harms 1999). Although the number of pigs clinically affected may be low, many do not recover. In herds surveyed by Harding et al. (1998), the case mortality rate was 81% ($\pm 23\%$) (Harding et al. 1998). Affected pigs often will continue to lose weight and may need to be killed. Veterinarians often report that several pigs in each pen in the barn are affected, but the disease does not seem to move through the barn from pen to pen. An increasing number of the more recent cases has been associated with outbreaks of porcine respiratory disease complex where porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), or *Mycoplasma hyopneumonia* are identified. These cases often present with morbidity rates of over 50%, with many

of the surviving pigs enduring a prolonged recovery. Because these cases affect larger numbers of pigs, cases that involve PCV2 associated with porcine respiratory disease complex are of the greatest concern.

GROSS LESIONS

The gross lesions observed in pigs with PMWS can be quite variable and, unfortunately, are not diagnostic. Systemic enlargement of lymph nodes is a commonly reported gross lesion. The lymph nodes are often tan and have a homogeneous appearance on cut surface. The lungs can be diffusely noncollapsed and rubbery and are often mottled red to pale (Rosell et al. 1999). These lesions are nonspecific and do little to differentiate PMWS from other viral infections, such as PRRSV or SIV, or even a bacterial septicemia, such as salmonellosis. Other less common gross lesions include icterus of the skin and organs, shrunken liver, or swollen kidney. Gastric ulcers of the pars oesophagea are often reported, although this, too, is a very nonspecific lesion.

MICROSCOPIC LESIONS

Currently, PMWS is defined by a set of specific microscopic lesions. The hallmark microscopic lesion is depletion of lymphoid follicles and of the perifollicular regions of the lymphoid tissue, with infiltrates of macrophages in the areas depleted of lymphocytes. Granulomatous inflammation of lymphoid tissue is often present. These lesions are regularly observed in the tonsils, lymph nodes, Peyer's patches, and spleen. The macrophages within these lesions will occasionally contain grape-like clusters of basophilic intracytoplasmic inclusion bodies, which are characteristic of PCV infection and are similar to those seen in other circovirus infections, such as psittacine beak and feather disease (Ritchie et al. 1989).

Many of the cases include histiocytic to granulomatous interstitial pneumonia with type 2 pneumocyte hyperplasia. Airway changes are common, including attenuation and necrosis of the epithelium with lymphocytes and macrophages infiltrating the subepithelial layers of the airways. Peribronchiolar and peribronchial fibrous tissue is also a feature and, in severe cases, fibrous tissue will obliterate the bronchiolar lumen. PCV2 antigen is consistently demonstrated in alveolar macrophages and in the alveolar septa, and occasionally within airway epithelial cells. The liver is variably affected, with initial lesions of multifocal individual hepatocellular necrosis and mild lymphohistiocytic periportal infiltrates, whereas the more advanced lesions include widespread necrosis with multifocal-to-diffuse granulomatous inflammation throughout the liver. Large amounts of PCV

antigen can also be demonstrated within these lesions. Granulomatous inflammation is also observed in other tissues, such as kidney and intestine.

EXPERIMENTAL REPRODUCTION OF PMWS

Experimental reproduction of PMWS has been challenging. Success in reproducing disease has been achieved using a coinfection model of PCV2 and porcine parvovirus (Ellis et al. 1999; Krakowka et al. 2000), as well as a PCV2 and PRRSV coinfection model (Allan et al. 2000; Harms et al. 2001). Based on immunohistochemistry, virus isolation, or polymerase chain reaction assays, 60% of the PMWS cases at ISU-VDL have concurrent PRRSV infection.

DIAGNOSIS

As previously noted, clinical signs and gross pathology are not specific and cannot be used for diagnosing PMWS with any certainty. Other infectious factors (PRRSV, SIV, salmonellosis, and ileitis) and noninfectious factors (environmental and nutritional) can result in similar clinical signs or gross lesions. Many cases of ill-thrift pigs received at the ISU-VDL with a differential diagnosis of PCV/PMWS are recently weaned pigs; however, very rarely are these recently weaned pigs diagnosed with PMWS, as most cases of PMWS involve pigs in the late nursery to early finishing periods.

A definitive diagnosis of PMWS requires histopathology with the identification of some of the key lesions mentioned previously, including lymphoid depletion and granulomatous inflammation in one or multiple tissues with or without the presence of intracytoplasmic inclusion bodies (Sorden 2000). These intracytoplasmic inclusion bodies are not consistent and cannot be relied upon in reaching a diagnosis. Immunohistochemistry has proven effective in identifying tissues with variable amounts of PCV antigen (Sorden et al. 1999). In situ hybridization for PCV has also been developed and is effective at detecting the presence of circovirus in tissues (Choi and Chae 1999).

Other diagnostic tools available include serology and polymerase chain reaction. Care must be taken in interpreting these results, because most herds will test positive for PCV or PCV2 by these methods. They may be used effectively to look at the ecology of the virus within the herd.

CONTROL MEASURES

Good husbandry practices seem helpful in limiting the damage done by PMWS, but are not completely effective in controlling the disease. Management strategies such as decreasing stocking density, all-in/all-out production

by facility, age segregation, and good sanitation are important aspects of control. It is unknown whether these practices affect PCV2 transmission and pathogenesis or whether they are simply effective because they control other infectious agents that may potentiate PCV2 infection and lead to PMWS. Many of the efforts to control PMWS in herds focus on controlling or modifying the other *coinfectors*. In the cases seen at ISU-VDL, the most common risk factor seems to be PRRSV infection. It appears that concurrent infections (e.g., parvovirus or PRRSV) exacerbate the clinical syndrome.

Current production practices have changed the dynamics of many of the common viruses of swine. Vaccination with killed PRRS product may increase the level of maternal protection, thereby moving PRRSV infection later in the production cycle. This may improve performance in the nursery, but may make PRRSV infection coincide with normal seroconversion of pigs to PCV2. Similar in-herd dynamics may be occurring with parvovirus infections as farms have moved toward more segregated production. PMWS might be controlled through vaccination, controlled exposure, managing maternal protection, or modifications in production practices, such as all-in/all-out or multisite production, to modify the disease dynamics of PCV2 within a herd, as well as for other pathogens.

PCV2 is quite stable, but several disinfectants are effective (Royer et al. 2001). Thorough cleaning and disinfection are important steps in decreasing or eliminating exposure to PCV2, although the ubiquitous nature and environmental stability of the virus make elimination difficult.

Vaccination is not currently an option, but development of a vaccine is being pursued by several research groups. Veterinarian and producer interest in autogenous vaccines for PCV is high, but few if any are currently in use. Culturing and growing the virus to levels sufficient for vaccine production are difficult. A modified-live vaccine from an attenuated virus or a nonpathogenic strain should be considered; however, a recombinant vaccine and a companion differential assay would be the most useful and potentially safer. A vaccine against PCV2 may be quite valuable depending on the extent of involvement of PCV2 in problems such as the porcine respiratory disease complex or variability in pig growth. Until we understand more about the factors that trigger disease in PCV2 infection, it will be difficult to devise effective vaccination strategies.

CONCLUSIONS

PMWS and PCV present new challenges to pork production and continue to test the current paradigms of disease pathogenesis. Creative and innovative approaches are needed to understand PMWS and devise ways to control the disease on farms.

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9.3 Postweaning Multisystemic Wasting Syndrome and Porcine Circovirus Type 2: The European Perspective

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and Mariano Domingo

SUMMARY

Postweaning multisystemic wasting syndrome (PMWS), a porcine circovirus type 2 (PCV2)-associated disease, is a recently described clinical condition affecting nursery and growing pigs. PMWS was recognized in Europe in 1996. Since then, most of the swine-producing countries in Europe have described cases of the disease. Clinically, the most representative clinical signs of PMWS include wasting, pallor of the skin, respiratory distress, diarrhea and, sometimes, icterus. No reproductive disorders associated with PCV2 infection have been consistently described in Europe. At present, little is known regarding the epidemiology of PMWS. Although PCV2 has been present in Europe since at least 1973, no explanations for the emergence of this disease have been established. Macroscopic lesions associated with PMWS are quite unspecific, but histopathologic lesions in lymphoid tissues (lymphocyte depletion with histiocytic infiltration) are almost unique for this disease. The criteria used for the diagnosis of PMWS include the existence of compatible clinical signs, the presence of characteristic microscopic lesions, and the detection of PCV2 within these lesions. Because no specific treatment for PMWS or vaccine against PCV2 is available, affected farms have attempted to make changes in management in order to reduce the burden of infection.

INTRODUCTION

In 1991, a new clinicopathological condition characterized by growth retardation, skin pallor, and high mortality was described in nursery pigs in Saskatchewan (Canada). The disease was named postweaning multisystemic wasting syndrome (PMWS) (Harding 1996), and since 1994 an increasing number of cases have been detected in Canada. In 1997, the presence of porcine circovirus (PCV) antigen was demonstrated in lesions of animals affected by PMWS (Clark 1997). Furthermore, nucleotide sequence analysis of the PCV associated with

PMWS revealed important differences compared with PCV derived from porcine kidney (PK-15) cells (ATCC CCL-33) (Hamel et al. 1998). Therefore, it was proposed to name these viruses as PCV type 1 (PCV1) for the cell culture-derived virus, and PCV type 2 (PCV2) for the virus associated with the new disease (Allan et al. 1999).

The first recognition of PMWS in Europe was in the spring of 1996 in Brittany (France) (named *maladie de l'amaigrissement du porcelet* or MAP). Clinical signs and lesions were similar to those reported in Canadian pigs (LeCann et al. 1997). In the spring of 1997, PMWS was described in Spain (Segalés et al. 1997). PCV was systematically detected in tissues of all French and Spanish cases of PMWS, and sequencing of those isolates revealed them to be PCV2 (Mankertz et al. 2000). Since 1998, PMWS cases have been also described in Denmark (Allan et al. 1999), the United Kingdom (Gresham 1999), Italy (Marcato et al. 1999), Germany (Soike et al. 2000), the Netherlands (Wellenberg et al. 2000), Belgium (Vyt et al. 2000), Greece (Kyriakis et al. 2000), Lithuania (Ohlinger et al. 2000), Austria (Ohlinger et al. 2000), Portugal (unpublished data), and Poland (unpublished data). Thus, at present, it seems that PMWS is a significant problem for several pig-producing countries in Europe.

CLINICAL PICTURE IN PMWS CASES

PMWS most commonly affects pigs of 2 to 3.5 months of age (Figure 9.3.1). Morbidity and mortality are variable depending on the farm and on the batches of animals. The usual rates are 4% to 15% and 70% to 80%, respectively. The on-farm descriptions of the syndrome are rarely detailed except when cohort studies are specifically designed for the purpose (Madec et al. 2000). However, there is enough evidence of a consensus among the authors on the critical period and on the list of signs observed on affected farms. Wasting is a major sign; other signs include skin pallor, respiratory distress, diarrhea and, sometimes, icterus. The question of whether skin damage, as seen in porcine dermatitis and nephropathy

syndrome (PDNS), is one of the clinical signs related to PMWS remains unanswered. In a study by Madec et al. (2000) in France, PDNS-affected pigs were observed in all the farms where PMWS occurred; however, the prevalence of PDNS was highly variable and usually low (less than 1%). Although unknown at this time, it can be hypothesized that the conditions leading to PMWS expression on the farms also predispose pigs to PDNS.

Other infections or diseases may be found on farms with PMWS, but a direct relationship with the syndrome is difficult to establish. Among these are Aujeszky's disease (pseudorabies), porcine reproductive and respiratory syndrome (PRRS), Glasser's disease, streptococcal meningitis, salmonellosis, postweaning colibacillosis, nonspecific diarrhea, dietetic hepatitis, and suppurative bronchopneumonia principally involving *Pasteurella multocida*, *Bordetella bronchiseptica*, and *Streptococcus suis*. Among all these diseases and infections, the respiratory form of PRRS has been a major concern for the European swine industry because of its clinical similarity with PMWS.

EPIDEMIOLOGY

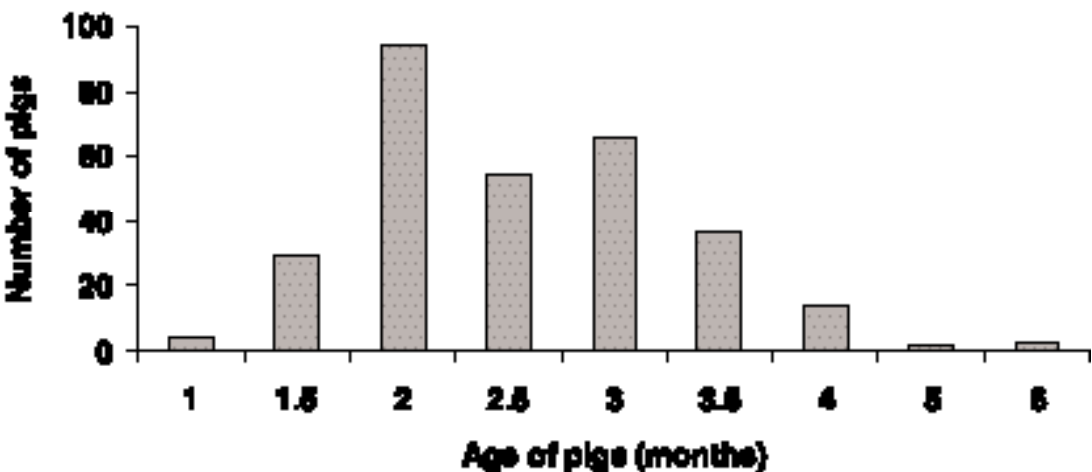
Descriptive Epidemiology

The on-farm course of PMWS and its epidemiology are not well documented. However, the main traits can be drawn out from the reports available (Ellis et al. 1998; Harding 1996; Madec et al. 2000; Segalés et al. 1997). The critical age for pigs to exhibit PMWS is from 8 to 13

weeks, although clinical signs and losses can occur outside these limits. Losses at later stages are frequently found on farms in the context of PMWS, but are attributable to PDNS. It remains to be determined why the critical step of pig farming regarding the expression of PMWS is either the second phase of the postweaning period and/or the first phase of growing-finishing period.

When PMWS occurs in farrow-to-finish or in farrow-to-feeder herds, no perceivable disturbance is observed in categories other than the postweaned-growing pigs. Reproductive performance, in particular, is maintained even in those herds with a high incidence of losses due to PMWS. Thus, reproductive function does not seem to be affected. In a group of severely affected farms, sow productivity expressed as the number of piglets weaned per sow per year was 24.4 on average, a level slightly above the average value for the whole country. No abortion storms or increases in stillborn or mummified fetuses were noticed either before or after the first manifestations of PMWS (Madec et al. 2000). At first sight, this observation does not corroborate a field case report where abortions were associated with PCV2 infection in Canada (West et al. 1999).

The individual expression of the disease seems to be a key point in this disease. In a given pen, only some individual pigs exhibit clinical signs. These pigs tend to die within a few days or fail to grow. Various treatments have failed to counteract the disease. On farms where the pigs could be properly identified and where they were not



9.3.1. Age distribution of pigs affected by postweaning multisystemic wasting syndrome (PMWS) ($n = 308$). These data correspond to all pigs necropsied and diagnosed with PMWS at the Veterinary School of Barcelona between May 1997 to May 2000.

mixed at the different stages, a litter effect was observed; certain litters were greatly affected whereas others kept in the same room did not exhibit problems (Madec et al. 2000). A recent report showed that castrated male pigs were more susceptible to PMWS than were females; furthermore, the investigators observed that pigs with lower birth weights, those with low weaning weights, or those that were lighter at the beginning of the fattening period tended to develop PMWS with higher frequency (Corrége et al. 2001).

However, some aspects of the descriptive epidemiology are very difficult to explain:

1. Persistence of the acute impact of the disease over a long period on the same farm raises questions about immunity acquisition in sows and, more globally, in the herd.
2. Changes in pig breeding and husbandry have been given as possible reasons for the emergence of this disease (Allan 2000). However, outbreaks of PMWS have also occurred on totally confined farms where no live pigs have been introduced for 2 years or more and where strict biosecurity measures were in place (F. Madec, unpublished data).
3. The appearance of PMWS in different countries around the world at almost the same time is a strange coincidence. An explanation is lacking for the emergence of PCV2-associated disease in the late 1990s, given that PCV2 has been present since at least 1973 in Northern Ireland, 1985 in Belgium, and 1986 in Spain, based on serological and *in situ* hybridization retrospective studies (Mesu et al. 2000; Rosell et al. 2000b; Walker et al. 2000).

Analytic Epidemiology

The circumstances leading to, and/or predisposing pigs to, the disease urgently need investigation, since they will provide the basis for preventive measures. Several recommendations have been proposed based on severe losses and clear deviations in zootechnical parameters observed in some case studies. When farmers could comply with most of the recommendations, positive responses were obtained, especially on severely affected farms (Guilmoto and Wessel-Robert 2000). There are at least two reasons for the delay in the start of analytic epidemiology:

1. PMWS presents a difficult clinical diagnosis. PMWS shows an expression pattern different from other infectious diseases and, accordingly, the approach needs to be adapted. The overall severity of the disorders varies markedly from farm to farm. Furthermore, al-

though wasting and/or death occur in the end, there are a variety of associated predominant clinical signs and gross lesions, depending on the farm.

2. PMWS has a confusing etiopathogenesis. PCV2 is recognized as playing a pivotal role in the syndrome. This viewpoint is supported by experiments showing protection through administration of PCV2 ORF2 (open reading frame 2) protein (Jestin et al. 1999). However, the role of PCV2 in the pathogenic cascade of events finally resulting in wasting disease is not yet clarified. In addition, serological surveys have shown that serum antibodies to PCV2 are widespread and have been so for several years, long before PMWS outbreaks were first described (Walker et al. 2000). These observations together with the quasi-simultaneous descriptions of PMWS worldwide raise questions about an exclusive etiologic role of PCV2 in PMWS. It might be a final, although decisive, factor acting in the last step of the pathological process. In other words, PCV2 is a necessary causal factor in PMWS, but it may not be sufficient. It may take advantage of specific synergistic circumstances present on certain farms, in certain individual pigs, at a certain time, to proliferate massively and then give birth to the disease.

PATHOLOGICAL FINDINGS IN PMWS-AFFECTED PIGS

At necropsy, the most striking lesions are noncollapsed lungs and enlargement of lymph nodes (mainly superficial inguinal, submandibular, mesenteric, and mediastinal) (Rosell et al. 1999). However, these lesions are not always present and cannot be used as the only marker of PMWS on a farm. Indeed, normal-to-atrophic lymph nodes have also been observed in pigs affected by this syndrome. The enlargement of lymph nodes is probably one of the earliest features of PMWS-affected pigs. In a low proportion, lymph nodes may have the presence of multifocal areas of necrosis that are visible macroscopically (Segalés et al. 2000a). The frequencies of the aforementioned findings and others in a study of 148 PMWS-affected pigs in Spain are summarized in Table 9.3.1.

Moderate-to-high numbers of PMWS-affected pigs have bronchopneumonia and gastric ulceration of the pars oesophagea. This is not a direct effect of PCV2. Bronchopneumonia is associated with bacterial infections, and gastric ulceration is of multifactorial origin. However, the lesion in the stomach causes internal hemorrhage and is the cause of death for a number of pigs with PMWS. It is also responsible for the pale skin that has been frequently

Table 9.3.1. Frequency of macroscopic lesions observed in 148 pigs affected by postweaning multisystemic wasting syndrome

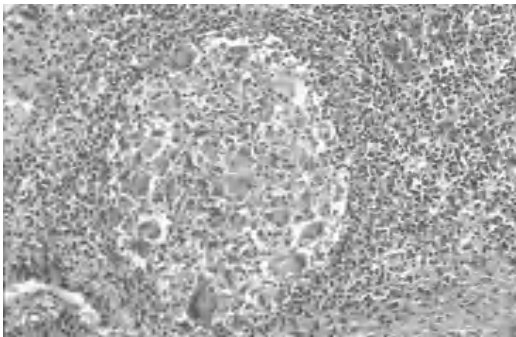
Macroscopic Findings	Frequency (Out of 148 Pigs)	%
Noncollapsed, rubbery lungs	103	69.8
Enlargement of at least one lymph node	101	68.2
Pulmonary consolidation (bronchopneumonia)	79	53.4
Gastric ulceration of pars oesophagea	56	37.8
White-spotted kidneys	27	18.2
Jaundice	9	6.1
Hepatic atrophy	8	5.4

associated with the disease (Segalés et al. 2000b). Chronically affected pigs may develop cachexia.

Regarding the aforementioned study of 148 PMWS-affected pigs, the most frequent histopathologic lesions are summarized in Table 9.3.2. The most characteristic microscopic lesions of PMWS are located in lymphoid tissues. A variable degree of lymphocyte depletion with loss of the follicular architecture is present in almost all pigs with PMWS; this finding is usually combined with a multifocal to diffuse, slight to very intense histiocytic and/or multinucleate giant-cell

infiltration (Figure 9.3.2). Another key finding is the presence of sharply demarcated, spherical, basophilic cytoplasmic inclusions of PCV2 in histiocytic cells (Rosell et al. 1999).

A subacute interstitial pneumonia is the most usual lung lesion in PMWS-affected pigs (Rosell et al. 1999). In some cases, it is possible to see large histiocytic and multinucleate giant cells in the thickened interalveolar walls and/or within alveoli. In chronic cases, bronchiolitis fibrosa obliterans may be present (Segalés et al. 2000a).



9.3.2. Mesenteric lymph node. Lymphocyte depletion with replacement by macrophages and multinucleate giant cells in a follicular area (hematoxylin-eosin).

Table 9.3.2. Frequency of microscopic lesions observed in 148 pigs affected by postweaning multisystemic wasting syndrome

Macroscopic Findings	Frequency (Out of 148 Pigs)	%
Lymphoid tissues		
Lymphocyte depletion	129	87.2
Histiocytic inflammatory infiltration	114	77.0
Intracytoplasmic inclusion bodies	67	45.3
Syncytial (multinucleate) cells	54	36.5
Multifocal coagulative necrosis	18	12.2
Lung		
Interstitial pneumonia	130	87.8
Liver		
Slight to moderate hepatitis	82	55.4
Intense hepatitis and destruction of parenchyma	11	7.4
Kidney		
Interstitial nephritis	67	45.3

Hepatic lesions have been described as lymphocytic-histiocytic inflammatory infiltration in portal zones, single-cell necrosis of hepatocytes, swelling and vacuolation of hepatocyte cytoplasm, and karyomegaly (Clark 1997). In some cases, however, it is possible to detect very severe lesions showing generalized perilobular fibrosis, with disorganization of liver plates and massive loss of hepatocytes; these lesions are associated with icterus and macroscopic lesions in the liver. Four stages of hepatic damage in PMWS-affected pigs have been established based on intensity and distribution of lesions (Rosell et al. 2000a).

Other microscopic lesions detected in PMWS-affected pigs include lymphohistiocytic inflammatory infiltrates in kidney, pancreas, intestines, and myocardium. Sporadically, moderate-to-severe granulomatous enteritis with blunting of villi has been observed.

DETECTION OF PCV2 AND PCV2 ANTIBODIES

Several methods have been developed to detect PCV2 in tissues and/or serum. Among them, in situ hybridization, immunohistochemistry, and polymerase chain reaction are the assays most commonly used routinely in Europe. When using in situ hybridization or immunohistochemistry, PCV2 nucleic acid or antigen can be found in the cytoplasm of histiocytes and other monocyte/macrophage lineage cells, such as alveolar macrophages, Kupffer cells, and dendritic cells of lymphoid tissues. Sporadically, virus can be detected in the cytoplasm of renal and respiratory epithelium, vascular endothelium, lymphocytes, and nuclei of monocyte/macrophage lineage cells and hepatocytes (Rosell et al. 1999). A very strong association has been observed between the amount of PCV2 nucleic acid or antigen and the severity of microscopic lymphoid lesions. PCV2 nucleic acid or antigen also can be found in tissues of clinically healthy pigs; in these cases, the amount of virus and the intensity of histopathologic lesions are very low (Segalés and Domingo 1999). Retrospective studies using in situ hybridization have shown the presence of PCV2 nucleic acid associated with PMWS microscopic lesions in Spanish pigs in 1986 (Rosell et al. 2000b). The polymerase chain reaction technique can be applied to any kind of sample, but a positive result should be interpreted with caution, since clinically healthy pigs or diseased pigs without PMWS may be infected with PCV2.

At present, no commercial serological techniques are available for detecting PCV2 antibodies. However, an immunoperoxidase monolayer assay and a competitive enzyme-linked immunosorbent assay have been used for research purposes (Rodríguez-Arrijo et al. 2000; Walker et al. 2000). Results using these tests have shown that PCV2 antibodies are widespread among pigs in Europe,

with very high seroprevalence within herds; in fact, it is tremendously difficult to find farms that are truly seronegative to PCV2. Furthermore, the presence of antibody to PCV2 in pigs does not appear to correlate with predisposition to PMWS (Rodríguez-Arrijo et al. 2000). These limited serological surveys have shown the presence of antibodies to PCV2 in Northern Ireland since at least 1973 (Walker et al. 2000) and in Belgium since at least 1985 (Mesu et al. 2000). No antibody to PCV2 has been detected in sheep, cattle, or horses (Allan et al. 2000).

DIAGNOSIS OF PMWS

As a general agreement, the final diagnosis of PMWS is established on the basis of three criteria: (1) presence of a clinical picture compatible with PMWS, (2) presence of characteristic histopathologic lesions, and (3) detection of PCV2 within the lesions in tissues of affected pigs (Sorden 2000). In all PMWS cases, PCV2 is present in a variable amount in at least one tissue and always closely associated with the microscopic lesions.

The most valuable tissues for establishing a diagnosis of PMWS are lymphoid tissues. However, for detecting PCV2 and other concomitant infections, lymph nodes, tonsil, spleen, intestines (with ileum, containing Peyer's patches), lung, liver, and kidney are all of value.

The differential diagnosis list for PMWS can be very large, depending on the predominant clinical sign(s) on each particular farm. The first and most important entity to be included in the list is the respiratory form of PRRS; however, the widespread seroprevalence to PRRS virus in most of the European countries makes the differentiation between PRRS and PMWS very difficult unless an appropriate battery of laboratory tests for PRRS virus and PCV2 are conducted at the same time. Moreover, all diseases or conditions that cause wasting have to be included in the differential diagnosis list (Harding and Clark 1997).

PMWS AND IMMUNOSUPPRESSION

Several observations have led to the speculation that pigs affected by PMWS may be immunosuppressed:

1. There is lymphocellular depletion of both follicle centers and parafollicular zones, together with histiocytic and multinucleate giant-cell infiltration affecting lymphoid tissues (Clark 1997; Rosell et al. 1999)
2. *Pneumocystis carinii* and *Chlamydia* spp., opportunistic pathogens commonly associated with immunosuppression, have been found in the lungs and intestine of PMWS-affected pigs (Carrasco et al. 2000; Clark 1997)

3. Many pigs with PMWS also have pulmonary and/or septicemic infections with bacteria such as *Pasteurella multocida* or *Haemophilus parasuis* (Madec et al. 2000; Segalés et al. 2000a).
4. Atypical lesions associated with Aujeszky's disease virus have been described in PMWS-affected pigs (Rodríguez-Arrioja et al. 1999)
5. Preliminary hematologic results showed some alterations in leukocyte populations in diseased pigs (Segalés et al. 2000b)
6. The strongest evidence of immunosuppression in PMWS pigs is a recent flow-cytometric analysis of peripheral blood leukocytes (Segalés et al. 2000c). Compared with clinically normal, non-PCV2-infected pigs, pigs with PMWS showed substantial changes in leukocyte subsets in the peripheral blood that were characterized by an increase in monocytes, a reduction in T-lymphocytes (mainly CD4+) and B-lymphocytes, and the presence of low-density immature granulocytes. Altogether, these changes would suggest an inability of acutely PMWS-affected pigs to mount an effective immune response.

PREVENTION

Preventive measures whose efficacy is validated by large-scale field studies are still lacking. Most reports involve a limited number of farms where PMWS was severe and where attempts were made to reduce its impact. The use of therapeutic drugs has shown real limitations. However, based on the knowledge that PMWS targets the defense mechanisms of pigs and the observation of obvious deficiencies in management in some cases, zootechnical changes have been proposed to reduce the burden of infection, whether from PCV2 or other pathogens (Madec et al. 1999). The changes mainly focus on improvement of hygiene and a reduction in stress at the different production stages. These measures include reducing the mixing of pigs, adequate pig flow (strict all-in/all-out procedures), reducing pig density, special care in castration, and improvement in air quality and comfort during the postweaning and growing periods. Significant positive results were obtained when these measures were applied (Guilmoto and Wessel-Robert 2000).

On certain farms, a more drastic strategy can be applied, i.e., depopulation-repopulation. Depopulation allows for the improvement of housing conditions and increases the level of hygiene. Despite indications that some farms are soon reinfected with PCV2, the impact of PMWS is reduced. In some cases, replacement stock was suspected as the source of reinfection (F. Madec, personal observations). Beside the zootechnical measures that are immediately available, current research on the etiolo-

gy of PMWS presents the hope for more direct intervention through vaccination.

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9.4 Postweaning Multisystemic Wasting Syndrome: Experimental Studies with Porcine Circovirus Type 2

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SUMMARY

Postweaning multisystemic wasting syndrome (PMWS) has recently emerged as an economically important disease of swine in many pig-producing countries. A novel porcine circovirus, termed porcine circovirus type 2 (PCV2) has been identified as the primary cause of PMWS. However, coinfection with other infectious agents, or nonspecific immunostimulation, can increase the severity of clinical disease in pigs experimentally infected with PCV2. The role of PCV2 in the pathogenesis of other conditions, including congenital tremor, porcine dermatitis and nephropathy syndrome, and infertility, is unknown.

INTRODUCTION

The Circoviridae are a family of small, nonenveloped animal viruses characterized by a single-stranded circular DNA genome of 1.76 to 2.31 kb (Todd et al. 2000). Members of this family include psittacine beak and feather disease virus, the porcine circoviruses, and chicken anemia virus. Porcine circovirus was originally identified as a contaminant of a continuous pig kidney cell line (PK-15) (Tischer et al. 1974). This cell culture-associated virus is currently referred to as PCV type 1 (PCV1). Apart from its isolation from stillborn piglets in Northern Ireland (Allan et al. 1995), it has not been associated with disease.

Postweaning multisystemic wasting syndrome (PMWS) was first identified in western Canada in 1991 (Clark 1997) and has subsequently been reported from the United States, Mexico, and many countries in Europe and Asia (Allan and Ellis 2000). It mainly affects weaned pigs and is characterized by progressive weight loss, respiratory signs, lymphadenopathy, jaundice and, occasionally, skin lesions (Harding and Clark 1997). Interstitial pneumonia, lymphadenopathy, hepatitis, and nephritis are prominent postmortem

findings. Histo-pathologic lesions comprise granulomatous inflammation, lymphocytic depletion, and syncytia formation in lymphoid tissues, and granulomatous lesions in many nonlymphoid tissues (Ellis et al. 1998). Characteristic amphophilic cytoplasmic and nuclear inclusions occur in lymphoid tissues and occasionally in nonlymphoid tissues. These lesions are associated with abundant antigen and nucleic acid of a recently characterized strain of porcine circovirus termed porcine circovirus type 2 (PCV2) (Allan et al. 1998; Ellis et al. 1998). Genomic analysis of PCV2 has revealed less than 80% sequence homology with the apparently nonpathogenic PCV1. In contrast, there is more than 96% intragroup nucleotide sequence homology among all PCV2 isolates so far sequenced (Meehan et al. 1998). These genomic differences are reflected in antigenic distinctions between PCV1 and PCV2, as indicated by reaction with panels of polyclonal and monoclonal antibodies (McNeilly et al. 2001).

PORCINE CIRCOVIRUSES AND NATURAL DISEASE

Although PCV1 has been associated with type A2 congenital tremors in piglets (Hines and Lukert 1994) and has been isolated from stillborn piglets (Allan et al. 1995), it is generally believed to be nonpathogenic.

In contrast, PCV2 has been identified as the primary cause of PMWS (Allan et al. 1999; Kennedy et al. 2000; Krakowka et al. 2000). It has also been associated with several other syndromes in pigs, including proliferative and necrotizing pneumonia (Hinrichs et al. 1999), sow abortion and infertility (West et al. 1999), porcine dermatitis and nephropathy syndrome (Rosell et al. 2000), and congenital tremor type A2 (Stevenson et al. 2001). However, its role in the pathogenesis of these conditions remains to be evaluated.

EXPERIMENTAL PORCINE CIRCOVIRUS INFECTIONS

Experimental infections of pigs with PCV1 have not resulted in clinical signs or significant lesions (Allan et al. 1995; Krakowka et al. 2001; Tischer et al. 1986). In contrast, lesions of PMWS have been induced by inoculation of gnotobiotic and conventional pigs with cell culture isolates of PCV2.

Krakowka et al. (2000) infected 1-day-old gnotobiotics intranasally with a cell culture isolate of PCV2, either alone or in combination with PCV1 or porcine parvovirus (PPV). Pigs that received PCV2 alone did not develop clinical signs, but had lymph node enlargement and mild histological lesions of lymphoplasmacytic cholangiohepatitis and myocarditis at necropsy 35 days post infection (DPI). Similar lesions developed in pigs infected with a combination of PCV2 and PCV1. In contrast, pigs inoculated with PCV2 and PPV had severe clinical signs and gross lesions of PMWS. Gross lesions in these animals included icterus, subcutaneous edema, thymic atrophy, hepatic mottling, and gastric ulceration. Histological lesions were moderate to severe and included angiocentric granulomatous inflammation in many tissues, hepatic necrosis, and lymphocytic depletion, and syncytia and inclusion body formation in lymph nodes. These results provided the first indication that PCV2 caused mild microscopic lesions in gnotobiotic piglets. They also demonstrated that concurrent PPV infection could potentiate the effects of PCV2 infection, resulting in clinically severe PMWS.

Experimental infection of four colostrum-deprived conventional pigs with a cell culture isolate of PCV2 at 1 to 2 days of age resulted in wasting in one of four inoculated animals (Allan et al. 1999; Kennedy et al. 2000). Severe PMWS-like lesions were seen in lymphoid tissues of this pig. At necropsy at 21 to 26 DPI, the three subclinically infected pigs in this group had mild-to-moderate histological lesions of PMWS in many lymphoid and nonlymphoid tissues, including lymph nodes, spleen, thymus, Peyer's patches, liver, lung, kidney, myocardium, pancreas, testis, brain, and salivary, thyroid, and adrenal glands. Lesions in all four pigs were associated with abundant PCV2 antigen. These results indicated that PCV2 could cause wasting and PMWS-like lesions in a proportion of conventional pigs exposed to this virus. As in dually infected gnotobiotics (Krakowka et al. 2000), the synergistic effects of PCV2 and PPV resulted in severe clinical signs and lesions in conventional pigs infected that received both viruses.

Magar et al. (2000) infected specific pathogen-free pigs with a cell culture isolate of PCV2 at 3 to 4 weeks of age. Although 1 of 11 infected pigs became unthrifty at 20 DPI, major clinical signs did not develop in any animal. However, gross lesions of pneumonia and enlargement of tracheobronchial, mediastinal, mesenteric, or

inguinal lymph nodes were apparent in several pigs. No histopathologic changes were seen in pigs necropsied at 6 or 13 DPI, but lesions typical of PMWS were present in a wide range of tissues of pigs killed 20 to 34 DPI. This study confirmed that PCV2 could cause lesions of PMWS in conventional pigs in the absence of other known swine pathogens.

Harms et al. (2000) inoculated 3-week-old, cesarean-derived, colostrum-deprived pigs with PCV2 alone or in combination with porcine reproductive and respiratory syndrome virus (PRRSV). In addition to spontaneous lesions of exudative epidermitis (that also developed in the other experimental groups), 4 of 19 PCV2-inoculated pigs became icteric. They also had lymphoid depletion and hepatitis associated with 40% mortality. Pigs dually infected with PCV2 and PRRSV developed a more severe disease characterized by severe and persistent pyrexia and dyspnea. Mortality in this group was over 90% and was associated with severe interstitial pneumonia, hepatitis, or both. These results provided further evidence that PCV2 alone could cause PMWS-like lesions in conventional pigs, and that the pathogenic effects of PCV2 may be increased by concurrent infection with PRRSV. Allan et al. (2000a) also found that PRRSV infection potentiated the effects of PCV2 in conventional pigs. In this study, PRRSV enhanced the replication and tissue distribution of PCV but not vice versa.

CONCLUSIONS

It can be concluded from the results of these experiments that PCV2 is the primary cause of PMWS, but that the expression of clinical signs and lesions in PCV2-infected pigs may be modified by concurrent PPV or PRRSV infections. All three viruses infect cells of the monocyte-macrophage series, and consequent activation of cells of this lineage may permit enhanced replication of PCV2. Allan et al. (2000b) proposed that these or other synergistic infections modulate the immune function of the host, leading to enhanced replication of PCV2 *in vivo*. This hypothesis is supported by the results of a recent experiment in which parenteral administration of an irrelevant antigen in incomplete Freund's adjuvant markedly enhanced the pathogenicity of PCV2 in gnotobiotic pigs (Krakowka et al. 2001). Immunomodulation by other infectious agents or immunomodulating drugs might therefore play an important role in outbreaks of PMWS in pig herds naturally infected with PCV2. However, the significance of these or other factors in the recent worldwide emergence of PMWS is unknown. Experimental evidence of viral persistence in the presence of serum antibodies (Krakowka et al. 2001) has implications for the epidemiology of PMWS, vaccine production, and diagnostic testing.

The role of PCV2 in the pathogenesis of other conditions, including congenital tremor, porcine dermatitis and nephropathy syndrome, and infertility, remains to be evaluated.

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9.5

A Clinician's Perspective on Postweaning Multisystemic Wasting Syndrome

Mateo del Pozo Vegas

SUMMARY

"Disease is not a tragedy if we respect the balance."

INTRODUCTION

Our production animals live within an environment full of microorganisms. Microorganisms are present not only in the environment, but also in the animals themselves. Spontaneous generation does not exist. In fact, in the majority of our farms, it is possible to isolate the most troublesome pathogens of swine: mycoplasma, *Actinobacillus pleuropneumoniae*, *Pasteurella* spp., even porcine reproductive and respiratory syndrome virus (PRRSV). However, not all positive farms develop clinical disease and, when outbreaks occur, they are not equally severe among herds. What determines whether a disease appears? Several authors have demonstrated that it is not merely the presence of the pathogen. Rather, in many cases, other factors are necessary for the expression of clinical disease. Very often, these factors involve the management of the animals.

On the other hand, we should be cognizant that in swine production we are dealing with populations, not with individual animals. Thus, a disease that affects a single animal or only a small group is not important as long as the productivity of the herd as a whole is unaffected. Therefore, we have learned to live with certain diseases by maintaining the *burden of infection* at a level sufficiently low so that, although the pathogen continues to circulate, clinical disease does not appear or (and this is the same thing), if it does, herd productivity is not affected.

Postweaning multisystemic wasting syndrome (PMWS) is a disease that has recently appeared, and there has been a great deal of discussion about it, particularly about its etiology. In this chapter, we do not enter into the debate about whether the causal agent is a new pathogen. Rather, we analyze the disease by using the available clinical and epidemiological data and, in a logical and scientific manner, attempt to determine what control measures we could put into place to reduce PMWS problems as much as possible.

THE DISEASE

In 1997, a new disease appeared in Spain. Affected pigs were 6 to 20 weeks of age and showed delayed growth, pallor, and icterus in some individuals, and finally died. Although morbidity was relatively low, mortality was high. The clinical signs of the disease were comparable to a syndrome that had appeared in Canada in 1991 and was described by Harding and Clark (1997) as *postweaning multisystemic wasting syndrome* or in France in 1995 and called *syndrome de dépérissement multisystemique du porcelet en post-sevrage*. Since the first descriptions of the disease, the syndrome has been diagnosed in other countries of Europe, including Spain, and America. Proinserga SA (Segovia, Spain) had the dubious honor of being one of the first farms in Spain where the disease was recognized (Segalés et al. 1997). In the first episodes of the disease, clinical signs included poor body condition, anemia, marked enlargement of the superficial inguinal lymph nodes and, in some animals, icterus. Occasionally, clinical signs include diarrhea, coughing, and nervous signs. Although similar clinical signs and lesions can be found in other diseases, the microscopic lesions were specific for PMWS. Characteristic gross and microscopic lesions of PMWS have been described in detail elsewhere. At present, PMWS can be found in all swine-producing areas of Spain.

Harding and Clark (1997) have reported morbidity and mortality of 50% in certain herds. However, most publications report morbidity ranging from 4% to 20% and mortality ranging between 70% and 90% in affected herds. On average, clinical signs persisted on farms for 1.5 to 2 years, with a higher propensity in herds with a high population density and that were not subject to a strict all-in/all-out program.

The fact that PMWS primarily affects pigs between 6 and 8 weeks of age and rarely younger pigs suggests that maternal immunity exerts a protective effect. In fact, the most marked clinical phase of the disease occurs when colostral immunity wanes.

The disease is evidently transmitted by contact, as has been demonstrated experimentally, although this is possible only during a narrow window of time. In fact, one of the observations made regarding herds with PMWS is that

only a few animals are affected by the disease; in the remainder, production parameters are not affected.

Sows seem to be the principal source of the disease, but they also provide protection to pigs through colostral immunity, i.e., the most severe clinical signs appear when the level of passive colostral immunity wanes. A study in France (Madec et al. 1999) examined the effect of sow age in relationship to PMWS in their litters but found no relationship.

PROINSERGA'S EXPERIENCE WITH PMWS

The first Spanish experience with the disease was in 1996 in a herd of 300 sows. Clinical signs included chronic wasting in pigs, beginning at about 10 weeks of age without association with anorexia, anemia, or icterus. The pigs did not respond to antibiotics. Morbidity ranged from 8% to 12%, with mortality of nearly 100% in affected animals. At necropsy, gastric ulcers were frequently found, as well as interstitial pneumonia, hard consistency in the liver in most cases or sometimes normal consistency with superficial foci of discoloration, and a generalized lymphadenopathy involving the superficial inguinal and mesenteric lymph nodes, giving the tissues a whitish, edematous, hypertrophic appearance. Clinically affected pigs were found on the farm for 2 years, but the disease on the farm is currently stable.

TREATMENT AND CONTROL

In general, when considering treatment and control of diseases of swine, several logical steps should be taken:

Diagnostic/Epidemiological Investigation

In general, it is not always possible to reach a precise diagnosis because a variety of infectious agents, nonspecific clinical signs, and the effect of environmental and management factors are present concurrently. This is especially true with this disease. Although the diagnosis of porcine circovirus type 2 (PCV2) is relatively simple, its role in the etiology of PMWS is not universally accepted. In any case, independent of circovirus, the diagnosis of the syndrome is based on clinical signs in conjunction with gross and microscopic lesions. Differential diagnosis should include PRRS, Glasser's disease, postweaning colibacillosis, *Lawsonia intracellularis*, carbadox/olanquindox toxicity, gastric ulcers, porcine dysentery (*Brachyspira hyodysenteriae*), and *Brachyspira pilosicoli*. PMWS has been diagnosed on both PRRSV-positive and PRRSV-negative farms, although more frequently on the former (80% vs 20%). This is even more dramatic in Spain, where PRRSV is widespread. However, besides PRRSV, an association between PCV2 and both porcine parvovirus and Aujeszky's disease virus has been postulated.

Treatment

No known vaccine or treatment will provide an immediate result. The actions taken should be based on good animal husbandry with the goal of reducing the burden of infection in the herd.

Control

An important point in the process of controlling this disease is to collect reference data and closely monitor the progress. Since this is a relatively unknown syndrome, this information will make it possible to assess the impact of changes. Those tests that do not give good results can be eliminated. Actions that have resulted in better control of the disease are based on epidemiological knowledge of the disease and primarily function to reduce the infectious burden in the herd. Although 100% disease control may not be achieved, animal productive losses can be lowered significantly by the following procedures:

FARROWING

1. Empty, clean, and disinfect the pits.
2. Wash and deworm the sows.
3. Cross-foster only the first 24 hours and foster only if absolutely necessary. Cross-foster only between sows of the same parity.
4. Maintain an adequate level of vaccination.

NURSERY

1. Use small pens with solid walls.
2. Empty, clean, and disinfect the pits.
3. Maintain three piglets per cubic meter at the beginning of the nursery phase.
4. Maintain a feeder trough space of 7 cm/piglet.
5. Maintain perfect ventilation and temperature.
6. Do not mix animals.
7. Other control measures: respect the "flows" (animals and air), use proper hygienic measures at any intervention (castration, teeth clipping, etc.), keep sick animals in isolation pens.

GROWERS AND FINISHERS

1. Use small pens with solid walls.
2. Empty, clean, and disinfect the pits.
3. Maintain space of at least 0.75 m³/pig.
4. Maintain correct ventilation and temperature.
5. Avoid mixing animals from different pens.
6. Do not mix animals from different lots.

CONCLUSIONS

It should be taken into account that wasting is not exclusively due to PMWS and can be associated with a variety of chronic processes. Although there is neither a vaccine nor a cure for PCV2, it is important to know that good swine husbandry and strict hygienic control measures will improve animal production significantly. As producers and veterinarians, we must seek to improve our knowledge of disease transmission and apply what we have learned.

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9.6 Porcine Dermatitis and Nephropathy Syndrome

Joaquim Segalés, Carles Rosell,
and Mariano Domingo

SUMMARY

Porcine dermatitis and nephropathy syndrome (PDNS) was first described in the United Kingdom in 1993. Since then, cases in Europe, North and South America, Oceania, and Africa have been described, suggesting a worldwide distribution of PDNS. The clinical detection of PDNS is relatively easy because of the presence of necrotizing skin lesions, mainly located on the hind limbs and perineal area. At necropsy, it is common to find that both kidneys are swollen and pale, with generalized cortical petechiae. Major histopathologic findings include a systemic necrotizing vasculitis, and necrotizing and fibrinous glomerulonephritis. These microscopic features together with the presence of immunoglobulin and complement factors in the damaged vessels and glomeruli suggest a type III hypersensitivity reaction as the possible pathogenic mechanism of disease. The antigen (or antigens) involved in this immune complex-mediated disorder is currently unknown. Theoretically, a wide spectrum of factors, including drugs, chemicals, food allergens, endogenous antigens, and infectious agents, may trigger an immune-mediated disease. At present, the role of infectious agents has been studied in more detail than other factors. Among them, porcine reproductive and respiratory syndrome virus and porcine circovirus type 2 have been strongly suggested as the possible antigen. However, definitive evidence of the participation of any of these viruses in PDNS is still lacking.

INTRODUCTION

Porcine dermatitis and nephropathy syndrome (PDNS) is a relatively new clinical-pathological condition that was first described by Smith et al. (1993) in the United Kingdom. The condition affected pigs weighing between 40 and 70 kg, and was of sporadic presentation with an overall prevalence of about 1% or less in affected groups. Macroscopically, this disease was characterized by skin lesions and enlarged, pale kidneys, with cortical petechiae. Acute glomerulonephritis and systemic necrotizing vasculitis were the most obvious microscopic findings.

Since 1993, the same disease has been described in other countries, including Canada (Hélie et al. 1995), the Republic of South Africa (Van Halderen et al. 1995),

Spain (Segalés et al. 1996), the United States (Ramos-Vara et al. 1997), Chile (Sierra et al. 1997), France (Solignac 1997), the Netherlands (Sierra et al. 1997), Argentina (Machuca et al. 1999), Australia (Cameron 1999), Italy (Gelmetti et al. 1999), and Greece (unpublished data).

The etiology of PDNS is unknown, but the microscopic lesions, and the presence of immunoglobulin and complement-factors in the damaged vessels and glomeruli (Hélie et al. 1995; Sierra et al. 1997), suggest a type III hypersensitivity reaction as the possible pathogenic mechanism for the disease.

CLINICAL AND PATHOLOGICAL FINDINGS

PDNS may affect nursery and growing pigs and, sporadically, adult animals (Drolet et al. 1999). The prevalence of the syndrome in affected herds is less than 1% (usually between 0.05% and 0.5%) (Segalés et al. 1998). Recently, higher prevalence was reported in the United Kingdom, with a case-fatality rate ranging from 0.25% to 20% in affected herds (Gresham et al. 2000), and in Spain, with 10% or higher prevalence (unpublished data). One study has shown that mortality among pigs 3 months of age or older was nearly 100%. However, only one-third of the affected pigs 1.5 to 3 months of age died, and the surviving pigs tended to recover and gain weight 7 to 10 days after clinical signs were first observed (Segalés et al. 1998). Many of the more severely affected pigs die within a few days after the onset of clinical signs. This situation has changed in farms with higher prevalence, and mortality rates have declined to less than 50% in most outbreaks.

PDNS-affected pigs exhibit anorexia, depression, prostration, a stiff gait and/or reluctance to move, and mild to no pyrexia. However, the most obvious sign in the acute phase of the disease is the presence of irregular, red-to-purple macules and papules on the skin, principally on the hind limbs and perineal area (Figure 9.6.1A), that tend to coalesce. A more generalized distribution is seen in the most severely affected animals. With time, the lesions become covered by dark crusts, and then gradually fade (usually in 2 to 3 weeks), sometimes leaving scars (Drolet et al. 1999).



9.6.1. A: Note the presence of irregular, red-to-purple macules and papules on the skin, especially the hind limbs and perineal area. **B:** Enlarged kidney with generalized petechial cortical hemorrhages. Note the increased size of the perirenal lymph node.

At necropsy, in addition to the skin, the kidneys, lungs, and lymph nodes are the most markedly affected organs (Segalés et al. 1998). Both kidneys are enlarged and pale, with petechial cortical hemorrhages (Figure 9.6.1B). Lungs are usually noncollapsing and tan mottled, with suppurative bronchopneumonia observed in some cases. Lymph nodes are usually enlarged (generalized lymphadenopathy) and dark red; the reddish appearance is usually confined to subcapsular and medullar sinuses, which suggests blood imbibing rather than lymph node hemorrhage. Other macroscopic lesions commonly associated with PDNS include serous effusions in body cavities, subcutaneous edema, and increased amount of synovial fluid (Drolet et al. 1999). In sporadic cases, focally or extensive necrosis of spleen (splenic infarcts) has been observed. Diarrhea, Glasser's disease, porcine reproductive and respiratory syndrome (PRRS), postweaning multisystemic wasting syndrome, conjunctivitis and gastric ulcers of pars oesophagea are sometimes coincidental features on the farms where the diagnosis of PDNS is established.

DEFINITIVE DIAGNOSIS

PDNS is relatively straightforward to diagnose based on clinical signs and macroscopic lesions. However, the definitive diagnostic criteria are the presence of glomerulonephritis and systemic necrotizing vasculitis detected by histological examination (Segalés et al. 1998; Thibault et al. 1998).

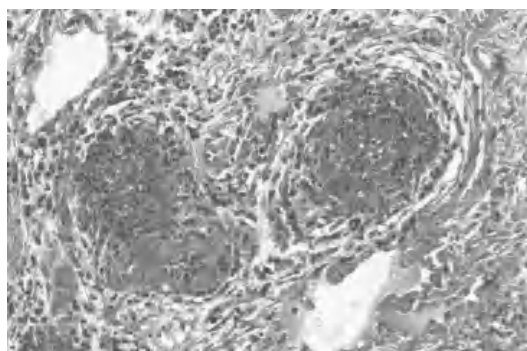
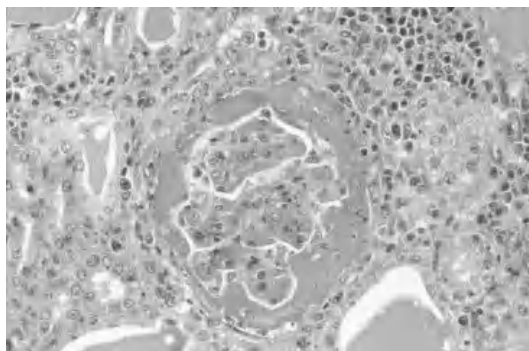
A severe, diffuse fibrinous glomerulitis is usually the most striking lesion in the majority of the pigs with PDNS (Figure 9.6.2A). Glomerular spaces contain precipitated fibrin, polymorphonuclear neutrophils, and erythrocytes. In a high proportion of acute cases of disease, all of the glomeruli are affected. In all of these se-

vere cases, pigs have high blood urea and creatinine levels, which indicate that renal failure is the cause of death of PDNS-affected pigs. Chronically affected animals show a mild-to-severe interstitial kidney fibrosis and glomerular sclerosis.

Leukocytoclastic necrotizing vasculitis has been observed in a variety of organs, including kidney (primarily renal pelvis but also glomeruli), mesentery, lymph nodes, spleen, lung, liver, heart, stomach, urinary bladder, meninges, and dermis (responsible for the ischemia and necrosis of the skin, leaving the macroscopically visible skin lesions). The necrotizing vasculitis tends to affect small to medium-sized arteries and capillaries (mainly in dermis), with fibrinoid necrosis of the tunica intima and media (Figure 9.6.2B). A severe, mixed inflammatory infiltrate throughout the arterial wall is also seen.

On the other hand, a mild-to-massive lymphocyte depletion in lymph nodes has been regularly observed in cases of PDNS. In about 50% of affected pigs, granulomatous inflammatory infiltrates, consisting of histiocytes and/or multinucleate giant (syncytial) cells, are seen in lymph node parenchyma, mainly within follicular areas (Rosell et al. 2000; Segalés et al. 1998). Another usual lesion in PDNS-affected pigs is a mild-to-moderate interstitial pneumonia, with edema and congestion of lung parenchyma (Rosell et al. 2000).

Not all affected pigs show macroscopic kidney and skin lesions. A few pigs that are considered PDNS cases do not have skin lesions. Others do not have kidney lesions, or very slight kidney lesions, and the renal failure associated with more severe cases. All of these are considered atypical cases of the syndrome, but they are considered PDNS cases because of the presence of the systemic necrotizing vasculitis. The numbers of atypical PDNS cases have clearly increased in the last 3 years,



9.6.2. A: Kidney. Glomerular space containing precipitated fibrin (fibrinous glomerulitis), with a mononuclear inflammatory infiltrate in the renal interstitium (interstitial nephritis) (hematoxylin-eosin stain). **B:** Skin. Necrotizing vasculitis with fibrinoid necrosis of the tunica intima and media, with a severe mixed inflammatory infiltrate (leukocytoclastic) throughout the arterial walls (hematoxylin-eosin stain).

especially on farms experiencing a high prevalence of the syndrome (unpublished data). The reason why they do not show the typical macroscopic distribution of lesions is unknown.

DIFFERENTIAL DIAGNOSIS

The low prevalence of PDNS in most swine herds may assist in its diagnosis. However, in herds where the prevalence of disease is higher than normal (more than 1%), an accurate differential diagnosis should be established.

In PDNS, the primary macroscopic lesions are present in the skin and kidney. The diseases and conditions that can be associated with red discoloration of the skin include classical swine fever, African swine fever, swine erysipelas, septicemic salmonellosis, infection with *Actinobacillus suis*, porcine stress syndrome, transient erythema (urine-soaked floors, chemical burns, etc.) and other bacterial septicemias. In addition, classical swine fever, African swine fever, and septicemic salmonellosis can also cause kidney lesions very similar to those observed in PDNS. Therefore, the major differential diagnosis to establish for PDNS is classical swine fever in European countries and septicemic salmonellosis in the United States and Canada. However, the differential diagnosis for PDNS will vary by country or geographical region, depending on the diseases causing skin and/or kidney lesions.

PATHOGENESIS AND ETIOLOGY

The microscopic lesions of PDNS are strongly suggestive of an immune complex-mediated disease (Smith et al. 1993). Immunoglobulin M (IgM), IgA, occasionally IgG, and complement factors C3 and C1q have been detected within renal glomeruli and affected vessel walls (Hélie et

al. 1995; Sierra et al. 1997). Immune complexes are composed of immunoreactants (e.g., immunoglobulins and complement factors) but also contain an antigenic component. The responsible antigen (or antigens) involved is currently unknown, but several authors have postulated several etiologic possibilities. Theoretically, the condition could be triggered by a wide spectrum of factors, including drugs, chemicals, food allergens, endogenous antigens, and infectious agents (Thomson et al. 1998).

Infectious agents have been the most studied—presumably antigens involved in PDNS pathogenesis. Until 1998, porcine reproductive and respiratory syndrome virus (PRRSV), *Pasteurella multocida*, *Streptococcus* spp., and lipopolysaccharides of gram-negative bacteria have been suggested as the possible antigens associated with PDNS (Drolet et al. 1999). Among these, PRRSV was probably the most closely studied. There are two reports in the literature, with 10 and 12 pigs, respectively, where PRRSV was suggested as a possible triggering antigen in PDNS (Segalés et al. 1998; Thibault et al. 1998). Investigators have speculated that PRRSV plays a role in PDNS for several reasons:

1. Most of the affected pigs are serologically positive to PRRSV (Segalés et al. 1998), and a certain proportion (Segalés et al. 1998), or all of them (Thibault et al. 1998), are also infected with the virus.
2. PRRSV has been shown to cause necrotizing vasculitis in experimentally infected pigs (Cooper et al. 1997).
3. In PRRSV infection, viremia coexists with antibodies (Rossow et al. 1994; Segalés et al. 1998), which could facilitate the development of immune complexes.

4. PRRSV antigen has been demonstrated within macrophages around affected cutaneous and renal blood vessels (Thibault et al. 1998).

However, PRRSV was not found within the injured vascular walls in any of the animals studied. Then, too, PDNS has been described in Chile, which is considered a PRRSV-free country (Sierra et al. 1997), and PDNS has been described on farms that were seronegative for PRRSV (unpublished data). These facts suggest that PRRSV may not play a causal role in the syndrome and that the association between PDNS and PRRSV infection may simply be coincidental.

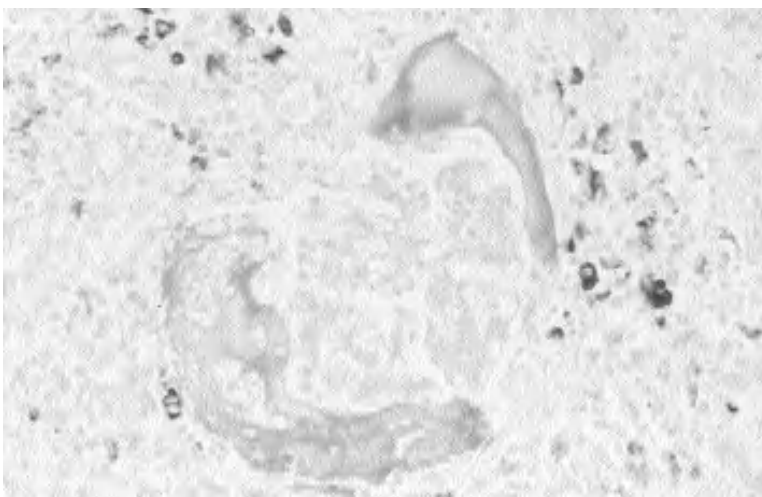
In 1997, a new disease affecting late nursery and early fattening pigs was described that was characterized by wasting, pale skin, dyspnea and, occasionally, jaundice (Clark 1997). This new disease, named postweaning multisystemic wasting syndrome (PMWS) (Clark 1997), had unique microscopic lymphoid lesions consisting of lymphocyte depletion and granulomatous inflammation (Rosell et al. 1999). Porcine circovirus type 2 (PCV2) is presently considered the cause of PMWS (Kennedy et al. 2000). Some years ago, pathological and epidemiological features suggested a possible link between PCV2 and PDNS:

1. Similarities in microscopic lesions between PMWS and PDNS; PDNS cases usually exhibit lymphocyte depletion, the presence of syncytial cells and granulomatous inflammatory infiltration of lymphoid tissues, and interstitial pneumonia (Rosell et al. 2000; Segalés et al. 1998).
2. Most countries having PMWS, including Canada, the United States, Spain, the Netherlands,

France, Greece, and the United Kingdom (Allan and Ellis 2000), also have reported cases of PDNS.

3. An increased incidence of PDNS has been observed in farms with PMWS in Spain, the United Kingdom, and France (Gresham et al. 2000; Madec et al. 2000; Rosell et al. 1999).

This indirect evidence prompted an investigation into the presence of PCV2 in tissues of PDNS-affected pigs. Among 30 pigs from Spain and three from Michigan (USA), PCV2 nucleic acid was detected in 31 (28 of 30 Spanish cases and all three US cases) by *in situ* hybridization (Rosell et al. 2000). The viral genome was primarily detected in lymphoid tissues, i.e., lymph nodes (perirenal, superficial inguinal, and mesenteric), Peyer's patches, tonsil, and spleen. In contrast to the high levels of PCV2 nucleic acid found in PMWS-affected pigs, PCV2 nucleic acid was generally low to very scant and mainly located in follicles of the lymphoid tissues. Only five pigs in the study showed amounts of PCV2-genomic material comparable to those found in cases of PMWS. In a smaller proportion of PDNS-affected pigs, viral nucleic acid was also detected in kidney (Figure 9.6.3). These results showed that the detection of PCV2 required screening several lymphoid tissues plus other nonlymphoid tissues, such as kidney, lung, and skin. The use of only kidney or only one lymphoid tissue could give negative results in pigs that are really infected. The target cells for PCV2 in pigs with PDNS were monocyte/macrophage lineage cells, dendritic-like cells, renal epithelial cells, and endothelial cells (Rosell et al. 2000). These tissues were not different from those observed in cases of PMWS (Kennedy et al. 2000; Rosell et al. 1999).



9.6.3. *Kidney. Darkly stained porcine circovirus type 2 nucleic acid within the cytoplasm of interstitial inflammatory cells of a kidney with typical lesions of porcine dermatitis-nephropathy syndrome (in situ hybridization).*

Similar results were recently reported in the United Kingdom, where PCV2 was isolated and detected by immunohistochemistry in pigs affected with PDNS from several farms in East Anglia, the region that had most of the cases of PMWS in the United Kingdom (Gresham et al. 2000). Furthermore, four PDNS cases examined in Northern Ireland also showed the presence of PCV2 in their tissues (Allan and Ellis 2000).

In addition to the PDNS cases in Spain, the United States, and the United Kingdom, a single pig with PDNS in Argentina (Machuca et al. 1999) and another in the Netherlands (unpublished data) were also infected with PCV2, as shown by *in situ* hybridization. All these data support the idea that PCV2 may be involved in the pathogenesis of PDNS. This is supported by the observation that PCV2 has been found in association with some of the lesions (mainly lymphoid lesions) observed in PDNS-affected pigs in all countries where the role of PCV2 has been studied. Recent data (unpublished results) have shown that pigs with subacute to chronic kidney lesions of PDNS (glomerular fibrin organizing to fibrosis, and the initial stages of chronic interstitial fibrosis) do not have PCV2 nucleic acid in their tissues when measured by *in situ* hybridization.

In the literature, more PDNS-affected pigs were infected with PCV2 compared with those infected with PRRSV. However, the same reasons that lead some investigators to question the role of PRRSV in PDNS also applies to PCV2. Again, PCV2 nucleic acid has not been demonstrated within the injured vascular walls. At present, the fact that PDNS and PCV2 infection have been found together may be a mere coincidence. Therefore, further studies are needed to evaluate the PCV2 infection status of PDNS pigs throughout the world and to establish whether PCV2 is involved in the pathogenesis of PDNS.

Very few studies have been conducted to identify potential precipitating factors for PDNS other than microbiologic agents. In one study, the potential role of vaccines, antimicrobials, and other biological or chemical products used on farms where PDNS cases were detected was studied, but no clear conclusions were forthcoming (Thibault et al. 1998).

TREATMENT AND PREVENTION

PDNS is usually a disease of low prevalence in swine herds and therefore of low impact on the profitability of pig farms (Duran et al. 1997). Only atypical cases with moderate-to-high morbidity and mortality rates may be of importance in terms of economic losses. Treatment, including attempts with a wide range of antimicrobial agents, has been unsuccessful in reducing mortality or accelerating the resolution of skin lesions (Segalés et al. 1998; Smith et al. 1993). The use of anti-inflammatory

drugs and multivitamin supplements together with the reduction of stress factors for the control PDNS and PMWS has been suggested (Baird et al. 2000), but the results have not been conclusive. Since the pathogenesis of PDNS is not known, no recommendations for its prevention can be made.

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10 PORCINE NIDOVIRUSES

10.1

Porcine Coronaviruses

Karol Sestak and Linda J. Saif

SUMMARY

The family Coronaviridae belongs to the order Nidovirales. Coronaviruses are pathogens associated with infections of veterinary importance causing a spectrum of clinical syndromes that vary depending on the host. Due to their large, inherently error-prone RNA genome, coronaviruses are well adapted to changing environmental selective pressures. The dynamic, quasi-species character of this virus family was recognized two decades ago with the emergence of porcine respiratory coronavirus (PRCV), a deletion mutant of transmissible gastroenteritis virus (TGEV). It was later shown that the loss of only two amino acids from the TGEV major surface attachment protein might result in a change from gastrointestinal to respiratory tropism. In addition to TGEV and PRCV, two other antigenically distinct coronaviruses have been isolated from pigs: hemagglutinating encephalitis virus (HEV) and porcine epidemic diarrhea virus (PEDV). Due to their emerging/reemerging nature and impact on swine production, this chapter deals only with TGEV, PRCV, and PEDV. Historical and current aspects related to epidemiology, pathogenesis, diagnosis, prevention, and control of these three porcine coronavirus species are discussed.

INTRODUCTION

The Coronaviridae family consists of two genera of RNA viruses that infect vertebrates including humans, domestic animals, and birds (Horzinek 1999). Coronavirus infections are usually associated with respiratory, gastrointestinal, cardiovascular, and neurological diseases (Lai 1990). The best-known representatives of this virus family include avian infectious bronchitis virus (IBV), mouse hepatitis virus (MHV), porcine transmissible gastroenteritis virus (TGEV), bovine coronavirus (BCV), human coronavirus (HCV), feline infectious peritonitis virus (FIPV), canine coronavirus (CCV), turkey coronavirus (TCV), and several other virus species of veterinary importance (Siddell et al. 1983; Lai 1990). From pigs, four antigenically distinct coronaviruses have been isolated: transmissible gastroenteritis virus (TGEV), hemagglutinating encephalomyelitis virus

(HEV), porcine epidemic diarrhea virus (PEDV), and porcine respiratory coronavirus (PRCV) (Pensaert 1989).

Historically, the first report of clinical disease in pigs caused by coronaviruses dates to 1946 (Doyle and Hutchings 1946) and TGE, which occurs throughout the world. According to serological surveys conducted in North America and Europe, a high seroprevalence (36% to 100%) to TGEV exists among swine [US Department of Agriculture (USDA) 1997; Pensaert et al. 1993]. Accurate TGEV seroprevalence assessments are complicated by the fact that antibodies to a natural deletion mutant of TGEV, PRCV, are indistinguishable from TGEV antibodies by routine serological diagnostic assays. According to the 1995 and the 1990 National Swine Surveys (United States), approximately 6% of swine operations reported problems with TGEV (USDA 1992, 1997). Diagnostic studies indicated that among the piglets that died from diarrhea before reaching the age of 1 month, 8% were positive for TGEV (Moon and Bunn 1993). A more recent serological survey showed that all of the 22 “medium to large” size swine herds studied in Iowa were positive for TGEV or PRCV antibodies, with 16 herds being specifically positive for TGEV antibodies (Wesley et al. 1997).

The emergence of PRCV from 1984 onward coincided with the disappearance of TGEV in Europe (McGoldrick et al. 1999). Based on several TGEV-PRCV cross-protection studies, it was suggested that repeated subclinical PRCV infections increased the level of immunoglobulin A (IgA) antibodies cross-reactive to TGEV in milk of lactating sows (Sestak et al. 1996). The TGEV infection in piglets born to such sows was characterized by reduced severity of clinical disease. In these situations, PRCV acts as a naturally modified-live vaccine to TGEV and induces active immunity in pregnant sows that is passively transferred to suckling piglets (Sestak et al. 1996).

Since the mid-1980s, a previously unrecognized porcine coronavirus spreading rapidly through Europe was identified (DeBouck et al. 1982). Epidemic spread, enteropathogenicity, and ability to cause diarrhea in swine of all ages were reported (Pensaert 1999). The agent was found to be antigenically distinct from TGEV, HEV, and other animal coronaviruses (Pensaert et al.

1981). The name porcine epidemic diarrhea virus (PEDV) was adopted. At present, PEDV has been identified in most swine-producing countries, except the Americas (Pensaert 1999).

EPIDEMIOLOGY

Transmissible Gastroenteritis Virus

Transmissible gastroenteritis can occur in three different forms, depending on the herd's health status (Bohl 1989). When the virus spreads within a fully susceptible herd with no previous history of TGEV, it is referred to as epizootic TGE, characterized by up to 100% mortality among newborn pigs, marked diarrhea and dehydration in weaned pigs, and inappetence, vomiting, and diarrhea in adult animals. Partial or total agalactia of lactating sows is common (Lanza et al. 1995). Epizootic TGE ends within several weeks. In herds where TGE is on the decline but the continuous introduction of susceptible animals occurs, infection becomes more chronic and is referred to as endemic or enzootic TGE (Bohl 1989). Mortality among endemic herds usually does not exceed 20%; however, the decline of colostral and milk antibodies contributes to the onset of diarrhea. A modification of endemic TGE is known as intermittent endemic TGE, where virus is introduced into a herd where only adult animals (sows) have been previously exposed and therefore can provide some passive immunity to their pigs (Bohl 1989). Because of better TGEV stability when kept cold and protected from the sunlight, TGE tends to be a seasonal infection with mainly a winter occurrence (Haelterman 1973). Transmission of virus by means of mechanical vectors or occasional hosts (dog, cat, fox, or starlings) can take place (Bohl 1989). In feces of young pigs, TGEV can be shed for up to 2 weeks and in the nasal secretions for 10 to 11 days (Kemeny et al. 1975).

Since its first description in an Indiana swine herd in 1946 by Doyle and Hutchings, TGE has been reported in all countries with an intensive pork industry (Bohl 1989; Doyle and Hutchings 1946; Saif and Wesley 1999). The economic losses caused previously by TGEV were significant, as reported from France, the United States, Czechoslovakia, England, and the Netherlands (Bohl 1989; Saif and Wesley 1999). In the United States, TGE remains a problem. TGEV was found in about half of the swine herds tested in 1987 and 1988 (Hill 1989; Polson et al. 1993) and was also responsible for 26% of all the cases of neonatal diarrhea reported to the Illinois Department of Agricultural Animal Diagnostic Laboratory (Hoefling 1989). Major economic losses to the swine industry occur from epizootic TGEV outbreaks that can cause 100% mortality among neonatal pigs (Saif and Wesley 1999), as well as from growth retardation and increased susceptibility to other infectious diseases in older TGEV-infected pigs (Hoefling 1989). In 1987 and 1988,

it was estimated that the pork industry in Iowa alone lost \$10 million as a result of TGEV infection (Hill 1989). A survey in 1990 conducted by the National Animal Health Monitoring System reported that 36% of swine herds in the United States were positive for antibodies to TGEV (Wesley et al. 1997). In 1995, 16 of 22 swine herds examined in Iowa were seropositive for TGEV (Wesley et al. 1997). The current economic impact of TGEV infections, since the occurrence of PRCV in the United States, has not been examined.

Porcine Respiratory Coronavirus

Since the 1980s, the significance of TGEV has diminished in Europe with the appearance of the TGEV mutant, PRCV (Laude et al. 1993). Possibly, one factor that contributed to the emergence of this porcine coronavirus with respiratory tract tropism was the intensification of pig production during the late 1970s (Pensaert 1989). In contrast to TGEV, PRCV does not cause mortality among pigs and infections are usually subclinical. Some strains were described that produce mild respiratory symptoms (Paul et al. 1997). Aerogenic virus spread was described, and seroconversion could not be distinguished from TGEV-induced seroconversion without the use of monoclonal antibodies (Pensaert et al. 1986; Simkins et al. 1993). Moreover, PRCV also became endemic in countries like Denmark and England where the incidence of TGEV was very low or absent (Brown and Cartwright 1986). In endemic areas, newborn pigs receive PRCV antibodies via colostrum and milk. This passive protection lasts 3 to 4 weeks and is gradually replaced with active immune response. Experimental passive-immunity studies suggested that multiple PRCV reinfections in endemic areas could contribute to the decline in TGEV outbreaks that have been observed; thus, PRCV could act as a naturally modified-live vaccine (Lanza et al. 1995; Sestak et al. 1996). In young and adult pigs, PRCV is excreted nasally for 10 to 11 days. Similarly to TGEV, PRCV exhibits an autumn-winter incidence (Pensaert 1989). Reinfections of pigs with PRCV were reported in France and Belgium, with an increased autumn incidence for several successive years (Jestin et al. 1987; Laval et al. 1991). Serological studies of the prevalence of PRCV infection among fattening pigs (Belgium) suggested that more than 50% of animals were seropositive (Pensaert 1989). In Iowa swine herds, it was suggested that the recent increases in TGEV/PRCV seroprevalence was most likely due to subclinical PRCV infections (Wesley et al. 1997). High seroprevalence to PRCV (61%) was recently reported from South Korea, suggesting an extensive distribution of this virus throughout the Korean swine population (Chae et al. 2000).

Porcine Epidemic Diarrhea Virus

Although PEDV has been isolated in most swine-raising countries in Europe and Asia (Kweon et al. 1993; Mostl

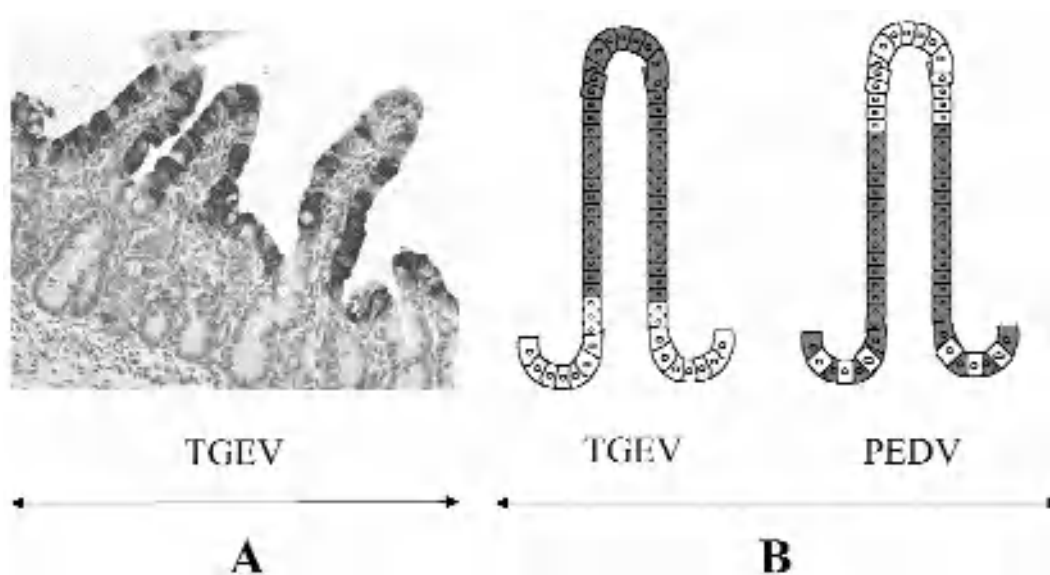
et al. 1990), no virus isolation has yet been reported from the Americas. Changing patterns of PEDV epidemiology have been observed in Europe, where PED is no longer epidemic but endemic and sometimes persistent (Pensaert 1999). This persistence is characterized by the presence of virus-specific antibodies and was preceded by the stage of acute epizootics during the 1980s (DeBouck et al. 1982). An epidemic epidemiological pattern has been observed during recent years in Asia, where massive and severe PED outbreaks clinically resembling TGE previously were associated with large economic losses (Hwang et al. 1994; Sueyoshi et al. 1995). Similarly to TGEV, PEDV transmission is maintained via feces or other virus-carrying fomites by the oral route of ingestion. In contrast to TGEV, PEDV appears to persist in swine farms, but mechanisms of this persistence have not been fully elucidated.

PATHOGENESIS

Transmissible Gastroenteritis Virus

The gateway for TGEV infection is the oral cavity, although the virus can be inhaled as well (Aynaud et al. 1991). After the virus is swallowed, it survives the low pH of the stomach and resists the proteolytic environment of the duodenum. In the small intestine, it infects the villous epithelial cells (Bohl 1989). Peplomer-shaped protrusions, i.e., the viral spike (S) glycoproteins bind to aminopeptidase N, a TGEV receptor expressed on the intestinal brush border (Delmas et al. 1993). The most prominent factor accounting for TGE pathology and diarrhea is destruction of villous epithe-

lium. A typical pattern is detection of TGEV in villous, but not crypt, epithelium (Figure 10.1.1). Only the enzymatically mature villous cells (absent in crypt epithelium) are infected. As a consequence of virus infection, discernible morphological changes in the intestinal epithelium were described, such as reduction and blunting of the villi (Saif and Wesley 1999). After 1 to 3 days of infection, the undifferentiated cells from the crypts start to migrate upward to replace the destroyed villous epithelium (Wege 1995). The time necessary for villous replacement depends on the age of animals (up to 10 days in the case of suckling pigs and 2 to 4 days in the case of weaned pigs) (Moon 1971). The reduction in enzymatic activity of the villous epithelium accounts for alterations in digestion, cellular transport, hydrolysis of lactose from milk, and subsequent development of a malabsorptive syndrome (Frederick et al. 1976). In contrast to the normal osmotic force in a healthy intestine, in TGEV-infected intestines, undigested lactose and the Na^+ accumulate in the gut lumen, which contributes to the withdrawal of body fluids and accounts for metabolic acidosis, diarrhea, and dehydration (Saif and Wesley 1999). TGEV infection of the respiratory tract has been described (Underdahl et al. 1975), and these virus strains (attenuated P115) were also found to replicate in lavaged alveolar macrophages (Laude et al. 1984). The TGE gross lesions involve the accumulation of undigested milk in the stomach and small intestine, thinner intestinal walls due to the villous atrophy and, in some cases, pneumonic lesions (Bohl 1989; Saif and Wesley 1999).



10.1.1. Epithelial cell (small intestine) tropism of enteropathogenic porcine coronaviruses. **A:** Detection of transmissible gastroenteritis virus (TGEV) antigens by immunohistochemistry. Courtesy of Dr. J. Hayes. **B:** Usual distribution of TGEV/porcine epidemic diarrhea virus (PEDV) antigens. Shaded cells are likely virus targets.

Porcine Respiratory Coronavirus

The loss of PRCV enteropathogenicity is explained by alterations in the spike (S) glycoprotein, which mediates attachment and thus plays a critical role during the early stages of cell infection (Ballesteros et al. 1997; Pensaert 1989). Aminopeptidase N, an enzyme expressed by the villous enterocytes of the small intestine, is known to be the major receptor for TGEV (Delmas et al. 1993). PRCV also uses aminopeptidase N as a cell receptor in the respiratory tract (Delmas et al. 1993). Two theories for the emergence of PRCV as a deletion mutant are (1) PRCV is a particular TGEV strain originally having a respiratory tropism, and (2) PRCV gained its ability to replicate in the respiratory tract because of the S-gene and possibly 3a-gene alterations (Pensaert 1989). Although some findings suggested that the 3a gene might be responsible for loss of PRCV enteric tropism (Paul et al. 1997), experiments with amino acid changes at the N terminus of TGEV S proteins suggested that the TGEV S gene is a determinant of enteric tropism (Ballesteros et al. 1997; Sanchez et al. 1999). However, it was still speculated that 3a-gene deletions could be a condition or prerequisite for the occurrence of the S-gene deletion. Recent characterization of British porcine coronavirus isolates suggests that virulent, enterotropic TGEV can have a large deletion in its 3a gene without any impact on S-gene completeness and virus tropism (McGoldrick et al. 1999). It was suggested that the severity of PRCV infections differs with the age of inoculated animals (Cox et al. 1990a). When animals younger than 5 weeks were inoculated by the nasal route, PRCV infected both the respiratory and intestinal tracts. However, because of a substantially lower extent of multiplication and infection of non-epithelial cells, the gut is not considered a target organ for PRCV (Saif and Wesley 1999). PRCV can be isolated from nasal mucosa, tonsils, trachea, and lungs and, with lower virus titers, also from the gastrointestinal tract (Cox et al. 1990b; O'Toole et al. 1989). Maximum antigen expression was demonstrated at postinoculation day 3 in epithelial cells of the pulmonary and bronchiolar alveoli (Cox et al. 1990b).

Investigation of the TGEV- and PRCV-shedding duration showed that PRCV-nasal shedding persisted (adult swine) until postinoculation day 10, whereas TGEV-fecal shedding persisted (suckling and weaned pigs) until postinoculation day 14, with TGEV-nasal shedding up to postinoculation day 11 (Laude et al. 1993; Saif and Wesley 1999). PRCV infections usually remain subclinical, although some investigators reported mild clinical signs of respiratory tract infections, such as sneezing, cough, dyspnea, and short-lasting fever (Cox et al. 1990a; Pensaert et al. 1986; Vannier 1990). Gross lesions have been described after experimental infection of gnotobiotic pigs and consist of catarrhal lobular bronchopneumonia, interstitial pneumonia with infiltration of macrophages, plasma cells, and lymphoblasts (Cox et al. 1990b; Van

Nieuwstadt and Pol 1989). Both PRCV and TGEV induce interferon- α secretion (Charley and Laude 1988; Van Reeth and Nauwynck 2000). It was suggested that dual infection of pigs with porcine reproductive and respiratory syndrome virus (PRRSV) and PRCV could result in more severe disease and growth retardation than only single PRRSV infection (Van Reeth et al. 1996).

Porcine Epidemic Diarrhea Virus

The severity of clinical PED depends on the immune status of the affected herd. In cases where PEDV is introduced into a nonimmune, fully susceptible population, clinical symptoms may resemble TGE, and mortality in neonatal piglets can reach about 80% (Pensaert 1999). This acute PED is characterized by watery diarrhea and dehydration in young piglets. In fattening pigs, an association between PED rate and stress was observed (Pensaert 1999). Subclinical, persistent PEDV infections are typical for populations with previous PED history that also possess virus-specific immunity. The mechanism of viral replication and consequent villous degeneration is similar to that described for TGE (Pospischil et al. 1981). The affected villous epithelial cells (Figure 10.1.1) can be seen in the small intestine and colon as early as 12 to 18 hours and as late as 5 days after inoculation (Pensaert 1999). When introduced into a seronegative herd, clinical and pathological signs associated with PEDV tend to be similar to those for TGEV, but less severe, except that the diarrhea may persist 2 to 3 weeks (Pensaert 1999).

DIAGNOSIS

Laboratory diagnosis of PRCV, TGEV, and PEDV infections usually involves one or more of the following: detection of virus, its genome, antigen components, or antibody response. PRCV antigen can be detected by a direct immunofluores (or immunoperoxidase) antibody test on formalin- or paraffin-fixed lung sections (Pospischil et al. 1969). An indirect immunofluorescence test has been used for the detection of virus antigen in nasal smears (Onno et al. 1989). Electron microscopy can be used to examine the cells of bronchiolar and alveolar tissues, including macrophages, for the presence of coronavirus particles (Cox et al. 1990b). Detection of TGEV/PRCV/PEDV-specific nucleic acid was performed by the use of dot-blot hybridization, reverse-transcription (RT) polymerase chain reaction (PCR), or RT-nested PCR (Benfield et al. 1991; Britton et al. 1993; Jackwood et al. 1993; Kim et al. 2000; Kubota et al. 1999; Kwon et al. 1998; Paton et al. 1997; Wesley et al. 1991; Woods 1997). A simple and reliable method to confirm TGEV infection is to detect TGEV antigens or virus contained within small intestinal fluids by enzyme-linked immunosorbent assay (ELISA) (Lanza et al. 1995). TGEV antigens can also be detected by immunofluorescence or immunoperoxidase techniques within virus-infected cells (Shoup et

al. 1996). However, this must be done during an early stage of infection (1 to 2 days after inoculation) since the infected enterocytes are rapidly destroyed and released from the villi (Pensaert et al. 1981). Clarified, diluted intestinal contents can be subjected to immunoelectron microscopy or ELISA (Horzinek et al. 1982; Saif and Wesley 1999).

During the mid-1990s, with the emergence of PRCV, the necessity for a new test arose, primarily because of export requirements for TGEV-seronegative animals. To meet this requirement, monoclonal antibodies and oligonucleotide probes specific for TGEV/PRCV were prepared, and differential ELISAs and RT-PCR assays were developed (Callebaut et al. 1989; Garwes et al. 1988; Have 1990; Kim et al. 2000; Sestak et al. 1999b; Simkins et al. 1993). These tests are used to detect and differentiate between TGEV- and PRCV-induced antibodies or viral RNA extracted directly from feces or nasal secretions of infected pigs.

In contrast, PEDV does not cross-react with TGEV/PRCV and exhibits a distinct pathogenesis in the intestinal tract. Because of its common host and cell tropism with TGEV, PEDV has to be considered when diagnosis is based solely on electron microscopy of fecal specimens (Kusanagi et al. 1992). PEDV can be confirmed by direct immunofluorescence or immunohistochemistry of the small intestine from piglets with acute diarrhea within 3 days after onset (Pensaert 1999). ELISA can be used for detection of PEDV antigens (Carvajal et al. 1995) or antibodies (De Arriba et al. 1995). Primers specific for the PEDV nucleocapsid (N) protein gene have been used for detection of virus in intestinal contents by RT-nested PCR (Kubota et al. 1999). Shedding of PEDV in feces of experimental pigs was detected between 3 and 11 days after inoculation (Pensaert 1999).

PREVENTION AND CONTROL

Application of general preventive measures such as “all-in all-out” herd turnover and the “black-and-white” system of sanitation helps to prevent infection with porcine coronaviruses (Bohl 1989; Stepanek et al. 1974). An efficient and historically the oldest immunization method to prevent TGE or PED is based on feeding the infectious gut materials from diarrheic piglets to pregnant sows and gilts approximately 3 weeks prepartum (Bohl 1989). Although effective active immunity of a sow and, subsequently, also passive immunity of suckling piglets can be induced by this method, it can also lead to uncontrollable perpetuation of other intestinal pathogens. The necessity to better characterize the potential vaccine dose and virus or antigen source led to the development of commercial vaccines.

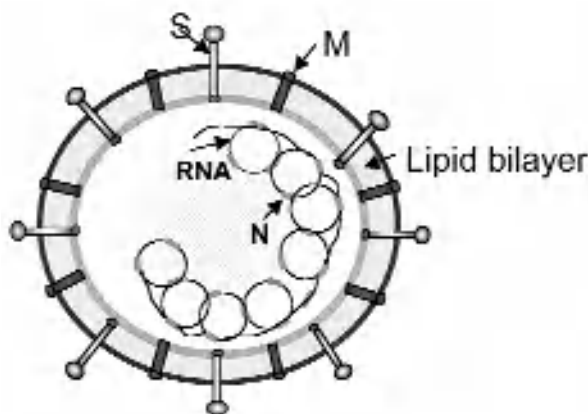
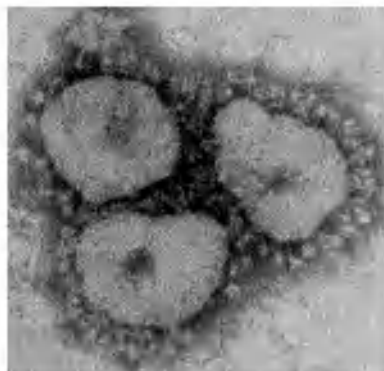
Current commercial TGEV vaccines consist of attenuated or killed virus that does not induce sufficient immune responses in the gut, resulting in irregular lev-

els of active and passive immunity. Commercial vaccines have been available since 1966 (Welter 1986). These vaccines were inactivated or modified-live virus, and were applied intramuscularly (IM) to sows before farrowing; they contributed mostly to systemic immunity (IgG) and to moderate or no reductions in the rate of piglet mortality (Bohl 1989). Several US companies reported the testing of attenuated live vaccines for IM administration (Welter 1986). It was found that passive immunity induced by a federally licensed, attenuated live vaccine for oral and IM use was overwhelmed after TGEV challenge exposure of suckling piglets (Moxley and Olson 1989). The immunity induced by these attenuated live vaccines functioned by means of stimulation of gut-associated lymphoid tissue, with secretory IgA production and prompting the gut-mammary homing pathway. An attenuated live vaccine is still one of the currently available commercial TGE vaccines licensed by Veterinary Biologics (USDA).

An important requirement for an oral TGEV vaccine is that it possess minimal pathogenicity for piglets while retaining the ability to deliver immunogenic antigens to gut-associated lymphoid tissue (Saif and Wesley 1999). The incomplete protection against TGEV induced by oral vaccines currently available is the result of their inability to infect the villous enterocytes of the small intestine. As a consequence, there is very low stimulation of intestinal IgA B-cell precursors (Saif and Wesley 1999; Sestak et al. 1999a).

With current vaccines being either too attenuated or applied at a dosage that is too low, protection is inconsistent (Saif and Jackwood 1990; Shoup et al. 1997; Van Cott et al. 1993; Saif 1996) and the search for more reliable vaccines continues. For the protection of suckling piglets, research continues to focus on the principle of colostral and lacteal intake of secretory IgA antibodies after immunization of sows with attenuated live vaccines (Park et al. 1998; Saif 1996; Sestak et al. 1996).

During this decade, emphasis has been on the construction of TGEV protein subunit vaccines. Among the three major structural proteins of TGEV (Figure 10.1.2), the S protein contains immunodominant epitopes that are recognized by virus-neutralizing antibodies (Delmas et al. 1986; Jimenez et al. 1986). Some of these epitopes were shown to be continuous domains (Delmas et al. 1990; Gebauer et al. 1991; Posthumus et al. 1990). Therefore, the objective of some studies was to design antigenic synthetic peptides derived from the S protein (Posthumus et al. 1991). It was found that the N protein and not S protein contains T-helper cell epitopes (Anton et al. 1995). A synthetic 15-mer peptide epitope derived from the N protein was shown to cooperate with the S protein for *in vitro* induction of TGEV-specific antibody (Anton et al. 1996).

**A****B**

10.1.2. *Transmissible gastroenteritis virus (TGEV), porcine respiratory coronavirus (PRCV), and porcine epidemic diarrhea virus (PEDV) exhibit typical coronavirus morphology. A:* Virions are pleomorphic, 60 to 200 nm, with club-shaped sparse spikes (S protein). In addition to S protein, membrane (M) and helical nucleocapsid (N) proteins are major structural components of the virus particle. The internal core contains the N protein and continuous mRNAs that are produced in host cells (TGEV and PEDV, small intestine villous epithelium; and PRCV, respiratory tract epithelium). **B:** In the electron micrograph, TGEV particles are indistinguishable from PRCV or PEDV particles.

To express the TGEV S, membrane (M), or N proteins, several prokaryotic and eukaryotic systems such as *Escherichia coli*, *Salmonella*, adenovirus, vaccinia virus, baculovirus, and plants were used (Britton et al. 1987; Chen and Schifferli 2001; Enjuanes et al. 1992; Godet et al. 1991; Gomez et al. 1998, 2000; Park et al. 1998; Pulford and Britton 1991; Shoup et al. 1997; Smerdou et al. 1996a,b; Torres et al. 1995, 1996; Tuboly et al. 1994, 2000). In some studies, protective antibodies were induced in inoculated animals, correlating with partial protection (Torres et al. 1995). In other studies, induction of protective antibodies was not reported (Gomez et al. 1998, 2000; Smerdou et al. 1996a,b; Tuboly et al. 2000), or they were detected as IgG virus-neutralizing antibodies (Park et al. 1998; Shoup et al. 1997). In the first attempts with prokaryotic expression systems, TGEV immunogens did not induce any neutralizing antibodies (Saif and Wesley 1999). Human adenovirus vectors were reported to undergo an abortive replication in the porcine gut and lose the TGEV (S) inserts (Torres et al. 1996). The baculovirus-expressed S protein induced virus-neutralizing antibodies to TGEV, as detected in the serum of rats and pigs (Shoup et al. 1997; Tuboly et al. 1995). However, the protective capability of these systemic antibodies was insufficient (Godet et al. 1991; Shoup et al. 1997; Tuboly et al. 1995). Similarly, when

baculovirus-expressed S protein with incomplete Freund's adjuvant was administered intramammary and IM to TGEV-seronegative, pregnant sows, only IgG antibodies to TGEV were detected in sows' colostrum and milk (Shoup et al. 1997). Moreover, there was no significant impact on morbidity or mortality after TGEV challenge exposure of litters from these sows (Shoup et al. 1997).

In studies using baculovirus-expressed TGEV structural proteins (S, N, and M) coadministered intraperitoneally with *E. coli* mutant thermolabile toxin (LT-R192G), immune responses associated with IgA antibodies to TGEV resulted in reduced TGEV shedding in the feces of challenged pigs (Sestak et al. 1999a). These results suggested that vaccines based on the three major TGEV proteins (S, N, and M) could stimulate both mucosal and systemic immune responses. Since the pathology of TGEV remains localized in the intestine, an effective vaccine should primarily elicit an intestinal immune response that can be targeted by oronasal immunizations with adequate doses and forms of attenuated vaccines (Saif and Jackwood 1990; Van Cott et al. 1993). TGEV vaccines might be improved further by the use of supplementary carrier systems such as immunostimulating complexes, biodegradable microspheres, or recombinant *Salmonella* expression and delivery vectors.

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10.2

Porcine Reproductive and Respiratory Syndrome Virus: Epidemiology

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SUMMARY

After the OIE List A diseases, porcine reproductive and respiratory syndrome virus (PRRSV) ranks as the most contentious and costly viral disease of swine. Although PRRSV was identified over 10 years ago, effective and reproducible strategies to prevent and/or control PRRS have not been found. Once the virus gains entry, most commercial herds become endemically infected and experience chronic losses or periodic outbreaks indefinitely. The ability of PRRSV to thwart the immune response and establish persistent infection is the primary problem. Given the inadequate immune response, controlling clinical losses by establishing and maintaining herd immunity is problematic. Vaccines that induce long-term protective immunity and simultaneously eliminate or reduce virus shedding are not yet available. And, if elimination is achieved, herds are vulnerable to reinfection with PRRSV through the introduction of carrier animals or by area spread.

INTRODUCTION

In the late 1980s, catastrophic clinical outbreaks of a previously unrecognized disease were reported across the United States (Keffaber 1989; Loula 1991). Initially reported in North Carolina, clinical signs included severe reproductive losses, extensive postweaning pneumonia, reduction of performance, and increased mortality (Hill 1990). Although the pattern of disease suggested an infectious agent, attempts to diagnose the cause of the outbreaks were unsuccessful and, in the absence of a specific etiology, it was termed *mystery swine disease*. In the late 1980s and early 1990s, mystery swine disease quickly spread throughout the major swine-producing areas of the United States (Quaife 1989).

In Europe, clinical outbreaks with signs similar to those of mystery swine disease were reported in November 1990 near Münster, Germany [Office International des Épizooties (OIE) 1992]. Again, the disease spread rapidly, and over 3000 outbreaks were documented in Germany in

May 1991. No link was found between outbreaks in the United States and those in Germany (Anonymous 1991). The disease appeared in the Netherlands in January 1991 and in Belgium in March (OIE 1992). It reached Great Britain in May 1991 (Edwards et al. 1992) and, by the end of October 1991, a total of 58 outbreaks had been confirmed. In Spain, three outbreaks were reported, and all animals were rapidly slaughtered (OIE 1992). In France, the first outbreaks appeared in Brittany in November 1991 (Baron et al. 1992; OIE 1992).

The agent of mystery swine disease was identified in 1991, when a previously unrecognized virus was reported as the cause (Terpstra et al. 1991a; Wensvoort et al. 1991). Soon afterward, the virus was isolated in the United States (Collins 1991) and Canada (Dea et al. 1992a,b). Both European and US virus isolates were shown to reproduce disease under experimental conditions (Collins et al. 1992; Terpstra et al. 1991a). European workers apparently introduced the terminology *porcine reproductive and respiratory syndrome* in early 1991 (Terpstra et al. 1991b).

ENTRY INTO DOMESTIC SWINE

Although first recognized as a new clinical entity in the mid-1980s, serological evidence of porcine reproductive and respiratory syndrome virus (PRRSV) infection was detected in "banked" serum samples originally collected in 1979 in Ontario, Canada (Carman et al. 1995). Whether this was the first and only introduction of PRRSV into domestic swine or only one of many is uncertain. The original source of the virus is not known, but it is reasonable to postulate spillover from another host species, as occurred with the Nipah and Hendra viruses. There may have been other incursions into domestic swine; introductions in which the virus was unsuccessful in reaching a swine population sufficiently large to support its continued circulation. Spontaneous elimination of PRRSV is recognized to occur in small, stable herds (Nodelijk et al. 2000), i.e., the industry standard prior to the 1980s.

Therefore, PRRSV entered the Canadian swine population well before the clinical syndrome was recognized. Similarly, in Europe, although clinical outbreaks of PRRS were reported in Germany in 1990, the serological evidence suggested that PRRSV was present in the former East Germany as early as 1988 (Ohlinger et al. 2000). Having entered the domestic swine population, the virus spread rapidly into other areas. In the United States, 1425 samples collected from Iowa swine in 1980 were seronegative, but seropositive samples were found in Iowa samples collected in 1985 and later (Zimmerman et al. 1997a) and in Minnesota samples collected in 1986 (Yoon et al. 1992). In Asia, antibodies against PRRSV were detected in serum from pigs imported into South Korea in 1985 (Shin et al. 1993) and in samples collected in 1988 in Japan (Hirose et al. 1995).

Thus, in a period of about 10 years, PRRSV entered and became endemic in a large proportion of the world's domestic swine population. The original source of the virus and the circumstances under which it came into the domestic swine population are not known. A wildlife reservoir is the most logical explanation, with feral swine the most reasonable candidate species. In Germany, however, Oslage et al. (1994) found only two seropositive animals among 482 wild boar samples collected in 1991 to 1992, and Lutz and Wurm (1996) found no positives among 768 wild boar samples collected in 1992–1993 and in 1995–1996. In the United States, there was no evidence of infection in feral swine serum samples collected between 1976 and 1993, and only two positive animals were found in 1994 (J. Zimmerman, unpublished data). Overall, the data suggest that feral swine acquire the infection from domestic swine, rather than the reverse. Since, PRRSV obviously existed prior to its entry into domestic swine, and feral swine were apparently not the source, another reservoir host must exist that has not yet been identified.

Adding to the puzzle of its origin, although PRRS appeared in Europe and North America at approximately the same time, the prototypic European and North American PRRSV isolates are genetically and antigenically quite different from each other, suggesting a long period of independent evolution.

CURRENT GEOGRAPHICAL DISTRIBUTION AND PREVALENCE

At present, PRRSV is endemic in nearly all swine-producing areas of the world. A few countries are believed to be free of PRRSV, among them Australia, Argentina, New Caledonia, Norway, Sweden, and Switzerland.

Reliable estimates of the prevalence of PRRSV infection are lacking for most parts of the world. The extensive use of modified-live PRRS vaccines in many countries has made it impossible to estimate the frequency of infection with wild-type viruses because antibodies

against vaccine viruses cannot be differentiated from antibodies against field virus. Transmission of modified-live vaccine strain viruses occurs in the field, which further compounds the problem. In the United States, the best prevalence estimates are based on a 1995 National Animal Health Monitoring System (NAHMS) study (United States Department of Agriculture 1997), in which 8038 serum samples were collected from 286 herds in 16 swine-producing states. Among herds not using modified-live PRRS vaccine, 129 (59.4%) of 217 herds were infected. Among 6376 unvaccinated animals, 41.3% were considered infected on the basis of serum antibody titers. When animals were identified by status, 23.5% of unvaccinated breeding animals and 51.7% of unvaccinated finishers were considered positive. As in the United States, reports from other parts of the world indicate that the prevalence of infection is high in endemically infected regions, generally ranging from 50% to 100% of herds tested.

SUSCEPTIBILITY TO INFECTION AND ROUTES OF SHEDDING

Swine are susceptible to PRRSV by a number of routes of exposure, including oral, intranasal, intramuscular, intraperitoneal, and vaginal. The virus is highly infectious and exposure to 10 or fewer PRRSV particles by either intranasal or intramuscular routes of exposure is sufficient to produce infection (Yoon et al. 1999). Benfield et al. (2000) estimated that a dose of 10 to 100 TCID₅₀ (50% tissue culture infectious doses) of PRRSV per milliliter of semen was required to achieve infection by artificial insemination. The infectious dose by oral exposure has not been measured.

Infection of susceptible animals results in the shedding of virus in saliva, nasal secretions, urine, semen, and mammary secretions. Virus is present at low levels, or perhaps intermittently, in the sections from many portals of exit for an extended period.

Virus has been shown to be present in saliva for ≥ 42 days post inoculation (PI), in nasal secretions for up to 21 days PI, in urine for up to 28 days PI, in semen for up to 92 days PI, and in the oropharynx for up to 157 days PI (Christopher-Hennings et al. 1995; Wills et al. 1997a,b). Virus was also detected in the colostrum and milk of susceptible dams exposed during gestation (Wagstrom et al. 2002). Conflicting evidence exists on fecal shedding, but the virus is highly labile and, if PRRSV were present in feces, it would be rapidly inactivated.

Boars shed virus in semen for variable periods of time (Christopher-Hennings et al. 1996; Swenson et al. 1994). Swenson et al. (1994) found infectious virus in the semen of experimentally infected boars for as long as 43 days after exposure. Using a reverse transcriptase-nested polymerase chain reaction (RT-nPCR), Christopher-Hennings et al. (1995) reported detection of viral RNA

in the semen of experimentally infected boars through day 92 after exposure and isolation of PRRSV from the bulbourethral gland of a boar euthanized 101 days after inoculation. As discussed by Christopher-Hennings et al. (1996), intermittent shedding of virus in semen may occur, and neither viremia nor serum antibody levels are reliable indicators of semen shedding. For that reason, they recommended that two or three semen samples be collected at weekly intervals and tested for virus by PCR. Transmission of PRRSV to females by artificial insemination with undiluted semen from experimentally infected boars has been demonstrated (Yaeger et al. 1993), as has transmission of PRRSV to females by using extended semen from experimentally infected boars (Gradil et al. 1996).

Shedding of virus by infected animals results in environmental contamination and creates the potential for transmission via fomites. However, PRRSV is labile and quickly inactivated by desiccation. For that reason, it does not generally persist in the environment or on materials commonly found on farms. Pirtle and Beran (1996) reported that the virus was rapidly inactivated on plastic, stainless steel, rubber, alfalfa, wood shavings, straw, corn, swine starter feed, and denim cloth. In addition, the virus has a narrow range of tolerance in fluids and is quickly inactivated in solutions such as urine or fecal slurry. However, PRRSV suspended in "clean" aqueous solutions at neutral pH under cool-to-frozen conditions can remain infectious for an extended period. For example, the half-life (inactivation of one-half of the virus population) in a pH 7.5 solution held at 39°F (4°C) was estimated to be 5.8 days (Bloemraad et al. 1994). On farms, infectious virus may persist in clean, standing water, i.e., in drinking cups, troughs, puddles, or lagoons. However, PRRSV is readily inactivated by standard disinfectants (Shirai et al. 2000), and thorough cleaning and disinfection procedures are adequate for inactivation of PRRSV in facilities and on equipment.

CARRIER ANIMALS

Clinically normal, but persistently infected, animals are a critical feature in the epidemiology of PRRSV and the most significant obstacle to the prevention and control of the disease. Zimmerman et al. (1992) first reported transmission between sows infected 99 days earlier and commingled susceptible animals. Albina et al. (1994) subsequently demonstrated transmission of PRRSV by pigs infected 15 weeks earlier. Wills et al. (1997b) isolated virus from oropharyngeal samples for up to 157 days after experimental inoculation. Benfield et al. (1997) reported detection of viral RNA for up to 210 days after farrowing in pigs exposed to virus in utero. Allende et al. (2000) recovered virus from one of four animals at 150 days PI, and Horter et al. (2001) detected infectious PRRSV in 51 (84%) of 59 animals necropsied between

days 63 and 105 PI, including 10 (91%) of 11 of animals at day 105 PI.

The detection of carrier animals is difficult using routine diagnostic procedures. No significant difference was found in the antibody response of carrier versus noncarrier animals on the basis of a commercial enzyme-linked immunosorbent assay (Horter et al. 2001). For identification of carriers, Horter et al. (2001) reported that an RT-nPCR on oropharyngeal scrapings was the most effective combination of assay and sample. The site of persistence appears to be lymphoid tissue, such as the tonsil (Horter et al. 2001; Wills et al. 1997b).

Carrier animals pose a problem for PRRS control because of the risk for transmission to susceptible herd mates. The fact that PRRSV routinely becomes endemic in herds suggests that, given time and the opportunity to interact with susceptible herd mates, carrier animals are routinely able to transmit the infection.

TRANSMISSION

The rapid spread of PRRSV throughout the world has demonstrated that transmission is one of the most remarkable characteristics of the virus. In the absence of vaccines or treatments capable of stopping virus shedding or eliminating infection in chronic carrier animals, strategies for the prevention, control, and eradication of PRRSV are forced to rely on a well-grounded understanding of the process of transmission.

PRRSV has been characterized as highly infectious, but not highly transmissible. It is infectious, i.e., the minimum infectious dose is low (Yoon et al. 1999), but contact with infected animals or virus-contaminated fomites often fails to result in transmission. In fact, a frequent observation in the field is that it is difficult to transmit the virus intentionally to virus-negative animals by housing them with infected animals. Then, too, it is common to find virus-negative animals within infected groups.

Transmission usually occurs by close contact between infected and susceptible animals. Airborne virus was once thought to be the primary route of transmission, but research data have not substantiated the role of aerosol transmission. Indeed, it has been shown that transmission declines markedly when pigs are separated by a space of even 1 meter (Wills et al. 1997c). The specific mechanisms of transmission between pigs are uncertain, but transmission probably occurs either through nose-to-nose contact or through fighting and contamination of wounds with virus. The presence of virus in the oropharyngeal region for several months after infection and the low minimum infectious dose make the movement of pigs within and between farms an ideal mode of PRRSV transmission.

Fomites do not tend to be important in the transmission of PRRSV because, as was discussed, the virus is rapidly inactivated under most environmental conditions.

However, compatible with the prolonged viremia that occurs during infection, transmission via contaminated instruments and needles is considered important (Otake et al. 2001).

NONPORCINE SPECIES

A number of species have been tested for susceptibility to PRRSV by experimental inoculation. Mice, rats (Hooper et al. 1994), and guinea pigs (J. J. Zimmerman, unpublished data) were not susceptible. Wills et al. (2000) reported that dogs, cats, skunks, raccoons, opossums, house sparrows, and starlings were also not susceptible to the virus. However, mallard ducks (*Anas platyrhynchos*) exposed to PRRSV in drinking water shed virus in feces for several weeks, and virus was recovered from fecal samples collected from 8 of 20 ducks 39 days after exposure (Zimmerman et al. 1997b). Mallard-to-mallard transmission was demonstrated by infecting ducks with feces from ducks shedding PRRSV, and swine were shown to be susceptible to mallard-derived virus. However, it seems highly unlikely that mallards are significant participants in the transmission of PRRSV. At best, mallards could serve as reservoirs of PRRSV, as they are known to do in the case of influenza viruses.

Recently, recovery of infectious virus from mosquitoes that had fed on experimentally infected swine was reported (Otake et al. 2001). This could be a very significant discovery, but it is not unusual to detect nonarthropod-borne viruses in mosquitoes. For example, encephalomyocarditis virus has been isolated from 13 species of mosquitoes, as well as ticks, fleas, and house flies (Zimmerman 1994), although these arthropods do not play a role in the transmission cycle. As Otake et al. (2001) point out, the data need to be interpreted cautiously until the work is completed.

TRANSMISSION WITHIN HERDS

Once infected, PRRSV tends to circulate within a herd indefinitely. Investigators have reported isolation of virus from nursery pigs up to 2.5 years after the initial PRRS outbreak (Joo and Dee 1993; Stevenson et al. 1993). Spontaneous elimination of PRRSV from commercial herds has been reported (Freese et al. 1993), but it appears to be an unusual occurrence and the circumstances under which it happens are not well defined. As described by Nodelijk et al. (2000), endemicity is, in part, a function of herd size. The key components that make endemicity possible appear to be persistent PRRSV infection in clinically normal carrier animals, the continual introduction of susceptible animals either through birth or purchase, and mixing of animals followed by fighting behavior associated with establishing social rank. Most typically, the virus is perpetuated by a cycle of transmis-

sion from dams to pigs either in utero or post partum, or by commingling susceptible animals with infected animals in later stages of production. In neonatal pigs, maternal antibodies may provide some immunological resistance to infection. However, the degree of protection is not very well characterized and appears to be insufficient to preclude infection.

Under conditions in which susceptible and infectious pigs are mixed, e.g., at weaning, a large proportion of the population may quickly become infected. Dee and Joo (1994) reported that 80% to 100% of pigs in three swine herds were infected by 8 to 9 weeks of age, and Maes (1997) found 96% of market hogs sampled from 50 herds to be positive. However, the pattern of infection often deviates from this description of rapid, uniform spread. Within infected herds, marked differences in infection rates between groups, pens, or rooms of animals are often observed in the field. Houben et al. (1995) found transmission to vary even within litters, with some littermates seroconverting as early as 6 to 8 weeks and other individuals as late as 10 to 12 weeks of age. In some cases, litters of pigs reached 12 weeks of age, the end of the monitoring period, still free of PRRSV infection. Thus, animals in endemically infected herds frequently escape infection for an extended period, as when Le Potier et al. (1997) reported seroconversion in young sows on farms using in-herd gilt replacements. Dee et al. (1996) concluded that the presence of susceptible animals in breeding herds provided a mechanism to maintain persistent viral transmission in chronically infected farms.

TRANSMISSION BETWEEN HERDS

The primary means of herd-to-herd transmission is the introduction of infected animals. Frequently, however, transmission is recognized too long after the fact to make it possible to determine the source of the virus accurately. Following outbreaks of PRRS in late 1990, Dee (1992) reported that, of 10 farms surveyed, eight had purchased breeding stock from the same source. Based on the PRRSV control program in France, Le Potier et al. (1997) reported that 56% of herds acquired the infection through infected pigs, 20% through infected semen, 21% through fomites, and 3% through unidentified sources.

Area spread is the transmission of virus between herds in the absence of pig movement or other known source of virus introduction. Area spread is important because many PRRSV-negative herds have become infected by area spread; relatively well-isolated herds, in some cases. As procedures for virus detection and monitoring have improved, it has been recognized that area spread generally occurs over shorter distances than once believed. This may be attributed to the fact that, until the existence of subclinical infection was recognized, the distance between clinical outbreaks was used to measure virus travel. Le Potier et al. (1997) found that 45% of

herds suspected to have become infected through area spread were located within 500 meters (0.3 miles) of the postulated source herd and only 2% were 1 km from the initial outbreak.

Area spread has been most commonly attributed to aerosols of infectious PRRSV traveling downwind from infected herds. However, transmission of PRRSV in aerosols under experimental conditions has been extremely difficult to achieve. Torremorell et al. (1997) attempted to transmit PRRSV from a group of acutely infected pigs to a group of susceptible pigs over a distance of 1 meter. They reported successful transmission in only one of two trials. Wills et al. (1997a) and Otake et al. (2001) described similar results across comparable distances, and Robertson (1992) reported no success in isolating virus from the air space in which acutely infected pigs were housed.

At present, the mechanism(s) of area spread have not been defined. This is an area that is important to pursue, because it is difficult to justify the cost of PRRSV elimination to producers when it is not possible to predict the likelihood of area spread or implement specific measures to protect against it. At present, alternate hosts or arthropods (Otake et al. 2001) appear to be the best areas of inquiry.

PRRSV IN PORK (MUSCLE TISSUE)

Questions regarding the ability of infectious PRRSV to persist in pork or pork products quickly surfaced among international trading partners in the early 1990s. Understandably, PRRSV-free countries had no wish to infect their swine populations through the importation of virus-contaminated pork products.

Several studies were undertaken to address this issue. Bloemraad et al. (1994) reported that virus was present, although at low titer, in muscle tissue collected from viremic pigs and that the quantity of virus was only slightly affected by storage for up to 48 hours at 4°C. Magar et al. (1995) collected meat samples from both experimentally infected pigs and from the carcasses of pigs from infected herds at an abattoir. In the experimental study, 6-month-old pigs were inoculated with PRRSV, and muscle tissue samples were collected at 7 and 14 days after inoculation. Virus was isolated from samples collected 7 days after inoculation, and viral antigens were detected by immunogold silver staining in scattered cells in muscle tissues. Neither virus nor viral antigens were detected in muscle tissue samples collected 14 days after inoculation. In the abattoir study, muscle tissue samples were collected from 44 carcasses from seropositive herds. No virus was isolated and no viral antigens were detected by immunogold silver staining. This same research group subsequently expanded upon this study by adding an RT-PCR assay as a detection method and increasing the sample size (Larochelle and Magar 1997). In the

follow-up study, 73 carcasses from four abattoirs were randomly selected and tested by virus isolation and RT-PCR. All samples were negative both by virus isolation and by RT-PCR. For that reason, the investigators concluded that meat does not retain detectable amounts of PRRSV and that transmission of virus through pork is unlikely.

Because of the ramifications for trade, significant interest continues in this area and additional research will undoubtedly be done to further assess the risks.

CONCLUSIONS

PRRSV emerged from an unknown source, entered the domestic swine population, and spread rapidly thereafter, primarily by animal movement. Viral genetic and antigenic differences suggest at least two independent introductions: one in Europe and one in North America. The time frame of these introductions cannot be firmly established for lack of historical samples, but PRRSV antibodies were detected in Canadian serum samples collected in 1979. Since its introduction, PRRSV has established its credentials as a highly successful pathogen. Epidemiologically, its most important characteristic is its ability to evade the immune response and establish persistent infection.

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10.3

Porcine Reproductive and Respiratory Syndrome: Virology

Kyoung-Jin Yoon

SUMMARY

Porcine reproductive and respiratory syndrome (PRRS) virus is an enveloped virus with a positive-sense, single-stranded RNA genome. The virus was unrecognized until the late 1980s, when devastating outbreaks of reproductive failure and respiratory disease (i.e., PRRS) emerged in North America and Europe. The isolation of a previously unrecognized virus was followed by confirmation of its etiologic role. It was designated the PRRS virus (PRRSV) and subsequently classified into the family Arteriviridae. Research during the previous decade revealed unique features associated with PRRSV or its infection. The main target cells of PRRSV are macrophage-lineage cells. The virus is capable of establishing the persistent infection resulting in chronic carriers. PRRSV is shed in semen from infected boars. Field isolates of PRRSV have been shown to be highly diverse both genetically and antigenically. All these characteristics have become significant impediments to prevention and control of PRRS and have become impediments in the trade of pigs and pork products.

INTRODUCTION

Since it emerged in the late 1980s, porcine reproductive and respiratory syndrome (PRRS) has become one of the most economically significant diseases of the swine industry, particularly in countries free of foot-and-mouth disease or classical swine fever viruses. The disease is characterized by clinical manifestations of reproductive failure in breeding animals and respiratory distress in pigs of all ages. Suboptimal productivity is the main concern in growing or finishing pigs affected by the disease. In the last decade, a significant amount of resource and effort has been invested in studying the virus and the disease, and in developing strategies for prevention and control. This chapter reviews what we know about the anatomy and characteristics of PRRSV.

TAXONOMY

Porcine reproductive and respiratory syndrome virus (PRRSV) is a small, enveloped RNA virus classified as a

member of the genus *Arterivirus* of the family Arteriviridae in the order Nidovirales. The order Nidovirales was proposed and approved in 1996 by the International Committee on Taxonomy of Viruses, and is comprised of the families Arteriviridae and Coronaviridae, which share a high degree of similarity in genomic organization and gene expression strategy. Other viruses in the genus *Arterivirus* include lactate dehydrogenase-elevating virus (LDV) of mice, equine arteritis virus (EAV), and simian hemorrhagic fever virus (SHFV). All of these viruses are enveloped and have an average diameter of 40 to 60 nm. Their genome is 13 to 15 kb in size, and gene expression is by the production of six or seven subgenomic nested mRNAs. These viruses also possess common biological properties, including primary replication in host macrophages and establishment of asymptomatic persistent infection in their hosts. A reverse transcription-polymerase chain reaction (RT-PCR) using degenerated primers for common replicase sequences [open reading frame 1b (ORF1b)] can detect all arteriviruses. Despite the many similarities among them, no serological cross-reaction has been demonstrated between PRRSV and other arteriviruses.

PHYSICOCHEMICAL PROPERTIES

PRRSV, which is spherical in shape and ranges 48 to 83 nm in diameter, contains an electron-dense icosahedral nucleocapsid that ranges 25 to 30 nm in diameter. The buoyant density of the virus is from 1.13 to 1.19 g/ml in cesium chloride gradients and 1.18 to 1.23 g/ml in sucrose gradients.

No chemical inactivating agent except chloroform has been tested in vitro against PRRSV. Treatment of virus particles with chloroform reduced virus infectivity by more than 99.99%, thereby demonstrating the presence of a viral envelope. It follows that any lipid solvents and detergents should have an adverse effect on virus infectivity.

The virus is stable for several months at -70°C and for at least 1 month at 4°C . At higher temperatures, the virus is rapidly inactivated. Complete inactivation occurs within 48 hours at 37°C and by 45 minutes at 56°C . In culture medium at pH 7.5, the estimated half-life (i.e.,

inactivation of half of the virus population) of Lelystad virus was 140, 20, 3, and 0.1 hours at 4°, 21°, 37°, and 56°C, respectively.

With respect to recovery of PRRSV from clinical specimens, virus viability can be maintained in serum at sub-optimal temperature (25°C), but not in tissues. In meat refrigerated at 4°C, virus, if present, was recovered for up to 48 hours. In the environment, infectious virus was rapidly inactivated by drying, but was recovered for up to 9 days from well water and 11 days from city water. Cumulatively, these data suggest that a humid environment is crucial for maintaining virus viability.

The stability of infectious PRRSV is also influenced by pH. Viral infectivity is reduced over 90% at a pH of less than 5 or greater than 7. Under constant temperature, increasing or decreasing the pH of the medium rapidly decreased the half-life of Lelystad virus. At 37°C and a pH of 6.0, the half-life of Lelystad virus was approximately 6.5 hours, but half-life declined to 0.65 hours at pH 5.0 and 1.28 hours at pH 8.5. At 4°C and a pH of 7.5, the half-life of Lelystad virus was estimated to be 140 hours as compared with 50 hours at pH 6.25.

Like other arteriviruses, PRRSV does not hemagglutinate red blood cells from mammalian or avian species, including human type O, sheep, goat, pig, cattle, horse, rat, rabbit, guinea pig, duck, goose, and chicken. However, Japanese investigators reported specific hemagglutination of mouse erythrocytes by PRRSV in the form of cell-free virus and lysate of infected cells. Hemagglutination was greatly enhanced by treatment of the virus with a nonionic detergent, such as Tween 80, followed by a lipid solvent, such as ether. Interestingly, hemagglutination inhibition titers of pig sera showed a positive correlation with neutralizing antibody titers. The same investigators who identified a hemagglutinin with 1.17 g/cm³ density that was associated with the virus speculated that the nucleocapsid protein may be responsible for hemagglutination.

GENOMIC ORGANIZATION AND GENE EXPRESSION

The genome of the virus is polyadenylated, single-stranded, nonsegmented, positive-sense RNA. Genomic RNA itself is infectious (i.e., producing infectious virus from a cell line transfected only with RNA). The genome is approximately 15 kb in size and consists of eight ORFs. ORF1a and 1b are located at the 5' end of genome and preceded by a 211-nucleotide-long noncoding leader sequence. This ORF comprises approximately 80% of the viral genome. ORFs 2 to 7 are located at the 3' end of genome, and the stop codon of ORF7 is followed by a 3' noncoding 114-nucleotide-long sequence and then a polyadenylated tail of approximately 20 nucleotides. Each of the ORFs (1 through 7) partially overlaps its neighboring ORF. Nucleotide analysis of the Lelystad

virus, the European PRRSV prototype, revealed a slippery sequence (UUUAAA) at the overlap region of ORF1a and ORF1b and a sequence that possibly forms an RNA pseudoknot. The slippery sequence is located immediately upstream of the UAG stop codon of ORF1a and is followed downstream by a potential stem-loop-forming sequence. Amino acid sequence analysis of PRRSV ORF1a showed the presence of (1) hydrophobic regions, (2) cysteine-rich domains, and (3) a putative serine protease consensus sequence. The predicted amino acid sequence of ORF1b is more conserved than ORF1a among PRRSV, EAV, and LDV. The ORF1b of these viruses shares four characteristic domains: (1) the RNA polymerase motif, (2) cysteine- and histidine-rich domains, (3) a helicase motif and nucleoside triphosphate-binding site, and (4) a conserved domain of yet-unknown function.

PRRSV genes are expressed by the production of a 3' coterminal nested set of six or seven subgenomic mRNAs. Such gene expression is similar to that of coronaviruses and toroviruses, rather than that of togaviruses. Each subgenomic RNA contains the same leader sequence (longer than 200 nucleotides) at its 5' end, which is identical to the 5' noncoding region of the genomic RNA. The size of each transcript in decreasing order is 15, 3.3, 2.7, 2.2, 1.7, 1.1, and 0.7 kb for mRNAs 1 to 7, respectively. An extra mRNA from an ORF located between ORFs 3 and 4, which has been designated mRNA3.1, was also demonstrated in isolates expressing seven subgenomic mRNAs. Although the larger viral transcripts (i.e., mRNAs) are polycistronic, i.e., contain more than one ORF, only the first ORF at the 5' end of each transcript is believed to be translated into a protein. It is not known how soon PRRSV-encoded mRNA is produced in infected cells, but viral polypeptide in infected cells can be detected 6 (MARC-145 and porcine alveolar macrophages (PAMs) to 20 hours (CL2621) after inoculation.

The product of each ORF has been identified. ORF1 encodes for RNA replicase, the only nonstructural protein of PRRSV that has been identified. ORFs 2 to 7 are thought to encode structural proteins. The numbers of amino acids encoded by ORFs 2 to 7 of PRRSV isolate VR-2332 are 256, 254, 178, 200, 174, and 123, respectively. In comparison, Lelystad virus ORFs 2 to 7 encode 249, 265, 183, 201, 173, and 128 amino acids. To date, the reported molecular masses of proteins expressed from each of ORFs 2 to 7 are 29 to 30, 45 to 50, 31 to 35, 25, 19, and 15 kD, respectively. Comparison of the amino acid sequences encoded by each PRRSV ORF to that of other members of the *Arterivirus* group indicates that PRRSV is more similar to LDV than to EAV.

VIRAL PROTEINS

Three structural proteins with molecular masses of approximately 15, 19, and 25 kD have been demonstrated

by numerous independent investigators in purified virions and lysates of infected cells. Endoglycosidase treatment and [^3H]glucosamine-labeling study revealed that the 25- to 30-kD protein is an *N*-glycosylated protein, whereas the 15- and 19-kD proteins are not glycoproteins. An additional viral protein with a molecular mass of approximately 22 to 23 kD that was identified in lysates of cells infected with PRRSV was less abundant than the 15-, 19-, and 25-kD proteins, but appeared to be immunologically recognized by infected pigs, as shown by the reactivity of sera from experimentally infected pigs to the protein by immunoprecipitation and/or immunoblotting. An additional glycosylated protein with a molecular mass of 42 kD has also been found in virus-infected MA-104 cells. It was hypothesized that this protein possessed a majority of the conformation-dependent epitopes, since seroreactivity of anti-PRRSV swine sera to the protein was shown by immunoprecipitation, but not by Western immunoblotting performed under reducing conditions. However, genomic sequence analysis suggested the existence of as many as six structural proteins. Compatible with this analysis, six proteins with apparent molecular masses of 15, 18, 25, 31 to 35, 45 to 50, and 29 to 30 kD have been identified in association with purified Lelystad virus. Using antipeptide sera specific for each ORF, investigators demonstrated that ORFs 2 to 7 encode proteins with molecular masses of 29 to 30, 45 to 50, 31 to 35, 25, 18, and 15 kD, respectively. Each of the six proteins was shown to react with sera from pigs experimentally infected with Lelystad virus, suggesting that all six are structural proteins. Discrepancies between North American isolates and Lelystad virus with respect to protein composition remain to be further investigated.

The functions of PRRSV proteins in structure and replication have not been completely determined. An electron-microscopic study using colloidal gold-labeled monoclonal antibodies (Mabs) and analysis of radioactively labeled purified virus indicated that the 15-, 19-, and 25-kD proteins are nucleocapsid (N), matrix (M) protein, and an envelope (E) protein, respectively. The other proteins (i.e., 29 to 30, 45 to 50, and 31 to 35 kD) are presumed to be glycosylated proteins and components of the viral envelope. In particular, the 31- to 35-kD protein has been demonstrated by immunoelectron microscopy to be a surface molecule of PRRSV. It was proposed that the membrane-associated proteins be designated as GP₂, GP₃, GP₄ and GP₅, where GP stands for *glycosylated protein* and the numbers indicate the corresponding ORFs.

An N protein of either 123 or 128 amino acids is predicted from ORF7 of North American or European isolates, respectively. Native N protein is 15 kD in molecular weight. A peptide of approximately 13.5 kD also has been detected by SDS-PAGE of purified virions and is thought to be a subproduct of the N protein. Despite

its relatively small size, at least five and possibly as many as seven antigenic determinants have been identified on the N protein. Monoclonal antibodies to the N protein identified conserved epitopes among all isolates (North American and European), as well as divergent epitopes present either in North American or in European isolates. The C terminus of the N protein is considered critical in the formation of antigenic determinants. Western immunoblot analysis of sera sequentially collected from experimentally infected pigs has suggested that the N protein is highly immunogenic. In general, the immunological response of infected animals is considered to be initially directed to a 15-kD protein and subsequently to other viral proteins in a time-dependent fashion. In contrast, one report showed that antibody to the envelope protein was detected in sera from pigs experimentally infected with, or naturally exposed to, PRRSV earlier than antibody against any other proteins.

The M protein has a calculated molecular mass of 19 kD and is expressed from ORF6. In vitro transcription and translation studies of ORF6 have also identified a nonglycosylated protein of 18 to 19 kD. The M protein contains 174 or 173 amino acids for the North American or European isolates, respectively. Hydrophilicity plots of the deduced amino acid sequence of the M protein of Lelystad virus indicated that the protein contained three membrane-spanning domains. Although the M protein is probably located in the viral envelope and contains a relatively large external domain, it is highly conserved among North American isolates and is also the most conserved structural protein between North American and European isolates. Monoclonal antibodies to the M protein have identified both common and unique epitopes among North American and European isolates. Western immunoblot analysis of convalescent sera from infected pigs by using cell lysate demonstrated that the M protein was highly antigenic and elicited a detectable antibody response as early as 10 days after infection.

Amino acid sequence analysis of ORF5 revealed that the E glycoprotein contained 200 amino acids for North American and 201 amino acids for European isolates, with two to four potential *N*-glycosylation sites, a signal peptide of 31 amino acids, and a molecular mass of 22.4 kD. In vitro transcription and translation of PRRSV ORF5 in the absence of microsomal membrane yielded a protein of 20 kD, whereas 25- and 17-kD proteins were produced from in vitro transcription and translation in the presence of microsomal membranes. The 25-kD protein is thought to be a glycosylated form and the 17-kD protein a nonglycosylated form from which the N-terminal peptide sequence is removed. The E and M proteins of PRRSV are linked by disulfide bonds. At present, a significant role for these disulfide bonds in interacting with cellular receptors has not been demonstrated, as is the case with EAV and LDV.

Molecular characterization of Lelystad virus suggested that ORFs 4, 3, and 2 encoded for potential virus envelope glycoproteins, but their identification in infected cell lysates or purified virions has been difficult. For this reason, it has been suggested that these proteins are either present at low levels in viral particles or are poorly immunogenic. Recently, it was demonstrated using polyclonal porcine anti-Lelystad virus antibodies, antipeptide sera, or Mabs that the products of these genes are part of the virus structure. ORFs 2, 3, and 4 encode for a 29- to 30-kD protein, a 45- to 50-kD protein, and a 31- to 35-kD N-glycosylated protein, respectively.

The role of each viral protein in cell-mediated immunity is unknown. With respect to humoral immunity, the N protein is not considered to be associated with the induction of neutralizing antibody and protective immunity. Experimentally infected pigs were shown to develop antibody specific for the N protein much earlier than detectable neutralizing antibody in sera. Furthermore, pigs vaccinated with a recombinant N protein that was proven to retain the intact antigenic structure of native N protein were found not to be protected against a challenge with Lelystad virus at 45 days after immunization. No Mabs against the N protein with neutralizing activity has been produced.

The membrane-associated proteins appear to be associated with virus neutralization. Several investigators demonstrated that antibody specific for the E protein was detected by radioimmunoprecipitation or immunoblotting close to the time at which virus-neutralizing activity appeared in serum. Furthermore, production of neutralizing antibodies to PRRSV was demonstrated in pigs inoculated with the protein product of ORF5, i.e., E protein. The presence of a neutralizing epitope on the E protein has been demonstrated with Mabs specific for the E protein. Preincubation of PRRSV with these Mabs blocked virus infection to a permissive cell line, MARC-145. Similarly, the 29-kD glycoprotein of EAV, the counterpart of the PRRSV E protein, is known to contain neutralizing epitopes and postulated to play a major role in virus-cell interaction.

In addition to the E protein, it was demonstrated that Mabs directed to GP₄ protein (i.e., the protein product of ORF4) neutralized infection of Lelystad to a permissive cell line (CL2621), indicating the presence of neutralizing epitope(s) in this protein. However, these Mabs could not block infection of CL2621 cells by a German isolate or the US isolate VR-2332, suggesting that the corresponding antigenic domain (amino acids 40 to 79) is a variable region of the protein.

Only limited information is available regarding the potential role of viral proteins in pathogenesis. It was reported that cells transfected with only ORF7 became rounded and detached from the surface of the cell culture vessel in a manner similar to cytopathic effect, suggesting that the ORF7 product (N protein) plays a role in

the cytopathogenesis of PRRSV. Expression of PRRSV ORF5 cloned into a vaccinia virus vector in mammalian cells induced cell death (i.e., apoptosis) *in vitro*, suggesting that the E protein might play an important role in the disease process. Studies on LDV showed that different degrees of glycosylation of the VP3 glycoprotein affected the neuropathogenicity of the virus. The presence of an extra glycosylation site hindered the attachment of LDV to neuronal cell receptors and resulted in decreased pathogenicity. It is logical to speculate that a similar mechanism could affect PRRSV pathogenicity, but it remains to be proven. Availability of full-length infectious transcripts will be useful for characterizing the role and function of viral proteins in structure, replication, and pathogenesis.

VIRAL REPLICATION

Monocyte/macrophage lineage cells, such as PAMs, peripheral blood monocytes, and pulmonary intravascular macrophages, are the only cells proven to support replication of PRRSV both *in vitro* and *in vivo*. The virus replicates preferentially in PAMs. Virus-specific cytopathic effects can be detected by light microscopy in PAM cultures between 24 and 72 hours after inoculation. The virus yield from alveolar macrophages ranges from 10^5 to 10^6 50% tissue culture infection dose (TCID₅₀/ml). Swine peripheral blood monocytes have been demonstrated to support PRRSV replication *in vitro*, but progeny virus yield in monocyte culture was found to be lower (10^4 TCID₅₀/ml) compared with virus yield obtained with PAM culture (10^5 TCID₅₀/ml). More recently, splenic macrophages, brain microglial cells, and pulmonary intravascular macrophages of swine were reported to support PRRSV replication *in vitro*. No other primary cells derived from swine have been found to support the replication of PRRSV *in vitro*. Swine testicle cells, spleen cells, lung cells, heart cells, endoepithelial cells, synovial cells, and fetal pig kidney cells were evaluated and found not to be permissive to PRRSV.

Replication of PRRSV has been demonstrated in established cell lines, such as the African green monkey kidney cell line MA-104 and its highly permissive clone MARC-145, as well as proprietary cell lines ATCC CL2621 and CRL11171. A variety of cell lines of human or animal origin has been evaluated for their permissiveness to PRRSV, but none was found to support virus replication. A comparative study found that PAMs were more susceptible to PRRSV than was the CL2621 cell in terms of the isolation rate of the virus from clinical specimens. Some PRRSV isolates only replicate in CL2621 or alveolar macrophages, but not both. However, the routine use of PAMs in the diagnostic setting may be limited by practical considerations. Alternatively, a more susceptible subpopulation of MA-104 cells (MARC-145) has been cloned. The virus replication cycle in MARC-145 cells was

reported to be 48 to 72 hours when the cells were exposed to virus at a multiplicity of infection of 0.01, resulting in maximum progeny virus titers of $10^{8.5}$ TCID₅₀/0.1 ml.

PRRSV is internalized into target cells through receptor-mediated endocytosis. The 210-kD putative receptor for PRRSV on porcine alveolar macrophages has been identified. It remains, however, to be determined why Mabs to the putative receptor protein did not react with a known permissive cell line. In the process of internalization, low pH in the intracellular compartment (i.e., endosome) was demonstrated to be crucial for virus entry. After internalization, viral replication occurs only in the cytoplasm of infected cells.

During replication, the virus produces a 3' coterminal nested set of seven subgenomic mRNAs. Each subgenomic mRNA is known to encode for individual viral proteins, i.e., N, M, E, GP4, GP3, GP2, and RNA replicase in order of the increasing size of transcripts. RNA polymerase is the only nonstructural viral protein identified to date that participates in the replication of the viral genome. It appears that viral replication does not require cellular DNA synthesis. Nascent viral antigen is located exclusively in the cytoplasm of infected cells. Viral antigens can be detected as soon as 6 hours after infection. Using gene-specific antisera to the proteins encoded by ORFs 5, 6 and 7 with indirect fluorescent antibody staining, the intracellular location of these proteins was demonstrated during virus infection of cells. The E and M proteins accumulated in the perinuclear area, whereas the N protein was found throughout the cell cytoplasm. The M and N proteins were found to be localized to the Golgi apparatus and in the perinuclear region and the nucleolus of infected MARC-145 cells. The nucleocapsid obtains an envelope by budding through the membrane of the smooth endoplasmic reticulum. Enveloped virions principally accumulate in the lumen of the endoplasmic reticulum, thereby causing its enlargement. In addition to enlargement of the endoplasmic reticulum, severe disruption of mitochondria and formation of double-layered vesicles have also been observed in infected cells as early as 4 hours after infection.

An ultrastructural study of porcine alveolar macrophages inoculated with PRRSV revealed that progeny virus was first observed in the cells at 9 to 12 hours, which suggested that the replication cycle of the virus in the cells was 9 to 12 hours. In a comparative study on the morphogenesis of PRRSVs, Lelystad virus replicated in PAMs faster than PRRSV isolate VR-2332, but VR-2332 replicated faster than Lelystad virus in cell line CL2621, although no difference in morphogenesis was observed. Progeny viruses are released from the cell initially by exocytosis and eventually by cell lysis. Most, if not all, PRRSV isolates produce cytolytic infections, but some investigators have reported the existence of noncytopathic PRRSV isolates.

The virus growth curve, as determined by detecting infectious virus in the supernatant of infected cells, may vary among isolates. In general, virus infectivity peaks 24 to 48 hours after inoculation and may be maintained for up to 60 to 70 hours after inoculation. The cytopathic effect typical of PRRSV in a continuous cell line is rounded clumps of cells that become pyknotic and detached from the monolayer 2 to 4 days after inoculation. The entire monolayer is eventually destroyed by 6 days after inoculation. By overlaying an appropriate solidifying reagent, i.e., agar or carboxymethylcellulose, so as to limit cell-to-cell spread of virus, "white" plaques are produced by virus infection. An isolate with smaller plaques was reported to be less pathogenic to pregnant sows than an isolate with large plaques or uncloned wild-type virus.

Cytopathological events similar to those observed in infected continuous cell lines occur in porcine alveolar macrophages infected with PRRSV, except that porcine alveolar macrophages do not form a monolayer. One study showed that cytopathic effects became evident in approximately 40% of PAMs at 40 hours after inoculation, but this may vary among laboratories. It is speculated that the permissiveness of PAMs to PRRSV infection may decrease in older animals.

It is not yet clear how PRRSV enters the pig and where the initial virus replication occurs. A model of pathogenesis has been proposed in which PRRSV infection is viewed as a multisystemic disease characterized by initial viremia with subsequent dissemination and replication of virus in multiple organs. In the proposed model, virus enters the animal through the nasal mucosae and/or the epithelia of the upper respiratory tract. Primary replication is postulated to occur in nasal mucosa, macrophages in the respiratory system, and/or lymphoid tissues. After primary replication, the virus spreads via blood circulation (viremia) to secondary replication sites in the lung, lymph nodes, heart, thymus, blood vessels, spleen, and elsewhere. Recent immunohistochemical examination of tissues from pigs experimentally or naturally infected with PRRSV demonstrated the presence of viral antigen in lungs, heart, lymph nodes, tonsils, nasal turbinate, thymus, spleen, intestine, kidneys, liver, adrenal glands, brain, and testes. At the cellular level, viral antigens were detected in cells of the macrophage lineage located within these tissues. In the testes, viral antigens and nucleic acid have been found in epithelial germ cells, such as spermatids and spermatocytes, as well as macrophages. Histopathologic changes due to tissue distribution and replication of virus are discussed elsewhere.

GENETIC AND ANTIGENIC VARIATION

Marked antigenic differences have been demonstrated among PRRSV isolates. Broad antigenic variation is a major concern in the development of effective diagnostic

tools, vaccines, and vaccination strategies against PRRS. The existence of antigenic variation among PRRSV isolates was initially demonstrated between European and North American PRRSV by the immunoperoxidase monolayer assay (IPMA). The investigators, who evaluated the reactivity of polyclonal porcine antibodies raised against Lelystad virus and isolate ATCC VR-2332 with PRRSV isolates from countries around the world, could distinguish between European and North American isolates based on differences in IPMA antibody titers. Significantly higher antibody titers were obtained when anti-Lelystad virus antibody was reacted with European isolates and anti-VR-2332 antibody with North American isolates, as opposed to anti-Lelystad virus antibody against North American isolates or anti-VR-2332 virus antibody with European isolates.

PRRSV field isolates have also been shown to vary in their susceptibility to neutralizing antibody. The antigenic relationship of 17 North American PRRSV isolates was compared by a one-way serum-virus neutralization (SVN) test using polyclonal porcine antibodies raised against PRRSV isolate ISU-P. Heterologous isolates' susceptibility to neutralization ranged from 13.2% to 81.1% relative to the SVN antibody titer with homologous virus, indicating that PRRSV isolates varied in their susceptibility to neutralization. It was also demonstrated that the susceptibility of individual isolates to neutralization by antibody was inversely correlated to virus yield in the presence of the same antibody; that is, PRRSV isolates with a low SVN index (i.e., resistant to neutralization) demonstrated a higher antibody-dependent enhancement of infection (i.e., higher yield of progeny virus in the presence of antibody). A similar variability in the susceptibility to neutralization was also observed among European isolates.

Antigenic diversity among PRRSV isolates has also been demonstrated using Mabs: 63 isolates from the United States and 57 European isolates from Denmark, France, Germany, Italy, Luxembourg, the Netherlands, Spain, and the United Kingdom were examined using a panel of three Mabs (SDOW17, VO17, and EP147) directed against the N protein. All three Mabs reacted with all 63 US PRRSV isolates. Mab SDOW17 reacted with the 57 European isolates, but neither VO17 nor EP147 reacted with any European isolates.

Additional studies have demonstrated that both European and North American PRRSV isolates are even more antigenically diverse than initially believed. The antigenic relationship of the 22 US PRRSV isolates from eight different states that were recovered from samples collected between 1989 and 1993 was evaluated by fluorescence microscopy using a panel of five Mabs (SDOW17, VO17, EP147, M146, M302) specific for the N protein. The 22 virus isolates were categorized into three groups based on their reactivity to the five Mabs. Of the 22 isolates, 18 reacted with all Mabs, three isolates react-

ed with all Mabs except EP147, and one unique isolate reacted only with VO17. More recently, the antigenic relationship among 70 North American isolates recovered from samples collected between 1989 and 1995 was studied using a panel of 21 Mabs, each of which represents a distinct epitope on the N, M, E, or 45-kD protein. Identified were 19 distinct antigenic groups. The investigators found that the antigenic variation was due to group-specific single amino acid substitution. The European Lelystad virus was distinct from any of the North American isolates tested and constituted a separate antigenic group. Similarly, the antigenic relationship of 18 United Kingdom and 7 continental European field isolates was evaluated using a panel of six Mabs specific for the N protein, and broad antigenic variation was also observed among these isolates.

Genomic sequence analysis of the PRRSV genome has provided genetic evidence for the antigenic diversity that was demonstrated by serological tests. These analyses suggested the existence of at least two distinct virus genotypes: the European (Lelystad virus) and the North American. By comparing partial genomic sequences, North American PRRSV isolates were found to have 87% to 95% homology in their nucleotide sequences, but were only 64% to 67% similar to European isolates. In these studies, amino acid homology between North American and European isolates was shown to be 55% to 80%. ORF7 was found to be the most conserved viral gene among North American isolates, with 95% to 100% homology, as compared with 57% to 59% amino acid sequence identity with Lelystad virus. In addition, genomic differences in ORF7 between North American and European isolates have been demonstrated by RT-PCR. Using a pair of primers for ORF7, the RT-PCR-generated product from the North American isolate was 35 base pairs smaller than the fragment obtained from the European isolate, or no amplified product from Lelystad virus was produced. ORF6 is also highly conserved among US isolates (up to 100% amino acid identity) and the most conserved between US and European isolates (70% to 81% identities). The amino acid sequence homology of ORF5 protein (E) varies from 88% to 97% among US isolates and from 51% to 59% when compared with the Lelystad virus. Although the E glycoprotein is the least conserved protein among isolates, it may contain a conserved epitope among North American PRRSV isolates. For the proteins encoded by ORFs 2, 3, and 4, homology of amino acid sequence among seven US isolates was reported to be 91% to 99%, 86% to 98%, and 92% to 99%, respectively. In addition, comparison of the Lelystad virus sequence with the US isolate ATCC VR-2332 indicated amino acid identity of 63%, 58%, and 68% for ORFs 2, 3, and 4, respectively. Similar results were obtained comparing the Lelystad virus with the US isolate VR-2385.

In a more recent study that compared sequences of 10 PRRSV isolates from Midwestern US swine herds to

VR-2332, 611 polymorphic nucleotide sites were identified in ORFs 2 through 7. The total nucleotide diversity of individual ORFs ranged from $3.8\% \pm 0.3\%$ for ORF6 to $9.7\% \pm 0.7\%$ for ORF5, with mean nucleotide diversity for all six ORFs of $6.0\% \pm 0.8\%$. Mean nucleotide divergence relative to VR-2332 ranged from 2.5% to 7.9% among those 10 isolates. With the additional nucleotide sequence information from a large pool of North American isolates, investigators concluded that the virus may be evolving by intragenic recombination, as well as by the accumulation of random neutral mutations that is known to be a major driving force for genetic variation among RNA viruses. Field observations and preliminary experimental data suggest that genetic variation continues and has significant implications for cross-protection, diagnosis, and virus persistence.

BIOLOGICAL CHARACTERISTICS

One of the most notable biological characteristics of PRRSV is a restricted cell tropism for macrophages, although epithelia in the upper respiratory system were shown to be infected by PRRSV. Because of this tropism, PAMs collected from infected pigs were shown to be a reliable sample for diagnosis of PRRSV infection, particularly in older animals. Immunohistochemical and in situ hybridization studies on tissues from PRRSV-inoculated 3- to 28-day-old specific-pathogen-free, colostrum-deprived or gnotobiotic pigs have shown that PRRSV replicates primarily in macrophages in the lungs and in macrophages and dendritic cells concentrated in germinal centers in lymphoid tissues. Hence, gross and microscopic lesions are most consistent in lung and lymph nodes. PRRSV antigen and nucleic acid have also been detected in vascular endothelial cells and intravascular and perivascular macrophages in segments of blood vessels in the heart, brain, kidney, and elsewhere. This may be manifested grossly as edema and microscopically as vasculitis and/or perivascularitis. Viral antigen has also been detected in nasal epithelial cells, nasal serous gland epithelial cells, cells lining airways, and microglial cells.

Infection of porcine alveolar macrophages can actually be enhanced in the presence of PRRSV-specific antibodies at subneutralizing levels. This phenomenon, termed antibody-dependent enhancement, may have implications for viral pathogenicity and vaccine development. Antibody-dependent enhancement of infection, first observed in vitro, has also been observed in vivo. Enhanced virus replication was demonstrated in fetuses by infecting them with antibody. Enhancement of virus replication was also demonstrated in pigs passively immunized with virus-specific immunoglobulin.

Notable characteristics of PRRSV also include (1) the ability of the virus to establish persistent infection in the presence of circulating antibodies and cause transplacental infection, and (2) high infectivity (i.e., low mini-

mum infectious dose). PRRSV is known to persist in pigs for over 150 days after initial exposure. Those animals become chronic carriers and may play a major role in transmitting the virus to pen mates. An exposure dose of 10 or fewer virions has been demonstrated to be sufficient to achieve infection in pigs.

Isolates may vary in their degree of virulence. Investigators demonstrated that significantly more PRRSV antigen was present in the lungs, lymph nodes, and tonsils of pigs experimentally infected with a high-virulence isolate as compared with the quantity detected in the same tissues of pigs infected with low-virulence isolates. The cell types in which antigen was detected and the distribution of PRRSV antigen-positive cells within tissues and organs were similar for the different virus inoculation groups despite differences in virulence among the isolates. Both a modified-live virus vaccine strain and field isolates were demonstrated to cross the placenta of inoculated gilts and infected fetuses in utero. However, the viruses varied in their pathogenicity as measured by the number of late-term dead fetuses. Piglets from gilts exposed to field isolates had a lower rate of survival and slower growth than pigs from control gilts. The genetic basis for virulence has been an area of study, but analysis of the amino acid sequences of ORFs 2 to 5 of five US isolates with differing virulence did not reveal the answer. It is worthwhile to note that the severity of clinical manifestation of PRRS varies among pigs with different genetic backgrounds.

CONCLUSIONS

PRRSV is a porcine arterivirus causing significant reproductive and respiratory problems in swine. Several outstanding characteristics of PRRSV have been identified. Among these, preferential replication in host macrophage-lineage cells, a high degree of genetic and antigenic diversity among field isolates, and the capacity of establishing persistence are a few. Persistence and semen shedding of the virus have become issues of concern for national and international trade. Tropism for immune cells and remarkable variability are of great concern regarding PRRS control by vaccination.

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10.4

Porcine Reproductive and Respiratory Syndrome: Diagnosis

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SUMMARY

Porcine reproductive and respiratory syndrome (PRRS) can be tentatively diagnosed based on clinical manifestations. However, the final diagnosis of PRRS should be confirmed by laboratory testing, including pathological examination. Various laboratory procedures have been developed for use in detecting virus (virus isolation), antigens (immunohistochemistry), or viral RNA (polymerase chain reaction). Serum antibody assays are used to monitor exposure to PRRS virus, vaccine compliance, or general herd immunity.

INTRODUCTION

A tentative diagnosis of porcine reproductive and respiratory syndrome virus (PRRSV) infection is suggested by clinical signs, i.e., reproductive disease in breeding stock and respiratory disease in pigs of any age. Reproductive problems associated with PRRS include poor conception rates, late-term abortions, and an increase in the rate of stillborn pigs, mummified fetuses, and weak, nonviable piglets. However, because of the similarity of clinical signs with those induced by other viral and bacterial pathogens, differential tests are required for a definitive diagnosis. Differential diagnoses should include Aujeszky's disease, postweaning multisystemic wasting syndrome, swine influenza, inclusion body rhinitis (cytomegalovirus), classical swine fever, leptospirosis, and infection with porcine parvovirus, hemagglutinating encephalomyelitis virus, or porcine enterovirus. There are no pathognomonic gross or microscopic lesions for the respiratory disease induced by PRRSV infection, although interstitial pneumonia is a common finding. Even when these lesions are demonstrated in conjunction with a compatible herd history, a definitive diagnosis of PRRSV infection requires the isolation of virus, detection of viral antigen or genomic material, and/or detection of antibody.

PATHOLOGICAL EXAMINATION

In late-term reproductive failure caused by PRRSV, lesions suggestive of PRRS are more common in congeni-

tally infected, live-born pigs that die or are killed within a few days after birth than in fetuses and stillborn pigs. Edematous or hemorrhagic enlargement of umbilical cords in fetuses or stillborn pigs suggests PRRSV infection, if present in a herd with a history compatible with PRRS. PRRSV infection is known to cause microscopic uterine lesions in pregnant sows. Lesions are characterized as multifocal lymphohistiocytic perivascular cuffs and variable amounts of edema in the myometrium and/or endometrium. In the case of systemic infection, the most consistent lesion in affected pigs of all ages involves lung and lymph nodes. Grossly, lungs are firm, noncollapsing, and sometimes edematous. Lymph nodes frequently are enlarged. Microscopically, interstitial pneumonia is the most characteristic histopathologic lesion of PRRSV infection. Lung, tonsil, lymph node, brain, and nasal turbinate are the preferred specimens for histopathologic examination and immunoassays on tissues.

Fetal Lesions

Few stillborn pigs or fetuses resulting from PRRSV-induced reproductive failure have gross or microscopic lesions specific for PRRSV infection. Lesions are more commonly seen in live-born PRRSV-infected littermates that die or are killed within a few days after birth. Most lesions in fetuses killed by PRRSV are nonspecific and a direct result of sterile in utero autolysis. The composition of abnormal PRRSV-infected litters that are either aborted or delivered full-term depends on the stage of gestation at which dams are infected with PRRSV and the virulence of the viral strain. PRRSV crosses the placenta in viremic pregnant swine more frequently during the last third of gestation. The proportion of fetuses in a litter that become infected, the mortality rate among infected fetuses and, for those that die, the time interval between PRRSV infection and fetal death may vary with strain. PRRSV-infected fetuses that live until parturition may be stunted and/or lack viability relative to noninfected littermates or cohorts. PRRSV-infected dams usually carry the pregnancy to near-term or term, i.e., late-term abortions (by 100 days of gestation or later) or full-term deliveries. Therefore, PRRSV-infected litters are composed of variable proportions of normal, live,

uninfected pigs; live, PRRSV-infected pigs of variable size and viability; and dead pigs of variable size and degree of in utero autolysis. Stillborn pigs or dead fetuses may be fresh, suggesting periparturient death, or they may exhibit mild-to-severe sterile postmortem autolysis leading to partial or complete mummification. Fetuses in PRRSV-infected litters are commonly coated with a thick brown mixture of meconium and amniotic fluid, which is a nonspecific finding that suggests fetal stress and/or hypoxia.

Because PRRSV-specific lesions are mild and predominantly microscopic, they can be detected only in fetuses with little or no autolysis. The most unique and diagnostically discriminating PRRSV-specific lesion in fetuses is in the umbilical cord. This lesion was first described in experimental inoculation studies using either North American or European strains of PRRSV. In affected cords, there is segmental-to-diffuse hemorrhagic and/or edematous enlargement up to three times normal diameter. Microscopically, there is moderate-to-severe segmental-to-circumferential necrosuppurative and lymphohistiocytic arteritis with variably severe transmural and periarterial edema and/or hemorrhage. The endothelium of affected segments of arteries is often swollen or absent. Other gross fetal lesions described in both natural and experimental infections include perirenal edema, splenic ligament edema, mesentery edema, ascites, hydrothorax, and hydroperitoneum. Microscopic lesions in PRRSV-infected fetuses are also rare and include (1) mild-to-moderate segmental arteritis in lung, heart, and kidney, (2) mild multifocal interstitial pneumonia, (3) mild periportal hepatitis, (4) mild perivascular myocarditis, and (5) mild multifocal encephalitis. Lung lesions are characterized by thickening of alveolar septa by mononuclear inflammatory cells, alveoli that contain necrotic mononuclear cells and cell debris, and occasional proliferation of type II pneumocytes. Occasionally, segmental pulmonary lymphohistiocytic vasculitis may cause multifocal hemorrhage. In affected hearts, lymphocytes, plasma cells, and macrophages multifocally surround subendocardial and interstitial blood vessels that may have segmental vasculitis. Similar inflammatory cellular aggregates may be found multifocally within the myocardium where there may be loss of myocardial fibers. In affected livers, lymphocytes, histiocytes, and/or eosinophils multifocally surround portal blood vessels and expand subcapsular connective tissue. In affected brains, the cerebellar white matter has multifocal lymphohistiocytic perivascular cuffs and glial nodules.

Although histopathologic changes have been described in fetuses from experimentally infected sows, the diagnostic value of fetuses in association with PRRSV-induced reproductive problem is usually considered very limited as compared with other viral infections, such as parvovirus. In particular, fetuses collected from aborted

sows, even if they are fresh, may be of no diagnostic value, since lesions, virus, and viral antigen are generally undetectable.

Maternal Uterine Lesions

Although pregnant sows may die during acute PRRSV infection, more commonly PRRSV-associated disease in sows is transient and nonfatal. Microscopic uterine lesions occur frequently in pregnant sows with reproductive failure caused by natural or experimental PRRSV infection. In some sections of affected uterus, there are multifocal lymphohistiocytic perivascular cuffs and variable amounts of edema fluid in the myometrium and/or endometrium. Less commonly, there is segmental lymphohistiocytic vasculitis in small vessels and microseparations between endometrial epithelium and placental chorionic epithelium that are filled with eosinophilic proteinaceous fluid and cellular debris.

Lesions in Acute Systemic Infection

Swine of all ages, if they are immunologically naive or have nonprotective immunity, are susceptible to PRRSV infection and may develop gross and/or microscopic lesions. The virulence of PRRSV strains differs, impacting the number of organ systems affected, the severity of lesions, and the clinical signs. Under experimental conditions, some strains of PRRSV cause no microscopic lesions or clinical disease when inoculated into conventional, specific-pathogen-free, caesarean-derived colostrum-deprived, or gnotobiotic pigs. Most experimental inoculation studies in which lesions have been described have been performed on suckling or weaned pigs that were 1 to 70 days of age.

In general, the same lesions have been described in swine of all ages, with the exception of a few additional gross lesions described only in pigs inoculated at 13 days of age or younger. Lesions unique to pigs that are 13 days of age or younger when inoculated with PRRSV include periocular edema on days 6 to 23 post inoculation (PI), scrotal edema on days 11 to 14 PI, and ventral cervical and inguinal subcutaneous edema on days 2 to 7 PI. The most consistent gross and microscopic lesions in swine of all ages inoculated with different strains of PRRSV are in lung and lymphoid tissues. Microscopic lesions are also found frequently in the heart and brain. Microscopic lesions have been described less frequently in kidney, nasal mucosa, and stomach.

Grossly, lungs have variably severe interstitial pneumonia and edema that may be visible from days 3 to 28 PI. Typically, interstitial pneumonia is most severe approximately 10 days PI. Mild lesions are limited to the cranial lungs or may be diffuse. Affected lung parenchyma is resilient, slightly more firm than normal, noncollapsing, and mottled gray-tan. Edema, when present, tends to gravitate, making the ventral portions slightly firmer than the dorsal portions. In severely affected

lungs, parenchyma is mottled or diffusely tan-red and noncollapsing, firm and rubbery, and heavy with edema fluid that may distend interlobular septa. Microscopically, lesions are also present from days 3 to 28 PI or later and may be in lungs either with or without gross lesions. Diffusely or multifocally, alveolar septa are thickened by infiltrates of macrophages, lymphocytes, and plasma cells and may be lined by hypertrophic and hyperplastic type II pneumocytes. Alveoli contain variable numbers of necrotic macrophages and amounts of cellular debris. Lesions are typically not described in the epithelium of airways, although some investigators described swelling of bronchial epithelial cells and limited immunohistochemical staining of airway epithelium, and loss of cilia on bronchiolar epithelial cells.

Lesions are in lymph nodes of most pigs, but differ in distribution and severity within affected pigs. Grossly, affected lymph nodes are enlarged from 2 to 10 times normal size approximately 4 to 28 days PI. Early after inoculation, enlarged nodes are edematous, tan, and moderately firm. Later after inoculation, nodes are firm and white or light tan in a nodular or diffuse pattern typical of lymphoid hyperplasia. Occasionally, nodes have multiple subcortical fluid-filled cystic spaces 2 to 5 mm in diameter. Microscopic lesions are predominantly in germinal centers. Early after inoculation, germinal centers are necrotic, depleted, and edematous, containing lymphocytes and/or macrophages with pyknosis or karyorrhexis and necrotic cellular debris. Later after inoculation, germinal centers enlarge and may coalesce, containing large numbers of blast-type lymphocytes. The cortices may contain small cystic spaces that are variably lined by endothelium and may contain proteinaceous fluid, lymphocytes, and prokaryocytes (i.e., multinucleated cells with up to 120 clustered, oval-to-circular, basophilically stippled nuclei, and scant-to-moderate amounts of lightly basophilic cytoplasm that contains vimentin intermediate filaments). Syncytial cells containing 2 to 10 nuclei with multiple prominent nucleoli and a moderate amount of eosinophilic cytoplasm have also been described at the margins of dense lymphoid areas in affected nodes. Although there are no gross lesions in other lymphoid tissues, microscopically there may be mild lymphoid necrosis, depletion, and/or hyperplasia in the thymus, in periarteriolar lymphoid sheaths of the spleen, and in lymphoid follicles in tonsil and Peyer's patches.

Mild-to-moderate multifocal, often subendocardial, lymphohistiocytic perivascular myocarditis may develop by day 9 PI or later. In affected segments of small arteries and veins, endothelial cells may be swollen and there are subendothelial, mural, and/or perivascular lymphocytes, macrophages, and fewer plasma cells. Less commonly, mild myocardial fibrillar necrosis and lymphocytic cuffing of Purkinje fibers are described.

Mild lymphohistiocytic leukoencephalitis or encephalitis involving cerebellum, cerebrum, and/or brain-

stem may develop by day 7 PI or later. The encephalitis is characterized by segmental cuffing of blood vessels by lymphocytes and macrophages and multifocal gliosis. In one reported field case of PRRS, neurological clinical signs and high mortality were consistently associated with severe lymphohistiocytic encephalitis and severe cerebral necrotizing vasculitis.

Renal lesions are occasionally described from days 14 to 42 PI and most commonly include mild periglomerular and peritubular lymphohistiocytic aggregates. In addition, in one inoculation study, mild-to-severe segmental vasculitis was described that was most severe in the pelvis and medulla. Affected segments of vessels had endothelial cell swelling, accumulation of subendothelial proteinaceous fluid, fibrinoid necrosis of medial smooth muscle, and sparse-to-dense intramural and perivascular aggregates of lymphocytes and macrophages.

In nasal mucosa, beginning as early as 12 hours PI, the epithelium multifocally has clumping and loss of cilia, swelling and exfoliation of epithelial cells, small cystic spaces, and squamous metaplasia. After day 7 PI, there are increased numbers of intraepithelial leukocytes and aggregates of lymphocytes and macrophages in the propria submucosa.

DETECTION OF VIRAL ANTIGENS

The frozen-tissue-section fluorescent antibody (FA) test and immunohistochemistry (IHC) test are commonly used for detecting PRRSV antigen in tissues. Both monoclonal and polyclonal antibodies have been employed for detecting viral antigens in frozen or fixed tissues. The direct FA test on frozen-tissue sections is inexpensive and rapid. It is specific, but is not always very sensitive. In particular, tissue quality (e.g., autolysis) affects test results. In contrast, IHC is useful for detecting viral antigen in formalin-fixed tissues. Two types of IHC have been developed: an immunoperoxidase test and immunogold silver staining. IHC is more sensitive than direct FA examination of frozen tissues, but takes more time and is more expensive than the FA test. A definitive diagnosis can be accomplished by detection of microscopic lesions characteristic of PRRS in conjunction with IHC or IFA tests positive for virus.

For direct FA examination, fresh or frozen tissues should be submitted. Tissues should be fixed in 10% neutral buffered formalin if submitted for IHC. Preferred tissues for these tests are tonsil, lung, lymph node, spleen, thymus, heart, and kidney. PRRSV antigens may also be detected in the adrenal gland, intestine, liver and, occasionally, in the brain. In the diagnostic setting, it should be taken into consideration that, when performing antigen detection tests such as FA and IHC, reliance on a single PRRSV-specific monoclonal antibody may lead to misdiagnosis because of the antigenic diversity that exists among PRRSV isolates.

VIRUS ISOLATION

PRRSV is known to replicate in only two types of cells: porcine macrophages and certain African monkey kidney cell lines. The cell line MA-104 is widely used for virus isolation in the diagnostic setting, especially an MA-104 clone identified as MARC-145. Other continuous cell lines, such as CL2621 and CRL11171, are reported to support virus replication *in vitro* and have been used for virus isolation from clinical specimens. However, these are proprietary cell lines and not available for routine diagnostic use. Porcine alveolar macrophages are also used for virus isolation, but are more expensive to use than continuous cell lines. In addition, porcine alveolar macrophages (PAMs) may harbor adventitious agents, and PAMs from individual pigs vary in their susceptibility to PRRSV. For these reasons, the MA-104 or MARC-145 cell lines offer certain advantages for routine diagnostic use; nevertheless, with the proper controls in place and the appropriate monitoring of donor pigs, PAMs may be used effectively in the diagnostic setting.

Although several cell types support PRRSV replication, the diagnostician's ability to isolate virus from samples is complicated by the fact that not all PRRSV isolates replicate in all cell types. PAMs are sometimes reported to be more susceptible to PRRSV than is the MARC-145 cell line, but not all laboratories agree on this point. In a comparative study, 98 tissues and 73 sera were assayed for the presence of PRRSV by using both PAM and CL2621 cells. Virus was recovered from 7 (7%) of 98 and 4 (4%) of 98 of tissue samples in PAMs and CL2621 cultures, respectively. Virus was isolated from 18 (25%) of 73 serum samples by using PAMs, but from only 2 samples (3%) in CL2621 cell cultures. Interestingly, 25 (30%) of 82 virus isolates made in CL2621 cells did not grow in PAMs, and 5 (28%) of 18 isolates from PAMs did not grow in CL2621 cells. Of the isolates, 28 (34%) that grew in PAMs did not produce cytopathic effect. These differences suggested that at least two cell types should be used for virus isolation whenever possible. Also, interference of antibody with virus isolation should be taken into consideration if virus isolation is attempted on serum samples using cell lines.

PRRSV has been isolated from many different clinical specimens, including serum, plasma, the buffy-coat layer of citrated blood, bone marrow, tonsil, lungs, lymph nodes, thymus, spleen, heart, nasal turbinate, nasal swabs, oropharyngeal scraping, placenta, saliva, urine, feces, and semen. For PRRS diagnosis, the usefulness of PAMs collected by pulmonary lavage from live animals or at necropsy has also been demonstrated. Virus can be detected in harvested PAMs either by virus isolation or by demonstrating the presence of viral antigens in the PAMs by the indirect fluorescent antibody technique. Bronchioalveolar lavage is routinely done in many diag-

nostic laboratories when pigs or whole lungs are submitted for PRRS diagnostic investigation.

In young pigs, viremia persists for a prolonged period (2 to 6 weeks). PRRSV is more stable in serum than in tissue. In older animals, viremia is of short duration, and virus isolation should be done on tissues and bronchioalveolar lavage fluids. Preferred tissue samples include lungs, tonsil, and lymph nodes. Oropharyngeal scrapings have been found to be a reliable sample for virus isolation when PRRSV-related problems are strongly suspected in growing/finishing pigs or adult animals, and difficulties are encountered in isolating PRRSV from the other aforementioned samples. Tissue must be fresh if virus isolation is to be successful, regardless of which tissues are submitted for diagnostic investigation.

Sample selection may also depend on the age of the pig from which specimens are collected and the stage of infection (i.e., acute, convalescent, or persistent). Serum, lung, and pulmonary lavage fluid are samples of choice for isolation of PRRSV in acutely infected animals. For virus isolation from persistently infected animals, tonsil and pulmonary lavage fluid are better samples than serum and lung. In cases of late-term abortion and early farrowing, samples should be collected from weak-born, presuckled pigs rather than mummies, aborted, or still-born pigs. Within affected litters, weak-born pigs are the most likely to be viremic, but the presence of high levels of colostrum-derived antibody to PRRSV may hinder attempts at virus isolation.

The thermal susceptibility of PRRSV in diagnostic specimens to different environmental temperatures has been evaluated in clinical specimens (lung, spleen, thymus, and serum) from five piglets experimentally infected with an Indiana isolate. The virus isolation rate from positive tissues was 47%, 14%, and 7% when tissues were kept at 25°C for 24, 48, and 72 hours, respectively, whereas virus isolation rates were 85% from tissues stored at 4°C and -20°C for the same period. In contrast, virus was isolated from all but one serum sample ($n = 5$) kept at 25°C for 72 hours. The data suggested that serum had a protective effect on the stability of PRRSV. The current recommendation is that tissues and clinical specimens for virus isolation be kept at 4°C (40°F) or lower after collection and during shipment to a diagnostic laboratory in order to enhance the likelihood of isolating virus. If long-term storage of samples is necessary, specimens should be kept frozen at less than -20°C.

DETECTION OF VIRAL GENOMIC MATERIAL

Polymerase chain reaction (PCR)-based tests have been developed for detecting PRRSV RNA in clinical specimens. As a general principle, PCR-based assays are believed to be highly sensitive and highly specific; however,

a recent study could not confirm this perception for PRRS PCR. Several reverse transcription-polymerase chain reactions (RT-PCRs) using specific primers complementary to the sequences of open reading frame (ORF) 7 have been developed to detect viral RNA in serum, infected cells, and tissue homogenates from infected pigs. Some RT-PCRs use a nested set of primers, i.e., RT-nested PCR (RT-nPCR). More recently, a commercial RT-PCR (Biovet, St. Paul, MN, USA) utilizing primers complementary to the sequence of ORFs 6 and 7 has been introduced. The development of an RT-nPCR that allows for the quantification of virus in a sample has been reported. An automated PCR-based test, the TaqMan PCR, has also been developed for detecting PRRSV (ORF6) in semen samples. Unlike nPCRs, TaqMan PCR employs one-step amplification in one tube, and PCR products are photometrically analyzed. With this test, some of the problems inherent to nPCR may have been avoided, such as carryover contamination causing false-positive results and the time and labor involved in the manipulation of amplified product for gel-based analysis. An RT-PCR that used degenerated primers complementary to ORF1b of known arteriviruses (PRRSV, lactate dehydrogenase-elevating virus, equine arteritis virus, and simian hemorrhagic fever virus) has been developed and found useful for detecting all of the arteriviruses identified to date.

PCR is useful for the detection of viral RNA in samples like semen or feces that are either cytotoxic for cell culture or cannot be evaluated by other methods. In fact, the primary diagnostic application of PRRSV PCR is for the detection of viral RNA in boar semen. PRRSV PCR also appears to be useful in detecting viral RNA in serum containing a high level of antibodies. At present, PCR is expensive relative to other methods routinely used in diagnostic laboratories and may not be the assay of choice for routine use. Furthermore, it should be borne in mind that a positive result on PCR indicates the presence of viral RNA and does not necessarily indicate the presence of infectious PRRSV.

An *in situ* hybridization (ISH) technique that uses a nonradiolabeled RNA probe specific for PRRSV ORF7 has been developed for detecting genomic material in fixed tissues. In a comparative study, it was found that, after the acute phase of infection, samples contained more cells expressing viral RNA than cells with virus antigen. This would explain the greater sensitivity of ISH in certain tissues. ISH may be useful for not only diagnostic purposes but also retrospective studies of PRRSV infection or research to elucidate the pathogenesis of PRRSV infection.

Although experimental data indicate that both RT-PCR and ISH techniques may be useful diagnostic tools, the diagnostic specificity and diagnostic sensitivity of these techniques have not been thoroughly evaluated in longitudinal studies.

DETECTION OF SERUM ANTIBODIES

The indirect fluorescent antibody (IFA) test, serum virus neutralization (SVN) test, immunoperoxidase monolayer assay (IPMA), and enzyme-linked immunosorbent assay (ELISA) have all been used for the detection of antibodies specific for PRRSV. The IFA test, SVN test, and ELISA are currently available in most North American veterinary diagnostic laboratories, whereas IPMA has been extensively used in Europe.

The IFA test is thought to have high specificity (99.5%) but unknown sensitivity for individual animals. An advantage of the IFA test compared with ELISA is that the magnitude of the antibody titer can be determined. A titer of 16 or 20, depending on the initial serum dilution for the test, is considered positive. The IFA test reliably detects specific antibodies for 2 to 3 months after infection. Since interpretation is subjective, the endpoints of IFA antibody titers often vary among technicians and laboratories. Furthermore, test results or endpoint antibody titers will vary depending on the degree to which the PRRSV strain used in the assay differs antigenically from the isolate that infected the pig. An IFA test developed for the detection of PRRSV-specific immunoglobulin M (IgM) antibodies was reported to be useful in detecting acute or recent PRRSV infection. A titer of 16 or 20 is considered positive. Importantly, research data demonstrated that high percentages of IgM-positive samples (81%) were from viremic pigs. False-positive results due to the possible nonspecificity of the test have been a concern.

The IPMA is also considered to be highly specific and sensitive. In one comparative study, the sensitivity of IPMA was better than that of a commercial ELISA. The standard IPMA uses virus-infected PAMs as antigen, although the test can be adapted to a platform using continuous cell lines. In a fourfold scale after a 1:10 initial dilution, a titer of 10 or less is considered negative and a titer of 160 or more positive. A serum sample with an antibody titer of 10 or 40 is considered suspect. Antibodies to PRRSV are usually detected by IPMA between days 7 and 15 after infection. Like the IFA test, the IPMA also reliably detects specific antibodies for 2 to 3 months after infection. Antigenic variability among isolates is also of concern with respect to test results.

The ELISA is also reported to be sensitive and specific. One disadvantage of the ELISA format reported during the developmental stage was an unacceptable background reaction in some negative pigs. Several formats of ELISA have been described: an indirect ELISA using a sample-to-positive ratio (S/P) system, an indirect ELISA using direct optical density values, and a blocking ELISA. In the commercial ELISA kit (HerdChek PRRS ELISA; IDEXX Laboratories, Westbrook, ME, USA), an S/P ratio of ≥ 0.4 is considered positive. Uniformity in the manufacturing of the kit and a high degree of

automation in performing the test in the laboratory result in less variation in the results from the commercial ELISA than in other tests. Other advantages of the commercial ELISA include (1) detection of antibody against both North American and European PRRSV strains, (2) fast turnaround, and (3) licensure by the US Department of Agriculture and AgCanada.

The SVN test is also considered to be specific, but appears to be less sensitive than the IFA test and the ELISA. The low sensitivity of the test is primarily due to the fact that neutralizing antibodies against PRRSV develop as late as 1 to 2 months after infection. Currently, a titer of 4 or higher is considered positive. Reportedly, the sensitivity of the SVN test was increased by adding a complement source, i.e., fresh normal swine serum or guinea pig serum, to the serum sample being assayed. Using this modification, SVN antibodies could be detected as early as days 9 to 11 after infection. Even so, the SVN test is best considered a research tool rather than a routine diagnostic test because of its laborious nature. As with the IFA test and the IPMA, test results are greatly influenced by the degree of antigenic relatedness between the isolate employed in the test and the isolate infecting pigs.

Although some research data suggested that SVN antibody specific for PRRSV plays a role in clearing the virus from the blood, the association between the presence of neutralizing antibody and protective immunity is uncertain. Prolonged viremia and persistent PRRSV infection have been demonstrated in the presence of circulating antibodies. This is an important issue for future research.

Using the IgM IFA, IgG IFA, and SVN tests, three categories of serological profiles have been described among pigs in herds exposed to PRRSV: noninfected pigs, acutely infected pigs, and pigs with antibody decay. Noninfected pigs can be identified as negative by all three tests. Acutely infected pigs were defined as pigs with IgM and/or IgG IFA titers of 64 or higher, but no detectable SVN antibodies.

INTERPRETATION OF SEROLOGICAL RESULTS

Antibodies specific for PRRSV often do not persist for the lifetime of an animal. In pigs exposed to PRRSV under experimental conditions, virus-specific antibodies were initially detected by IPMA, ELISA, and the IgG IFA and SVN tests at 5 to 9, 9 to 11, 9 to 13, and 9 to 28 days after inoculation, respectively. PRRSV-specific IgM antibodies are detected within 5 days PI and persist for 21 to 28 days PI. Depending on the assay, antibody levels reached their peak value by 30 to 50 (IFA test), 35 to 50 (IPMA), 30 to 50 (ELISA), and 60 to 90 days (SVN test) after inoculation, after which they began to decline. Antibody titers were estimated to approach undetectable levels by 4 to 5 months (IFA test), 4 to 10 months

(ELISA), 11 to 12 months (IPMA), and 12 months (SVN test) after infection. The same time frame would be expected in animals without a history of previous exposure to PRRSV that are vaccinated with modified-live virus (MLV) vaccine.

Several problems or limitations should be taken into account when interpreting PRRS serology. Serological information from a single sample is not sufficient for diagnosing clinical PRRS in an individual animal because PRRSV infection is highly prevalent in swine herds. Positive results may or may not mean that PRRSV caused clinical disease. Possible presence of maternal antibody should be considered when interpreting serological results. Passive maternal antibody was detected in the serum of a piglet tested 4 days after birth. In some instances, no maternal antibody was detected in sera of piglets born to infected dams. Maternal antibody specific for PRRSV has been reported to persist as long as 4 to 10 weeks of age and occasionally up to 16 weeks of age in pigs nursing immune dams.

Since antibodies often do not persist for the lifetime of an animal and because of the relatively short duration of IFA and/or ELISA antibodies, it is generally recommended that young pigs, rather than breeding stock, be tested to determine the herd's PRRSV infection status. In single-site, farrow-to-finish swine herds, the seroprevalence of PRRSV infection is usually expected to be highest in the grow-finish unit. Serum from 10 to 15 finishing pigs is often sufficient to determine whether the herd has been exposed to PRRSV. For multisite production systems, each stage of production represents a single population, so each site should be sampled.

Negative PRRS serology on samples at one point in time has several possible interpretations: (1) the pigs were not infected with virus, (2) the pigs were recently infected with virus and have not yet seroconverted, (3) the pigs were infected with virus but have become seronegative, or (4) the result was negative because of low sensitivity of the test or because of laboratory error.

Therefore, if using single point samples, PRRS serology must be used in conjunction with valid population-sampling methods to determine whether a herd has been exposed to PRRSV, not whether individual animals are infected.

Diagnosis of PRRSV infection as the cause of reproductive failure or respiratory disease can be achieved by showing seroconversion using paired serum samples or a rising antibody titer in paired serum samples. However, sows may be exposed to virus 2 or more weeks prior to the onset of clinical signs, in which case rising antibody titers may not be observed. The presence of PRRSV antibodies in fetal fluids or in blood collected from stillborn and weak piglets before ingestion of colostrum is also indicative of PRRSV infection. A definitive diagnostic evaluation of PRRS with respect to clinical disease requires that serological information be interpreted in

combination with results from virus isolation and/or detection of antigenic or genomic material. It is important to bear in mind that the current serological assays used in the diagnostic setting cannot routinely differentiate vaccine-derived antibodies from field-isolate-derived antibodies.

DIFFERENTIAL TESTING

Serological assays cannot routinely differentiate antibodies to field isolates from vaccine-derived antibodies. However, characterization of virus isolates is possible by several methods. Monoclonal antibodies specific for PRRSV may be used to characterize the antigenic relationship among PRRSV isolates. Panels of monoclonal antibodies can easily differentiate European isolates from North American isolates and vice versa. Using this technique, no evidence of European-type PRRSV was found in US Midwestern swine herds. Monoclonal antibody analysis was previously used to differentiate a commercial MLV vaccine from field isolates. Although monoclonal antibody SDOW17 was once considered a universal diagnostic reagent, it was shown that the PrimePac PRRS vaccine virus (Schering-Plough Animal Health, Elkhorn, NE, USA) did not react with monoclonal antibody SDOW17. There are also field isolates that do not react with SDOW17.

Molecular biology has also made it possible to characterize PRRSV isolates by using PCR, a restriction fragment-length polymorphism (RFLP) analysis, and sequencing. A PCR-based technique has been employed to differentiate North American from European isolates. Although its usefulness was demonstrated, PCR is not routinely used for that purpose. The RFLP analysis involves PCR amplification of ORF5 from isolate or clinical specimens and restriction endonuclease digestion followed by electrophoresis of the products to visualize cutting pattern. RFLP patterns have no known association with specific viral characteristics, such as virulence, and in that sense the results have limited usefulness. The current use of RFLP is for differentiating a vaccine strain (RespPRRS/Repro or Ingelvec; Boehringer-Ingelheim Vetmedica, St. Joseph, KS, USA) from wild types. However, considering the high rate of mutation of RNA viruses, the utility of this assay in the long run is uncertain. With these limitations in mind, sequence analysis has the merit of detecting minor differences in nucleotides between isolates. Restriction endonuclease cutting sites can also be predicted from the sequence data.

CONCLUSIONS

As is the case for other diseases, diagnosis of PRRS requires a multidisciplinary approach. Although PRRSV is known to cause a unique disease, it should be taken into consideration that PRRS is a syndrome, i.e., a complex of

diseases. It follows that PRRSV may not always be the primary cause of clinical outbreaks that appear PRRS-like and that other viruses and bacterial agents capable of causing similar clinical signs should be included on the differential list.

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10.5

Characteristics of the Cell-Mediated Immune Response of Swine to Porcine Reproductive and Respiratory Syndrome Virus

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SUMMARY

Studies were conducted to ascertain the characteristics of the immunity induced by infection with wild-type virus or immunization with a modified-live virus (MLV) vaccine against porcine reproductive and respiratory syndrome virus (PRRSV). A striking observation resulting from this research was the protracted development of an interferon-gamma (IFN- γ) response to this virus. For instance, weak PRRSV-specific IFN- γ -secreting cells were initially detected in the blood of pigs at 3 weeks after exposure to either wild-type virus or an MLV vaccine. Only after a gradual and continuous increase during the ensuing 4 to 9 months did this antiviral immunity approach the level induced by a pseudorabies virus, MLV, by 2 to 4 weeks after vaccination. In contrast, a detectable humoral immune response was elicited against both virulent and attenuated PRRSV within 2 weeks after exposure, although a noticeable titer of virus-neutralizing antibodies was not apparent until 6 to 9 weeks later. Interestingly, the frequency of PRRSV-specific IFN- γ -secreting cells remained constant after the first 10 months after infection, whereas 80% of the pigs became seronegative by 23 months after infection. To add to the complexity of the regulation of the porcine immune response to PRRSV, an attempt to enhance the immunogenicity of the PRRS MLV by coadministration of a proven stimulator of porcine immunity failed. Therefore, based on the unusual host response to PRRSV infection, the rational development of effective vaccines will require an understanding of the mechanisms that regulate the kinetics, quality, and intensity of porcine immunity against this pathogen.

Given the economic losses attributed to the disease caused by this virus, this information is urgently needed.

INTRODUCTION

An evaluation of the potency of vaccines currently available in the United States against porcine reproductive and respiratory syndrome virus (PRRSV) has unfortunately revealed that they induce an inadequate immune response. For example, only half of the offspring of sows vaccinated during pregnancy with either of two modified-live virus (MLV) vaccines survived challenge with a lethal dose of virulent PRRSV. The percentage of survivors was further reduced fivefold when their mothers had instead been immunized with an inactivated virus vaccine (Osorio et al. 1998). Since vaccination against PRRSV is one of the major strategies to control its associated disease, the poor performance of these vaccines under experimental conditions is a concern. In fact, the current status and usage of PRRSV vaccines have been characterized as "chaotic" (Lager and Mengeling 1997). Moreover, use of MLV is also controversial, as illustrated by the apparently adverse outcome resulting from the immunization of pigs with a commercial vaccine in Denmark (Botner et al. 1997).

Clearly, the rational development of an effective PRRSV vaccine requires knowledge of the immunobiology of this pathogen. Our approach for resolving the inadequacy of current vaccines has been to examine the characteristics of the immunity induced by infection with either virulent wild-type virus or immunization with attenuated or inactivated virus. Based on our studies of the kinetics, intensity, and quality of the humoral and cellular immunities, the immune response to either

infection or MLV vaccination with this virus appears to be unconventional.

MATERIALS AND METHODS

Four studies that involved monitoring the porcine humoral and cellular immune responses to PRRSV exposure utilized Yorkshire × Landrace cross-bred pigs. Prior to virus infection or vaccination, all pigs were ascertained to be PRRSV free. For the third study, pigs were also verified as pseudorabies virus (PRV) free.

In the initial experiment, twelve 5-month-old boars were intranasally inoculated with $10^{3.8}$ tissue culture infectious dose 50% (TCID₅₀) of the Nebraska PRRSV isolate 2068-96 and kept for more than 400 days. In the second study, four 6-week-old pigs were similarly inoculated. These, as well as two uninfected control animals that were housed in a separate isolation room, were maintained for more than 6 months. The first vaccination experiment entailed a 2-month examination of two groups of five 10-week-old pigs that were immunized twice at a 2-week interval with either a commercially available MLV vaccine against either PRRSV (RespPRRS; Boehringer Ingelheim Vetmedica, Ames, IA, USA) or PRV (PRV/Marker Gold; Schering-Plough Animal Health, Lennexa, KS, USA). For the last study, groups of five 8-week-old pigs were immunized twice 4 weeks apart with either the RespPRRS vaccine in the presence or absence of the oil-in-water adjuvant Imugen II (Bayer Corporation, Pittsburgh, PA, USA) or the inactivated virus vaccine (PRRomiSe, Bayer) and then monitored for an additional 2.5 months.

In all studies, the intensity of the cellular immune response was measured by using both a standard lymphoproliferation assay and an ELISPOT protocol (Zuckermann et al. 1998b) that enabled enumeration of the frequency of PRV- or PRRSV-specific interferon-gamma (IFN- γ)-secreting cells. In both assays, the *in vitro* recall response to PRRSV or PRV was induced by stimulation with homologous virus strain VR-3223 (PRRSV) or Kaplan (PRV). Humoral immunity to PRRSV was measured with either a commercial enzyme-linked immunosorbent assay (ELISA) (HerdChek Porcine Reproductive and Respiratory Syndrome Antibody Test Kit; IDEXX Laboratories, Westbrook, ME, USA), a Western immunoblot assay against baculovirus-derived recombinant proteins, or a serum-virus neutralization assay. Significant differences between groups were evaluated using the *t* test.

RESULTS

A humoral response was readily detected by ELISA within 2 weeks after inoculation with either a wild-type PRRSV or an MLV vaccine. Although reactive with PRRSV, these antibodies did not neutralize virus infectivity. Only by 8 to 9 weeks after virus exposure were virus-

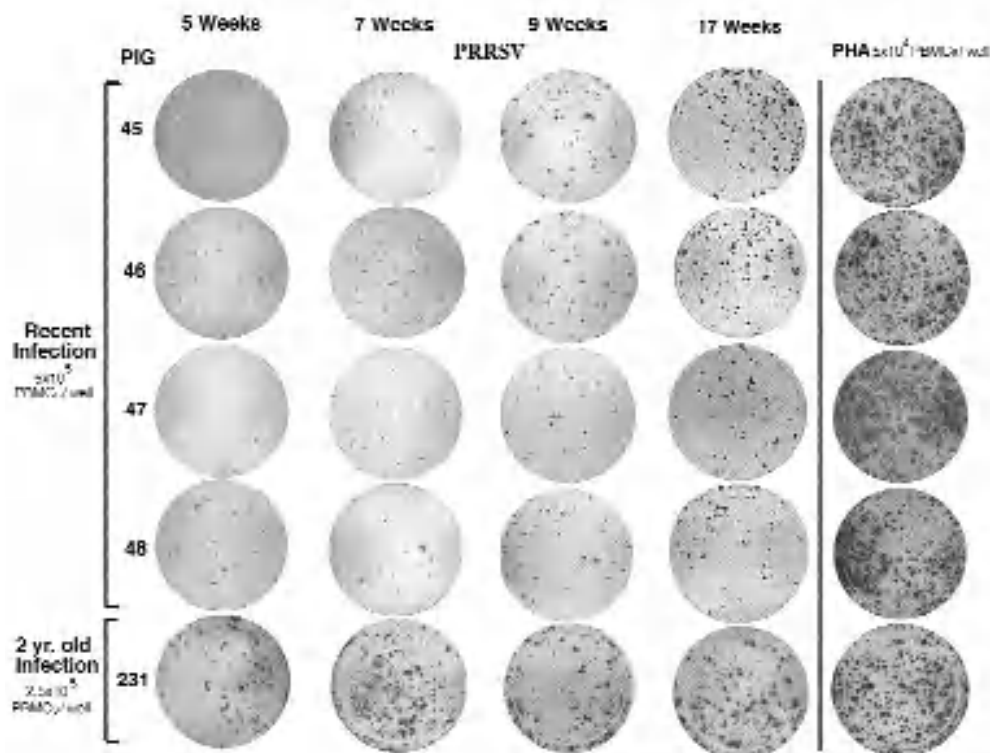
neutralizing antibodies detected. The generation of neutralizing antibodies was more readily induced by PRRSV infection than by vaccination, implying a possible correlation between the elicitation of virus-neutralizing antibodies and the degree of virus virulence. In contrast, the presence of a cellular immune response, as evidenced by the presence of PRRSV-specific IFN- γ -secreting cells, was not observed until 4 to 5 weeks after inoculation (Figure 10.5.1) or vaccination (data not shown). Even then, the frequency of virus-specific IFN- γ -secreting cells was approximately five- to eightfold lower than that induced by immunization with a PRV MLV vaccine (Zuckermann et al. 1998a). However, in animals inoculated with wild-type PRRSV, the frequency of virus-specific IFN- γ -secreting cells increased gradually so that by 9 to 10 months after infection it was comparable to the levels present in pigs at 2 to 3 weeks after the administration of a PRV MLV vaccine (Meier et al. 2002). Moreover, once obtained, this degree of cellular immunity remained constant in all of the animals infected with wild-type virus up to the termination of the experiment at 690 days (Figure 10.5.1, bottom row). In contrast, after peaking at 11 weeks after infection, the titer of PRRSV-specific antibodies in the serum of these animals decreased such that by the end of the experiment 80% of the pigs were considered to be seronegative based on ELISA results.

In an attempt to increase the immunogenicity of the PRRS MLV vaccine, the adjuvant Imugen II was mixed with vaccine and injected into the pigs. Although this modification had been previously observed to enhance the porcine immune response to PRV immunization (F. A. Zuckermann and S. Martin, unpublished observations), the degree of host immunity elicited by PRRSV vaccination was not altered.

DISCUSSION AND CONCLUSIONS

As previously observed (Yoon et al. 1995), the titer of PRRSV-specific antibodies declined within a few months after exposure to the virus to levels undetectable by several serological assays. Thus, due to their inherently low sensitivity, the use of such assays for diagnostic purposes is questionable. Instead, the ELISPOT applied in this study to detect individual IFN- γ -secreting memory T cells responding to PRRSV antigen is very sensitive and can be used to detect frequencies as low as 0.001% of the total lymphocyte population in porcine peripheral blood (Zuckermann et al. 1998b). Clearly, the IFN- γ ELISPOT can be a powerful alternative for the evaluation of swine herd exposure to PRRSV in those cases where the serological test results are negative.

By using the ELISPOT assay, we have previously shown that, in the case of PRV, a high frequency of virus-specific IFN- γ -secreting cells is associated with the presence of protective immunity (Zuckermann et al.



10.5.1. Gradual development of the interferon-gamma (IFN- γ) response of swine to porcine reproductive and respiratory syndrome virus (PRRSV). Four 8-week-old PRRSV-free pigs (nos. 45, 46, 47, and 48) were infected intranasally with 10^5 TCID₅₀/2 ml (1 ml/nostril) of wild-type PRRSV strain 12068-96. The IFN- γ response of these pigs to infection was monitored using an IFN- γ ELISPOT assay. Peripheral blood mononuclear cells (PBMCs) were isolated from these pigs at the indicated times following infection, plated at 5×10^5 per well, and cultured for 20 hours in the presence of homologous PRRSV (multiplicity of infection = 1). Samples from these four infected pigs were negative in the IFN- γ ELISPOT assay from weeks 0 to 4 (not shown). Two uninfected pigs that served as negative controls had a negative IFN- γ response to stimulation with PRRSV throughout the experiment (not shown). As a positive control of the assay, PBMCs isolated from two pigs (nos. 231 and 153) that had been infected 2 years earlier with the same strain of PRRSV were tested simultaneously at each time point. Representative data for pig 231 are shown (bottom row). Cells from these positive control pigs were plated at 2.5×10^5 per well and stimulated with PRRSV as before. As a positive control for the functionality of the isolated cells, 5×10^4 PBMCs per well from each sample were plated at every time point tested and stimulated with the T-cell mitogen phytohemagglutinin (PHA). A representative PHA response from each pig is shown in the right column. A gradual increase in the frequency and intensity in the IFN- γ spots generated by the PBMCs from infected pigs can be appreciated. The consistency and quality of the ELISPOT test at each time point can be judged from the bottom row (pig 231) and the PHA response from each of the samples.

1998a,b). Although the exact immune mechanisms responsible for protection against PRRSV infection are unknown, it would be expected that a similar association regarding immunity to PRRSV exists. This concept is supported by the demonstration that pretreatment of target cells with IFN- γ increases their resistance to PRRSV infection (Bautista and Molitor 1999; Rowland et al. 2001). Moreover, convalescent sows exposed to a secondary infection of PRRSV at 5 to 6 months after the primary one did not exhibit disease symptoms (Lager and Ackermann. 1994). Based on the results presented

here, this point of time after virus infection would correspond to the ascent of cellular immunity and the decline of humoral immunity, especially that associated with antibodies unable to neutralize the virus.

If indeed the inadequate stimulation of the generation of IFN- γ -secreting cells and virus-neutralizing antibodies by PRRSV is a principal cause of the suboptimal performance of the MLV vaccine under experimental conditions (Osorio et al. 1998) and field conditions (Lager and Mengeling 1997; Mengeling et al. 1997), then an enhancement of the immunogenicity of the vaccine

should be beneficial. In view of our failure to accomplish this via the inclusion of an adjuvant proven effective in conjunction with a PRV vaccine (Meier et al. 2002), it is likely that PRRSV has an intrinsic property responsible for the protracted development of protective immunity. One possible mechanism for this "delaying tactic" would be modulation of cytokine production. In this regard, the apparent frequency of PRRSV-specific IFN- γ -secreting cells, as determined by using the ELISPOT assay, can be increased nearly twofold by exposure of the lymphocytes to the T-helper-1 response-promoting cytokine, interleukin 12, or antibody capable of inhibiting the T-helper-2 response-promoting activity of interleukin 10. Similarly, inclusion of exogenous interleukin 10 resulted in a decrease in the apparent frequency of PRRSV-specific IFN- γ -secreting cells. An alternative, but not mutually exclusive, possibility is that the virus induces the elimination of antigen-presenting cells in lymphoid tissues. Indeed, PRRSV has been implicated in the destruction of lymphoid tissue either by directly causing the lysis of infected cells or by indirectly inducing apoptosis (Sirinarumit et al. 1998; Sur et al. 1998). To resolve this issue, we are currently examining the potential effect of PRRSV on accessory cell function and antigen presentation as well as on the regulation of the porcine immune response by cytokines *in vivo*. Such information is essential for the development and formulation of a highly immunogenic PRRSV vaccine.

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10.6

Bacterial Infections Are Potentiated by Porcine Reproductive and Respiratory Syndrome Virus Infection: Fact or Fiction?

Joaquim Segalés and Monte B. McCaw

SUMMARY

Since 1987, clinicians and scientists have consistently observed an increase in bacterial diseases in conjunction with porcine reproductive and respiratory syndrome virus (PRRSV) outbreaks. Most early studies attempting to demonstrate the potentiation of secondary disease by PRRSV infection used a common experimental design: PRRSV inoculation of a weaned pig, followed by challenge 5 to 10 days later with any of a number of bacterial pathogens. Most of these experiments were unsuccessful. On the other hand, switching the order of inoculation, i.e., pathogenic bacteria followed by PRRSV, reproduced the additive disease effects observed in the field. Furthermore, alternate models of clinical disease, such as in utero infection, have shown that PRRSV can cause lesions in lymphoid tissues of piglets and produce alterations in peripheral blood leukocyte populations and leukocyte function suggestive of an immunosuppressive effect. These observed changes may explain why bacterial infections cause much more severe disease in PRRSV-infected pigs. Concurrent infection with other potentially immunosuppressive agents, such as porcine circovirus type 2, may aid, or be necessary for, the expected increase in susceptibility to disease following PRRSV infection under field conditions.

INTRODUCTION

Respiratory diseases are the most common and costly diseases in nursery and finisher pigs (Straw 1992). On the one hand, a multitude of microorganisms have the ability to cause or exacerbate respiratory disease in swine (Done et al. 1993; Halbur et al. 1993). On the other hand, adoption of intensive swine production methods and facilities likely has contributed to the development of res-

piratory disease complexes. These complexes are comprised of multiple microorganisms acting together rather than individually, as in the past. Currently, the most important of these complexes is porcine respiratory disease complex (PRDC) of finishing pigs, which involves at least porcine reproductive and respiratory syndrome virus (PRRSV) and *Mycoplasma hyopneumoniae* (Dee 1996).

FACTORS THAT POTENTIATE BACTERIAL DISEASE IN SWINE

Only some of the porcine reproductive and respiratory syndrome virus (PRRSV)-respiratory pathogen combinations that cause disease in the field have been recreated and studied experimentally. One organism may aid the others through direct synergistic effects on mucosal barriers, by affecting the immune system, and/or by initiating the release of cytokines or chemokines during an inflammatory response.

Some respiratory viruses of swine may potentiate bacterial infections, thereby causing disease that the pigs could otherwise resist. Classical viral-bacterial disease complexes of swine are summarized in Table 10.6.1. Environmental factors such as chilling or ammonia concentrations in the air can increase a pig's susceptibility to bacterial diseases, and bacterial infections or their products can facilitate infection and respiratory disease by other bacteria.

Researchers have found that potentiation of bacterial infection via concurrent viral infection may occur by many different mechanisms (Galina 1995):

1. Aid bacterial adherence.
2. Inhibit chemotaxis.
3. Damage mucociliary clearance mechanism of respiratory tract.

Table 10.6.1. Virus and bacteria respiratory disease complexes reproduced under experimental conditions

Virus	Bacteria	References
Influenza	<i>Haemophilus suis</i>	Shope 1931
Adenovirus	<i>Mycoplasma hyopneumoniae</i>	Kasza et al. 1969
Classical swine fever	<i>Pasteurella multocida</i> type A	Pijoan and Ochoa 1978
Aujeszky's disease	<i>Pasteurella multocida</i> type A	Fuentes and Pijoan 1987
Aujeszky's disease	<i>Actinobacillus pleuropneumoniae</i>	Ramirez 1990
Aujeszky's disease	<i>Streptococcus suis</i> type II	Iglesias et al. 1992
Aujeszky's disease	<i>Haemophilus parasuis</i> (serovar 4)	Narita et al. 1994

4. Interfere with function of alveolar and pulmonary intravascular macrophages.
5. Interactions between the virus and host immunity:
 - a. The more virulent the virus, the greater the suppression of antibacterial defense mechanisms.
 - b. Host immunity against the virus decreases its ability to address secondary bacterial infections.
 - c. Host antiviral immune responses often do not protect against heterologous virus infection and therefore do not decrease secondary bacterial infection rates.

Documented examples of primary bacterial infections aiding the development of respiratory disease caused by another bacteria include

1. *Bordetella bronchiseptica* infection facilitated *Pasteurella multocida* type D ability to cause atrophic rhinitis (De Jong 1992).
2. *Bordetella bronchiseptica* infection increased the occurrence of suppurative meningitis caused by *Streptococcus suis* type 2 (Vecht et al. 1989).
3. *Mycoplasma hyopneumoniae* infection aided the development of pneumonia caused by *Pasteurella multocida* type A (Ciprian et al. 1988).
4. *Mycoplasma hyopneumoniae* infection increased the development of pleuritis and pneumonia caused by *Actinobacillus pleuropneumoniae* (Yagihashi et al. 1984)

DOES PRRSV INFECTION FACILITATE SECONDARY BACTERIAL DISEASE?

Since PRRSV appeared in 1987, clinicians have consistently observed an increase in a variety of bacterial diseases associated with PRRS outbreaks. These PRRSV and bacterial disease complexes may last from 6 months to 2 years in affected herds (Pijoan et al. 1994). Reports from

veterinary diagnostic laboratories support the perception that PRRSV potentiates diseases caused by infection with other swine pathogens. The Iowa State University Veterinary Diagnostic Laboratory reported 385 confirmed cases of PRRSV infection during 1994; in 85 cases (48%), other pathogenic viruses or bacteria were also isolated (Halbur et al. 1995a). Similarly, from 221 confirmed cases of PRRS at the South Dakota State University Veterinary Diagnostic Laboratory, 133 (60.2%) had concurrent pulmonary infections with other pathogenic bacteria or viruses (Zeman 1996). As a result of these clinical and diagnostic observations, the international veterinary community claimed that PRRSV infection was immunosuppressive (Drew 2000).

Early experimental or pathological studies suggested that PRRSV infection may be either immunosuppressive or induce immunomodulation (Done and Paton 1995; Molitor et al. 1992):

1. PRRSV replicated in monocytes and macrophages.
2. PRRSV-infected pigs had a marked decrease in alveolar macrophage numbers in bronchiolar lavage fluid 7 days after infection.
3. PRRSV decreased superoxide production following stimulation of infected macrophages.
4. PRRSV infection induced nasal mucosa inflammation and necrosis.
5. PRRSV infection caused transient leukopenia.
6. PRRSV-infected pigs had an enhanced antibody response against Aujeszky's disease virus (pseudorabies) and *Brucella abortus* following immunization versus uninfected controls.
7. PRRSV-infected pigs displayed an enhanced delayed-hypersensitivity response to dinitrofluorobenzene.
8. Experimental infection with a European strain of PRRSV followed by challenge with a virulent strain of *Streptococcus suis* 5 days later resulted in a significantly higher rate of suppurative meningitis versus bacteria-only infection controls.

Most early studies attempting to demonstrate potentiation of secondary disease by PRRSV infection followed a similar experimental design: PRRSV inoculation of weaned pigs followed by challenge 5 to 10 days later with any of a variety of bacterial pathogens. However, rather than confirming the field observations of enhancement of bacterial disease, most of the experiments were unsuccessful in demonstrating a potentiation of disease by PRRSV preinfection. Experimental studies that examined the effects of PRRSV preinfection and other pathogenic agents are summarized in Table 10.6.2. Variations in relative virulence among PRRSV isolates appeared to occur in the field (Halbur et al. 1995b). These same variations in virulence may explain why many failed to recreate the desired clinical syndrome following challenge of experimentally infected pigs, particularly if the isolates of PRRSV used actually had relatively mild virulence. Successful reproduction of *Streptococcus suis* meningitis and death following intranasal challenge of PRRSV-infected weaned pigs has been achieved using the highly virulent Iowa State University PRRSV strain (VR-2385) (Thanawongnuwech et al. 2000).

ALTERNATE MODELS OF PRRSV INFECTION IN CLINICAL DISEASE

The majority of experimental models reported before 1998 were based on bacterial challenge after possible pulmonary immunosuppression caused by PRRSV infection. These attempts were generally unsuccessful in reproducing field observations and, therefore, may not have accurately recreated field conditions (Segalés 1996). Recent studies suggest alternative explanations for the

interactions between PRRSV and other pathogens in infected pigs. Studies on the interactions between PRRSV and *Mycoplasma hyopneumoniae* infections demonstrated that the bacterial infection potentiated the lesions resulting from PRRSV infection. Pigs infected with *Mycoplasma hyopneumoniae* alone had minimal lesions of pneumonia. Mycoplasma-infected pigs subsequently challenged with PRRSV had significantly increased lesions of interstitial pneumonia for a much longer time than did pigs infected with PRRSV alone (Thacker et al. 1999). When PRRSV challenge in weaned pigs is followed by infection with *Salmonella choleraesuis*, similar findings of an additive effect resulting in more severe signs of disease were observed. Pigs infected with *Salmonella choleraesuis* and then PRRSV showed signs of dyspnea, diarrhea, and decreased weight gain. However, severe disease, prolonged salmonella shedding, and increased mortality were not observed unless pigs were also given a 5-day regimen of dexamethasone, following the PRRSV challenge, to mimic the effects of stress (Wills et al. 2000). Therefore, the order of infection between pathogenic bacteria and PRRSV may be important in recreating the additive disease effects observed in the field.

Other studies based on in utero PRRSV infection models have found a profound impact on both viral persistence and the immune functions of live-born piglets. These piglets displayed a very prolonged viremia and had severe lesions in lungs and lymphoid tissues. PRRSV was detected by polymerase chain reaction up to 210 days of age in the serum of piglets born to sows inoculated intranasally at day 90 of gestation (Benfield et al. 1998). PRRSV has also been detected in tonsils and lymph nodes of these pigs for prolonged periods. Therefore, piglets surviving in utero infection could remain a

Table 10.6.2. Experimental studies on the association between porcine reproductive and respiratory syndrome virus (PRRSV) and secondary agents published prior to 1999

Bacterial/Viral Agent	Effect of PRRSV	Days After Inoculation ^a	References
<i>Streptococcus suis</i>	Increase in meningitis	5	Galina et al. 1994
	None	2 and 7	Cooper et al. 1995
<i>Pasteurella multocida</i>	None	2 and 7	Cooper et al. 1995
	None	7	Carvalho et al. 1997
<i>Haemophilus parasuis</i>	None	7	Cooper et al. 1995
	None	7	Solano et al. 1997
<i>Salmonella choleraesuis</i>	None	7	Cooper et al. 1995
<i>Actinobacillus pleuropneumoniae</i>	None	7	Pol et al. 1997
<i>Mycoplasma hyorhinis</i>	Increase in polyserositis ^b	5	Kubo et al. 1995
<i>M. hyoneumoniae</i>	None	7	Van Alstine et al. 1996
Classical swine fever virus	None	3	Depner et al. 1999
Porcine respiratory coronavirus	Mild increase in clinical signs	3	Van Reeth et al. 1996
Influenza virus	Mild increase in clinical signs	3	Van Reeth et al. 1996
	None	7	Pol et al. 1997
Transmissible gastroenteritis virus	None	14	Wesley et al. 1998

^a Days after inoculation with PRRSV.

^b Very few pigs per group were used in this experiment.

risk for infection of pen mates throughout the nursery and finishing phases of production. The critical question is whether, under field conditions, individual pigs infected with PRRSV in utero could carry the virus into boar studs or breeding herds. This possibility remains to be explored.

In utero infection by PRRSV may result in profound immunosuppression in the surviving piglets. These piglets have severe lesions in various lymphoid tissues, including the thymus, lymph nodes, spleen, and bone marrow (Feng et al. 2001). Macroscopically, the weight of the thymus of in utero-infected pigs may be only one-half to one-third normal. Histologically, severe lymphocyte depletion was observed in the thymic cortex, resulting in the loss of the typical corticomedullary structure of the thymus. In the spleen, lesions were observed in the periarteriolar-associated lymphoid sheaths. Lymph nodes showed hypertrophy and hyperplasia, with necrosis and apoptosis of lymphocytes, and the presence of multinucleate giant cells, and hypoplasia was seen in bone marrow (Feng et al. 2001). At North Carolina State University, piglets infected with PRRSV in utero showed a reversal of peripheral blood CD4+/CD8+ lymphocyte ratios and significantly elevated levels of peripheral blood mononuclear cell expression of interleukin 6, interleukin 10, and interferon- γ at 0 and 14 days of age when compared with noninfected controls. Interleukin-12 levels were similar among infected and noninfected pigs, which resulted in a significant increase of the interleukin-10/interleukin-12 ratio (Feng 1999). In summary, the histopathological lesions, alterations in peripheral blood lymphocyte subpopulations, and cytokine expression profiles suggest that in utero PRRSV infection results in piglet immunosuppression.

Based on the previous supposition that in utero infection by PRRSV may be immunosuppressive, piglets were challenged intranasally with *Streptococcus suis* at 5 days of age. All piglets born to PRRSV-infected dams were viremic at 5 days of age. Of 22 in utero infected piglets, 20 (91%) infected with *Streptococcus suis* developed meningitis, whereas only 5 (22%) of 23 non-PRRSV-infected, *Streptococcus suis*-inoculated piglets developed meningeal inflammation. No meningitis was observed in the control group or in utero PRRSV-infected nonchallenged piglets (Feng et al. 2001). These data strongly suggested the possible immunosuppressive role of in utero PRRSV infection and its ability to potentiate the effect of secondary bacterial infections.

In utero PRRSV infection may play a role in post-weaning pig respiratory disease complexes observed in the field. If in utero PRRSV infection causes immunosuppression and enhanced susceptibility to secondary bacterial disease, this may explain why herds with reproductive PRRS or with asymptomatic circulation of the virus among the breeding stock often have severe respiratory disease complexes in their nursery and/or fatten-

ing pigs. *Management changes to reduce exposure to bacteria to eliminate losses* (McREBEL) is based on the assumption that piglets infected at birth with PRRSV were more susceptible to secondary bacterial disease (McCaw 2000). McREBEL was designed to minimize piglet exposure to high levels of bacteria caused by repeated cross-fostering between litters and excessive handling. McREBEL procedures have controlled secondary bacterial disease among suckling and weaned pigs during acute outbreaks of PRRS in large herds (McCaw 2000). The increased incidence of endemic bacterial disease in nurseries or fattening units may result from bacterial infections after maternal immunity against these agents has waned. Without either the protection of passive immunity or the ability to mount an adequate active immune response, in utero PRRSV-infected piglets would be unable to control bacterial infections that typically colonize pigs, but do not disseminate or result in disease. If this assumption is correct, from a practical standpoint, the cessation of PRRSV circulation among breeding stock is an essential factor for controlling nursery/finisher pig PRRS. This assumption is supported by field reports that depopulation of PRRSV-infected nurseries was unable to control the endemic disease problems until circulation of the virus had been stopped in the breeding herd (Dee et al. 1993; Keffaber et al. 1992).

Finally, simultaneous infection with PRRSV and other viruses may be necessary to suppress the pigs' immune system sufficiently to make them susceptible to secondary bacterial disease. A new virus, porcine circovirus type 2 (PCV2), has been associated with a new disease named postweaning multisystemic wasting syndrome (PMWS). It has been suggested that PMWS-affected pigs are also immunosuppressed (Segalés et al. 2000). PRRSV and PCV2 infections can occur concomitantly in swine herds (Segalés et al. 2002). The globally observed increase in secondary bacterial respiratory diseases initially attributed solely to PRRSV may also occasionally be a result of immunosuppression caused by dual virus infections, including PCV2.

PRRSV POTENTIATES SECONDARY BACTERIAL DISEASE: FACT OR FICTION?

The studies and reports discussed in this chapter suggest three general conclusions:

1. Bacterial challenge 5 to 10 days after experimental PRRSV inoculation of weaned pigs does not consistently cause disease. However, some highly pathogenic PRRSV strains are able to consistently potentiate secondary disease following bacterial challenge.
2. In utero infection causes lesions in lymphoid tissues of piglets and alterations in peripheral blood leukocyte populations and function that

may be compatible with the expected immunosuppressive effect of PRRSV infection. These observed changes may explain why *Streptococcus suis* infection caused much more severe disease in these pigs.

3. Concurrent infection with other potentially immunosuppressive agents may aid, or be necessary for, the observed increase in susceptibility to disease following PRRSV infection under field conditions. A global effort to more accurately detect all organisms involved in respiratory diseases of nursery and finishing pigs is necessary to determine which agents are truly potentiating secondary diseases.

Does PRRSV infection potentiate other agents that infect nursery or fattening pigs resulting in disease, as presumed by veterinarians and scientists? Is PRRSV actually only one of many agents acting simultaneously on pigs to result in disease? Many diagnosed cases of PRRS have concurrent respiratory pathogen infections, suggesting the resulting disease is multifactorial. Additionally, management practices and environmental conditions may play a significant role in the expression of disease following these infections. To determine which of the aforementioned factors are the initiators or which are the most important to control for the prevention of disease is extremely difficult, if not impossible, in individual cases of respiratory disease.

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10.7

Serological Associations Between Porcine Reproductive and Respiratory Syndrome Virus and Other Swine Pathogens in Mexico

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SUMMARY

It has been suggested that porcine reproductive and respiratory syndrome virus (PRRSV) enhances secondary infections in pigs by means of immunosuppression or immunomodulation. To investigate a possible association between PRRSV and other viral and bacterial pathogenic agents found in swine in Mexico, a serological model was followed. For this study, 3600 four- to six-month-old fatteners were bled and tested for antibodies against infectious various agents. As indicated by the odds ratio, it was found that pigs with antibodies against PRRSV were also more likely to be antibody positive for swine influenza virus, porcine respiratory coronavirus, and blue eye disease virus. In 1000 sows, PRRSV-infected animals were also more likely to have antibodies against swine influenza virus, blue eye disease virus, and Aujeszky's disease virus.

INTRODUCTION

Serological surveys done in swine in Mexico have demonstrated that porcine reproductive and respiratory syndrome virus (PRRSV) infection is widely spread, reaching up to 80% of the farrow-to-finish farms (Diosdado et al. 1997). Clinical signs in PRRSV-infected herds vary from none to severe outbreaks of respiratory and reproductive disease, with the isolation of numerous bacterial and viral swine pathogens. Therefore, it has been suggested that PRRSV infection causes immunosuppression or immunomodulation in swine that allows a higher multiplication of concurrent microorganisms (Drew 1999; Molitor 1994; Molitor et al. 1992).

PRRSV enters the body by the oronasal route, inhibits tracheal ciliary movement, and induces lysis of alveolar, intravascular, and interstitial macrophages and of dendritic cells of the upper respiratory tract and lungs, resulting in suppression of interferon- α production in the lungs (Albina et al. 1998; Park et al. 1996; Thanawongnuwech et al. 2000). Viremia occurs by 6 to 12 hours after infection and, in sows, the virus may reach the placenta, inducing abortions, premature farrowing, and increased levels of stillbirths, mummies, and mortality during the suckling period, due to agalactia and the birth of weak piglets.

Particularly in the respiratory tract, the pathogenesis of PRRSV may predispose animals to infection with other respiratory pathogens. In fact, more severe clinical disease and growth retardation has been demonstrated under experimental or field conditions from dual infection with PRRSV, swine influenza virus (SIV), or porcine respiratory coronavirus (PRCV) (Kay et al. 1994; Pol et al. 1997; Van Reeth et al. 1996). Concurrent infection with PRRSV and blue eye disease virus (BEDV) has not been studied, but there was not an apparent synergism between PRRSV and a porcine paramyxovirus isolated in Germany (Groschup et al. 1993).

Synergism between PRRSV and respiratory bacteria has been studied by a number of investigators. Thacker et al. (1999) reported that the chronic inflammatory response elicited during infection by *Mycoplasma hyopneumoniae* increased the severity and duration of PRRSV-induced pneumonia, but this effect has not been demonstrated in all experiments (Van Alstine et al. 1996). Stevenson et al. (1993) reported that nursery mortality due to salmonellosis increased during PRRS outbreaks in the field. Experimentally, when pigs were infected with *Salmonella*

choleraesuis, PRRSV, and chemically stressed with dexamethasone, a severe disease resulted characterized by unthriftiness, rough hair coat, dyspnea, diarrhea, and death. Wills et al. (2000) concluded that the severity of clinical signs during PRRS outbreaks was the result of interactions among concurrent infections and stressors. Experimentally, Galina et al. (1994) and Thanawongnuwech et al. (2000) found that PRRSV infection predisposed pigs to infection and disease caused by virulent strains of *Streptococcus suis* serotype 2, but other investigators did not observe this effect when infecting animals with *Streptococcus suis* or *Haemophilus parasuis* (Cooper et al. 1995; Segales et al. 1999; Solano et al. 1997).

Regarding the interaction of PRRSV with other viral infections, it was reported that PRRSV did not potentiate a classical swine fever virus (CSFV) infection in pigs (Depner et al. 1999) or increase the severity of transmissible gastroenteritis virus (TGEV) infections (Wesley et al. 1998). Recently, it was reported that proliferative necrotizing pneumonia was the result of coinfection between PRRSV and porcine circovirus type 2 (Pesch et al. 2000).

In addition to experimental and field studies, serology has been used to demonstrate a possible interaction among different microorganisms. Groschup et al. (1993) and Diosdado et al. (1999) found that finishers with higher levels of antibodies against PRRSV had also higher levels of antibodies against PRCV and SIV.

STUDIES IN MEXICO

To investigate a possible association between PRRSV and other pathogenic viral and bacterial microorganisms in swine in Mexico, the model described by Groschup et al. (1993) was followed. To conduct this study, a serological survey was done on 240 farrow-to-finish farms, sampling 15 pigs from 4 to 6 months of age from each farm and collecting a total of 3600 sera. In addition, sows were sampled on 100 farrow-to-finish farms. Ten sows from each farm were sampled, for a total of 1000 serum samples. The serum samples were tested in the following serological assays:

- 1. *Actinobacillus pleuropneumoniae* (APP): Slide agglutination test with serotype 1 antigen (Pleurotest; Ciprolab, Mexico).
- 2. Aujeszky's disease (pseudorabies) virus (ADV): Enzyme-linked immunosorbent assay (ELISA) (HerdChek PRV gpI Antibody Test Kit; IDEXX Laboratories, Westbrook, ME, USA).
- 3. Blue eye disease virus: Hemagglutination inhibition (HI) test.
- 4. *Leptospira interrogans*: Microagglutination test with 11 serovars.
- 5. *Mycoplasma hyopneumoniae* (Mhyo): ELISA (Chekit Hyoptest; Dr. Bommeli AG, Liebefeld-Bern, Switzerland).

- 6. Porcine parvovirus (PPV): Hemagglutination inhibition (HI) test.
- 7. PRRSV: ELISA (HerdChek PRRSV Antibody Test Kit; IDEXX).
- 8. *Salmonella*: Slide agglutination test with *S. choleraesuis* and *S. typhimurium* antigens.
- 9. Swine influenza virus: Double-diffusion test with group A nucleocapsid antigen.
- 10. Transmissible gastroenteritis virus and porcine respiratory coronavirus: ELISA (Svanovir TGEV/PRCV-Ab; Svanova Biotech, Uppsala, Sweden).

The association between PRRSV and other microorganisms was evaluated by calculating the odds ratio (OR) and 95% confidence interval (CI) (Groschup et al. 1993; Thrusfield 1995). In finishers, there was a significant association between PRRSV and antibodies against the following agents:

BEDV	OR = 2.95 (95% CI 1.16, 7.50)
PRCV	OR = 9.00 (95% CI 1.22, 82.53)
SIV	OR = 5.80 (95% CI 2.38, 14.55)

In finishers, no significant association was found between PRRSV and antibodies against the following agents:

ADV	OR = 1.95 (95% CI 0.97, 3.94)
APP	OR = 1.03 (95% CI 0.88, 1.20)
Mhyo	OR = 1.17 (95% CI 0.96, 1.43)
<i>Salmonella</i>	OR = 1.10 (95% CI 0.97, 1.24)

In sows, there was a significant association between PRRSV and antibodies against the following agents:

ADV	OR = 2.28 (95% CI 1.64, 3.18)
BEDV	OR = 1.86 (95% CI 1.16, 3.00)
SIV	OR = 3.54 (95% CI 1.29, 10.20)

In sows, no significant association was found between PRRSV and antibodies against the following *Leptospira* agents:

PRCV	OR = 2.14 (95% CI 0.68, 6.97)
TGEV	OR = 0.64
<i>L. bratislava</i>	OR = 1.30 (95% CI 0.93, 1.82)
<i>L. canicola</i>	OR = 0.95
<i>L. grippotyphosa</i>	OR = 1.48 (95% CI 0.18, 32.70)
<i>L. hardjo</i>	OR = 1.05 (95% CI 0.53, 2.12)
<i>L. hebdomadis</i>	OR = 2.97 (95% CI 0.40, 61.59)
<i>L. icterohaemorrhagiae</i>	OR = 1.37 (95% CI 0.95, 1.97)
<i>L. panama</i>	OR = 1.33 (95% CI 0.87, 2.02)
<i>L. pomona</i>	OR = 0.86 (95% CI 0.55, 1.34)
<i>L. pyrogenes</i>	OR = 0.37
<i>L. tarassovi</i>	OR = 1.24 (95% CI 0.75, 2.06)
<i>L. wolffi</i>	OR = 1.54 (95% CI 0.79, 3.06)

An association between PRRSV and parvovirus could not be evaluated because all sows had antibodies against PPV. However, the levels of parvovirus or BEDV HI antibody titers were not statistically different between PRRSV-positive and PRRSV-negative sows.

The serological survey was done in fatteners and sows separately because the former usually suffer acute infections whereas sows are endemically infected. This results in different levels of infection and immunity.

Due to the constant mixing of animals during weaning, grower, and finishing periods, fatteners by 4 to 6 months of age have developed antibodies against pathogens. The serological association between PRRSV and SIV and PRCV has been reported elsewhere (Done and Paton 1995; Groschup et al. 1993). This suggested that infection of the respiratory tract by PRRSV predisposed pigs to secondary respiratory viral infections. BEDV also induces respiratory clinical signs in pigs, so it was interesting to find that there was also a positive association with PRRSV. These results might be due to a transient inhibition of the respiratory tract defenses and the lack of interferon production, thereby allowing the replication of other respiratory viruses in the herd (Albina et al. 1998). Groschup et al. (1993) did not find a serological association with a porcine paramyxovirus isolated in Germany, but this may be due to the lack of pathogenicity of the German isolate compared with Mexican BEDV strains. In fact, BED has been mistakenly diagnosed as PRRSV infection due to similarities in clinical signs.

An association with antibodies against respiratory bacteria was not observed, which may indicate the absence of an interaction or may reflect differences in sensitivity and specificity of the serological tests. For instance, the *Salmonella* antigen used in the assay detected antibodies against *Salmonella* spp. and not specifically to pathogenic *S. choleraesuis* or *S. typhimurium*.

The sow population was tested because it represents the primary source of pathogenic microorganisms on the farm. Significant associations with respiratory viruses (SIV, BEDV, and ADV) were found. In contrast, no association was detected between PRRSV infection and infection with PPV, TGEV, PRCV, or any of the leptospiral serovars. PRRSV-infected animals did not have a higher level of antibodies to TGEV relative to PRRSV-negative animals, as Wesley et al. (1998) had reported.

It has been suggested that the serological association might result from a PRRSV-induced nonspecific polyclonal B stimulation enhancing the humoral response to infection with other agents or vaccinal antigens (Vezina et al. 1996). However, PPV or BEDV antibody titers were not higher in PRRSV-positive as compared with PRRSV-negative sows, indicating that this was not a factor.

CONCLUSIONS

It was concluded that finishers and sows with PRRSV antibodies also more frequently had antibodies against respiratory viral pathogens. Since there was no evidence of an enhancement of antibody titers against parvovirus or BEDV in PRRS-seropositive animals, this probably reflects differences in infection rates rather than PRRSV-induced enhancement of the humoral response.

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10.8

Control of Porcine Reproductive and Respiratory Syndrome in Large Systems: Strategies for the Future

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SUMMARY

Porcine reproductive and respiratory syndrome virus (PRRSV) was identified just over a decade ago. Even so, basic PRRSV information, particularly in the area of immunity and transmission, is conspicuous by its absence. Controlling clinical cases in commercial production systems is a constant problem for producers and veterinarians. Although vaccines are available, the protection they confer is inconsistent. In this chapter, we discuss strategies for controlling PRRS, with an emphasis on methods for establishing and maintaining herd immunity.

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) has been creating havoc in swine production for over a decade, yet there is still little consensus among producers and veterinarians regarding control procedures in production systems. The confusion stems from the lack of concrete information in two areas: reproductive and respiratory syndrome virus (PRRSV) transmission and protective immunity.

At the time of this writing, the US swine industry has four commercial vaccines, but no one has yet described how PRRSV, by either field infection or vaccination, elicits protective immunity. Furthermore, there is no practical way to evaluate cross-protection between the modified-live virus (MLV) vaccines and heterologous field strains of PRRSV. Vaccine has been used extensively in some swine production systems, but with mixed results. Some systems report good results; others have blamed vaccines for creating even more severe disease problems. These highly dissimilar

results suggest one of two possibilities: either a lack of cross-protection or no protection at all. If vaccines were cross-protective, many herds should have improved dramatically when vaccinated. Since the problems the industry has had with PRRS have continued in the face of multiple vaccinations, it is easy to conclude that, in many cases, the vaccine has not created heterologous protection; that is, if the field virus were antigenically similar to the vaccine strains, the MLV products would have created protection. In addition, there are many examples of farms returning to normal production without any vaccine use after severe breaks.

Therefore, the long-term stability of PRRSV-infected farms depends on consistent acclimation. *Acclimation* means preparing PRRSV-naïve gilts for entry into the sow herd through the development of active immunity against the virus. For farms to maintain long-term stability, it is important that all PRRSV-susceptible sows in the herd develop active immunity and that all replacements are immune prior to entry. The use of vaccine in these cases may actually create populations of sows that have not been exposed to the field strains within the herd. These unprotected populations can result in small rebreake that manifest as viremic pigs in the nursery. In some cases, vaccine may have been given credit for reducing the severity of subsequent breaks in the sow herd when there was actually a decrease in the susceptible population due to previous field-virus circulation.

The following description details what may have happened over the past decade in the industry and helps explain why problems have escalated in the last 5 years.

A CLINICIAN'S PERSPECTIVE ON CYCLIC OUTBREAKS OF PRRS

In the initial breaks, the rapid stabilization of the sow farm resulted from the rapid spread of the virus through an entirely susceptible population. If the original source of virus were incoming gilts, the continued introduction of these previously exposed, naturally protected replacements stopped the virus spread due to the absence of susceptible animals in the population. Ultimately, the stabilization of the sow herd depended on one of three things:

1. The entry of previously exposed, naturally protected gilts meant no susceptible population would redevelop.
2. The acclimation (infection) of PRRSV-naïve replacements using viremic nursery pigs maintained herd immunity.
3. The virus stopped circulating completely.

Over time, these stable farms would eventually produce PRRSV-negative pigs, but would then move these susceptible animals into positive nursery/finishing systems, where they would become infected. After a few years of unacceptable nursery/finishing performance, the industry implemented procedures to clean up the downstream pig flow. Among these methods were partial/complete depopulation (Dee and Joo 1994; Dee et al. 1997a,b), all-in/all-out facilities (Dee et al. 1993), and/or unidirectional pig flow (Dee and Phillips 1998). If the PRRSV-positive replacement gilt source were successful in eliminating PRRSV, the result was naïve, totally susceptible gilts entering the sow farm. The sow herd dynamics then began to change as the population started to differentiate into resistant and susceptible classes in regard to PRRS immunity. Some farms rolled through this phase and became PRRSV negative. Other herds, after an uncertain length of time or number of naïve animal introductions, began to recirculate virus and eventually rebroke with classical, clinical PRRS. There were even rebreaks in cases where systems believed they were properly acclimating gilts by exposure to infected sows or nursery pigs. This was because the gilts they received were already positive, but to a heterologous strain of PRRSV. These gilts were susceptible to the *resident* PRRSV strain, but the acclimation process failed, perhaps because the contact sows or nursery pigs to which they were exposed were no longer shedding virus. The use of MLV vaccines confused the issue even more by creating antibodies that could not be differentiated from field virus. Therefore, seropositivity or seroconversion to PRRSV could not be used to confirm exposure to field virus during acclimation.

Some of these farms ended up in cycles: clinical outbreaks on the sow farm prompted procedures to acclimate gilts, which led to a clinically quiet phase, the

production of negative pigs, and depopulation in nursery/ finishing stages. Success in controlling PRRSV circulation led to the introduction of PRRSV-naïve animals into the sow herd, where the cycle eventually started over again. These cycles revolved around the absence of homologous protection against the PRRSV strain endemic in the herd, which, in turn, was based on two factors: the use of modified-live PRRSV vaccine, and all-in/all-out gilt acclimation.

METHODS OF CONTROL OF PRRS IN SWINE UNITS

A few definitions are necessary before discussing an appropriate system for control of PRRS in large sow units. For simplicity, only gilt sources are discussed, but the same concepts apply to boars. For clarity, *PRRSV naïve* indicates that animals have never been infected with either field strain or vaccine strain of PRRSV. In contrast, *PRRSV negative* is usually defined by serum antibody status. It is not uncommon for infected or vaccinated animals to eventually test negative by serology.

Incoming animals can be put into two categories as derived by source: PRRSV-naïve sow farms or positive sow farms. Naïve sow farms, by definition, produce PRRSV-naïve replacement gilts. These gilts may be going to either PRRSV-positive or naïve commercial farms. If entering naïve herds, these animals only need to be isolated; there is no need for off-site acclimation. If these animals are going to a PRRSV-positive sow farm, they must first be acclimated to a PRRSV homologous to the virus endemic on the farm before entering the sow herd. It is essential that these animals match the status of the sow herd before introduction.

The second category is animal sourcing from positive sow farms. Individual animals from these farms may be PRRSV naïve or PRRSV positive, depending on the production system and the success of the source sow farm's PRRS stabilization protocol. It is important to understand that the same principles applied at the commercial level to create negative nursery/finisher populations are occurring upstream at the source farm. The success of their program and the consistency of that success are paramount to the success of the acclimation procedures in place in the commercial herd.

With these definitions in mind, we can center the discussion on introducing animals into recipient sow farms.

The ideal procedure is to receive naïve replacements from naïve source farms. This allows the option of acclimating the replacements to specific sow herds and their farm-specific strains of PRRSV. It also decreases the chance of clinical breaks resulting from the introduction of a different PRRSV into the herd or of a new virus emerging through recombination of PRRSV strains. The changes due to recombination may be much more dangerous than changes due to mutation.

When naive gilts are to be introduced to a positive sow farm, they must first develop immunity to the resident strain of PRRSV. This is the point at which differences in opinion arise. A procedure that has been widely publicized involves the use of a commercially available PRRS vaccine in combination with cull-animal exposure in all-in/all-out off-site facilities to acclimate incoming animals. The acceptance of this procedure has varied depending on past experience and perceived success. The system that will be described in this chapter is quite different. This system is based on two simple premises: (1) field-virus infection results in effective long-term protection, and (2) successful PRRS acclimation will decrease the amount of virus circulating on the sow farm.

If acclimation runs all-in/all-out and relies on exposure to cull sows to introduce virus to each group, naive gilts will eventually be put into the sow farm because cull sows will not consistently shed and/or transmit virus. If gilts are vaccinated upon entry to the isolation/acclimation site, it will be impossible to know if field-virus exposure has occurred. Previous experiences suggest that commercial vaccines have not been completely cross-protective (Benson et al. 2000). Without the ability to determine definitively whether field-virus infection has occurred, it is questionable whether successful PRRS acclimation has occurred.

When introducing naive gilts into positive farms, the goal is exposure to a homologous field virus in an off-site acclimation facility. For the purposes of this chapter, the definition of acclimation is infection of a susceptible animal with a specific disease organism.

Natural infection and recovery is the surest way to elicit protection against PRRSV and can be accomplished by one of three methods: (1) exposure of pigs to viremic cull sows or nursery pigs, (2) adding naive pigs to a PRRSV-infected, continuous pig flow, or (3) injection of live virus into the pigs.

In the first method, the long-term risk is that shedding of PRRSV on the sow farm eventually stops, thereby resulting in acclimation failure. Continuous-flow acclimation is one way to address this eventuality. PRRSV will continue to circulate in the population as long as susceptible gilts are continually introduced, but maintaining virus circulation is a function of population size, mixing procedures, and gilt introduction interval. Of these three, introduction interval is likely the most significant factor. It is critical that the virus continues to circulate and successfully infects the next group. If too much time is left between introductions of gilts, the virus may stop circulating and populations of naive gilts will again enter the sow herd. In practice, the clinical disease experienced during acclimation is least severe in 10- to 14-week-old pigs. Vaccination programs for the common secondary infections can minimize the clinical aspects of these diseases. It is important to have at least 90 to 120

days of off-site isolation/acclimation in these continuous-flow systems to allow for complete recovery from PRRSV.

A theoretical concern with continuous-flow acclimation is viral mutation; that is, over time the virus goes through many replications. This increases the chance for mutations that may create instability in the sow herd due to cross-protection failure. In addition, it is impossible to control all of the other infectious diseases circulating in the continuous-flow population well enough to avoid increases in mortality and culling rates. Over time, some of these systems will become progressively worse and partial/complete depopulation may be necessary. If an acclimation/isolation site is to be depopulated, it is important to maintain a source of virus to restart PRRSV circulation. This can be accomplished by harvesting serum from viremic pigs and later injecting it into naive gilts or by leaving a few viremic pigs in the facility. Blood testing is required to confirm exposure and infection when acclimation is restarted.

The last method deals with intentionally exposing naive gilts to live virus by injection. This is equivalent to using a live autogenous vaccine. This procedure may be necessary to allow sufficient "cool down" after infection if isolation/acclimation is limited to 60 days. In continuous-flow systems, it may require 3 weeks to expose every pig to virus, whereas with injection the cool-down period starts immediately. Other advantages of this system include control of the dose and exact time of infection. If this system is used in conjunction with all-in/all-out pig flow, exposure to other pathogenic microorganisms can be reduced, which leads to decreased mortality and culling. This procedure also requires less testing to confirm infection of the entire population because a few PRRSV-positive gilts are sufficient to establish that the inoculum was infectious at the time of injection. It should be acknowledged, however, that there are ethical and safety issues concerning this method that should be discussed prior to its implementation.

Regardless of the method employed, the ultimate goal of acclimation is to get naive replacement gilts infected and consequently protected against an endemic PRRSV prior to entry into a positive sow farm. Acclimation needs to consistently achieve the goal of infection/protection now and well into the future.

What if the gilt source is a positive sow farm? All replacements from PRRSV-positive source farms should be viewed as positive gilts until proven otherwise. It is true that groups of gilts from positive farms are PRRSV naive, but farms do rebreak. What does this mean to the recipient sow farm? It means that if the stability of the source farm is disrupted, virus may find its way downstream to your farm. This virus will not create significant clinical problems as long as it is sufficiently similar to the PRRSV that infected your farm and susceptible sow populations have not developed due to failure to acclimate naive gilts.

It is risky to receive naive replacements from a positive source farm if that farm was not the original source of your farm's PRRSV—someday the gilts may not truly be naive due to a breakdown in the source farm's stability. If this strain of PRRSV is sufficiently different, the result will be instability in the recipient herd. Naive gilts from positive sow farms need to be isolated and tested to prove they are actually PRRSV naive. After their naive status has been confirmed, they must be exposed to a homologous virus and prepared for entry into the sow herd. This program may consist of isolating weaned pigs in a nursery and, if they test negative (naive) at 10 to 12 weeks of age, proceeding to acclimate them. The quality of diagnostic testing techniques available today determines the risk of receiving these animals. If the original gilt source sends positive and negative groups over time, as long as the virus is similar and continues to circulate in acclimation there should not be a problem.

Acclimation may not be needed if the source farm was the original and only source of your PRRSV and it continues to allow PRRSV to circulate through the finishing phase. Acclimation may consist only of a cool-down period of 30 to 60 days to decrease the risk of introducing a large level of virus into the sow farm. This system worked well until source farms started implementing techniques to create PRRS-negative nursery/finishers—which then become your replacement gilts. Their reasoning was perfectly understandable when looking at nursery/finishing performance, but it created instability problems on the recipient commercial farms. This instability was due to a change in replacement gilt status from infected/protected to PRRSV naive and the recipient farms failure to adjust to the changing gilt status and take measures to assure acclimation.

In summary, receiving gilts into positive farms creates two challenges. First, naive gilts must be acclimated to the resident PRRSV. Second, if the gilts are PRRSV positive, the virus to which they were exposed must be the same as the virus endemic in the recipient herd to assure protection. Sufficient cool-down time is important to allow the virus load to decrease. If the gilt source was not the original source of the endemic PRRSV, additional problems may arise due to inadequate cross-protection. Receipt of seropositive gilts makes it impossible to determine whether farm-specific PRRSV exposure occurs.

CASE EXAMPLES

The following two cases contrast differing gilt acclimation procedures and provide evidence for the ideas described in this chapter.

Case 1

Gilts from PRRSV-positive sow farms were introduced to acclimation facilities every 5 weeks. Six different ages of

gilts were housed on the site, but in different barns. At 10 weeks of age, gilts were exposed to live virus via intramuscular injection. A random sample of gilt sera was obtained at the time of exposure, 6 weeks after exposure, and 16 weeks after exposure. In this fashion, 58 groups of gilts were monitored, and enzyme-linked immunosorbent assay mean sample-to-positive (S/P) ratios (Herd-Chek PRRSV Antibody Test Kit; IDEXX Laboratories, Westbrook, ME, USA) were as follows for the three bleedings: 1.41, 1.51, and 1.55.

Subsequently, the acclimation system was changed. The intramuscular live virus exposure was discontinued, and gilt flow on the site was modified so that PRRSV exposure occurred by housing animals for 10 weeks in a continuous-flow barn. After 10 weeks, like-aged gilts were brought together into one barn for a cool-down phase. As before, serum samples were obtained from 29 groups at the time of exposure, 6 weeks after exposure, and 16 weeks after exposure, and mean S/P ratios were, respectively, 1.21, 1.50, and 1.21. The lack of a decline in mean S/P value over time seen in the first acclimation method was believed to be less desirable than that seen in the second. The second acclimation method resulted in the production of PRRSV-negative groups of nursery pigs from these sow farms whereas the first method resulted in PRRSV circulation in the nursery phase. Subsequent, nursery production parameters as measured by average daily gain and mortality were improved under the second method.

Case 2

Two production systems (A and B), consisting of six farms each, used different gilt sourcing and acclimation procedures. System A used multiple sources of PRRSV-positive gilts. System B used PRRSV-naive gilts and acclimated them between 10 and 13 weeks of age to the farm-specific strains via older, previously exposed, seedler pigs. Gilts were introduced to the sow farms every 3 to 4 weeks in both systems. Serum samples from a random sample of 30 sows from each farm were collected bimonthly for seven periods over a year. Sequential S/P values for system A were 1.24, 1.22, 1.37, 1.05, 1.09, 1.20, and 1.25. Sequential results for system B were 1.48, 1.16, 1.22, 0.91, 0.89, 0.94, and 0.81. System A had PRRSV circulation in the nursery, as demonstrated by seroconversion, whereas system B did not. System B was characterized by the absence of abortion storms, whereas every farm in system A had an abortion storm at least once per year. Clearly, this evidence supports farm-specific acclimation of PRRSV-naive replacement gilts.

CONCLUSIONS

This overview is not intended to give answers to individual farm or system PRRS problems. The intent is to stimulate thinking and to challenge popular paradigms. The

following is a list of conclusions that may help formulate solutions for each case that is presented:

The purpose of acclimation is to infect animals with farm- or herd-specific virus and stimulate protective immunity. There are various methods to achieve this, but it should always be done separately and apart from the sow herd, regardless of the method used.

1. Replacements should originate from PRRSV-naïve source farms.
2. Immune protection against homologous field virus is excellent.
3. PRRSV infections in healthy 10- to 16-week-old pigs can be uneventful.
4. Infection/protection in acclimation must be measurable and continual.
5. Many groups of infected pigs coming from PRRSV-stable sow farms will show no clinical signs through the finishing phase.
6. Farms can stay endemically infected if PRRSV-naïve replacements are introduced directly into the sow herd. These farms typically have low farrowing rates, high sow mortality, elevated preweaning deaths, and an increased number of stillborns.
7. History suggests that stable, PRRSV-positive sow farms that introduce naïve replacements into the breeding herd eventually become clinical again without a satisfactory explanation for an external source of virus.
8. Introduction of animals carrying a PRRSV strain other than the one already endemic in the herd may lead to clinical expression of the disease.

We are continuously learning about this virus, but never quite fast enough. An important point is that information to solve specific farm or system problems may come from many sources, but the solutions will come from within.

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