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**Preliminary characterization of the transmission of porcine
reproductive and respiratory syndrome virus**

by

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**A Dissertation Submitted to the
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ABSTRACT

The objective of this dissertation is to provide a more complete characterization of the transmission of porcine reproductive and respiratory syndrome virus (PRRSV). Three papers are presented within the dissertation. The first paper focuses on modes of transmission between infected and susceptible animals. Portals of exit and duration of shedding of virus from infected animals are investigated in the second paper. The final paper concentrates on the occurrence and duration of infection in swine.

In the first study, five trials were conducted to study transmission of virus to pigs placed in different degrees of contact with inoculated pigs. The study demonstrated that although direct contact is probably the most efficient mode of transmission it was not required for transmission to occur. Transmission across distances of 102 cm were demonstrated. The failure of transmission to occur in pigs separated by relatively short distances questioned the role of aerosols in PRRSV transmission.

The second paper reports the results of 2 trials in which weekly samples were collected from inoculated and control pigs. While the pigs were anesthetized, serum, saliva, conjunctival swab, urine by cystocentesis, and feces were collected. Following anesthesia, the endotracheal tube was rinsed in saline and the rinse retained. All samples were assayed for

PRRSV. Virus was isolated through day 14 post inoculation (PI) from urine, day 21 PI from serum, day 35 PI from endotracheal tube rinse, and day 42 PI from saliva. No virus was recovered from conjunctival swabs or fecal samples. Recovery of PRRSV from saliva has not been reported previously. Virus-contaminated saliva, especially when considered in the context of social dominance behavior among pigs, probably plays an important role in PRRSV transmission.

In the third study, serum samples were collected from 4 inoculated pigs every 2 to 3 days until day 42 PI and then approximately every 14 days until day 213 PI. Oropharyngeal samples were collected at the time of serum collection on days 56 to 213 PI. Viremia continued up to 23 days. Persistent infection with PRRSV was demonstrated by isolation of virus from oropharyngeal samples for up to 157 days after challenge.

INTRODUCTION

Dissertation Organization

This dissertation begins with an abstract which provides a summary of the general objectives and conclusions. This is followed by a general review of the literature and a statement of the problem. The next 3 chapters are made up of papers submitted for publication. The last chapter of the dissertation is a general discussion summarizing the results presented in the 3 papers and discussing conclusions drawn from them. A list of references cited will be included at the end of each of the chapters.

Review of Literature

History of PRRS

Porcine reproductive and respiratory syndrome (PRRS) is a recently identified viral disease of swine. The first outbreaks of the disease were reported in 1987 (Keffaber, 1989). The disease had been reported in 11 states in the United States and 2 provinces of Canada by 1990 (Hill, 1990). In Europe, the syndrome was first recognized in Germany in 1990 (Leyk, 1991). As in the United States, the disease spread rapidly, appearing in Belgium (Varewyck, 1991), England (White, 1991), Holland (Wensvoort et al., 1991), and Spain (Plana et al., 1992) by 1991.

The disease was referred to by a variety of names prior to the identification of its etiologic agent. Reflecting the unknown etiology of the

disease, it was first known as Mystery Swine Disease (Dial et al., 1990). Other early names for the disease include disease '89, pig plague '89, SMEDI-like syndrome, and swine reproductive failure syndrome (Keffaber, 1989). As the clinical parameters of the disease became more defined, other names were suggested including swine infertility and respiratory syndrome (SIRS) (Hill, 1990). In Europe, it was referred to as abortus blauw, porcine epidemic abortion and respiratory syndrome (PEARS) (Terpstra et al., 1991), blue eared pig disease (Edwards et al., 1992), and Seuchenhafter Spätabort der Schweine (infectious late abortion of swine) (Justel, 1991). At the First International PRRS Symposium in 1992, it was agreed to adopt the name porcine reproductive and respiratory syndrome (PRRS) (Collins, 1992).

The etiologic agent of PRRS, first isolated and described in the Netherlands, was designated the Lelystad virus (Wensvoort et al., 1991). Confirmation that the virus was the cause of PRRS was achieved by experimentally reproducing clinical signs of reproductive disease and re-isolating the virus from sows inoculated with cell-propagated Lelystad virus (Terpstra et al., 1991). Isolation of a PRRS-producing virus in the United States was reported in 1992 (Collins et al., 1992). Clinical signs of PRRS were reproduced by inoculating sows with the virus, thus confirming its role in the etiology of the syndrome (Christianson et al., 1992). A case control study demonstrated a strong association between clinical diagnosis of PRRS

in a herd and seroconversion to porcine reproductive and respiratory syndrome virus (PRRSV) (Morrison et al., 1992). Isolation of the virus was later reported in France, Denmark, Taiwan, Canada, Germany, Great Britain, Spain, Japan, and Belgium (Baron et al., 1992; Bøtner et al., 1994; Chang et al., 1993; Dea et al., 1992; Ohlinger et al., 1991; Paton et al., 1992a; Plana et al., 1992; Shimizu et al., 1994; Vynckier and Pensaert, 1993)

Virus characterization

Lelystad virus was characterized as a non hemagglutinating, enveloped RNA virus only able to replicate *in vitro* on primary cultures of porcine alveolar macrophages (PAM) (Wensvoort et al., 1991). Further analysis revealed the virion was a 45-55 nm diameter particle containing a 30-35 nm nucleocapsid and having a buoyant density of 1.19 g/ml in cesium chloride. The virus isolated from swine in the United States was a fastidious, non hemagglutinating, enveloped RNA virus able to be propagated on a continuous cell line (Benfield et al., 1992). The virus was further characterized as a 62 nm pleomorphic, but predominantly spherical, virion containing a 25-35 nm core, and having a particle density of 1.18-1.19 g/ml in cesium chloride. Incubation at 37° C for 48 hours or 45 minutes at 56° C caused inactivation of the virus. Physical similarities, in conjunction with

comparable clinical presentation, suggested the European and North American viruses were the same or related viruses.

Following further characterization of PRRSV, investigators noted that it shared similarities with members of the newly proposed virus family Arteriviridae in morphology, structural proteins, nucleotide sequence, genomic size and organization, replication strategy, and predilection for replication in macrophages (Plagemann and Moennig, 1992; Meulenberg et al., 1993). The formation of this new family of viruses had been proposed because prior classifications did not adequately reflect the relatedness of lactate dehydrogenase-elevating virus (LDV), equine arteritis virus (EAV), and simian hemorrhagic fever virus (SHFV). Similarities in virion size and structure, size and polyadenylation of RNA, structural proteins, *in vivo* predilection for macrophages, growth cycles, and ability to establish asymptomatic persistent infections justified inclusion of these viruses in a new family. (Plagemann and Moennig, 1992).

Clinical presentation

The clinical signs expressed with PRRSV infection are dependent upon the previous exposure history of the herd, signalment of the infected animal, concurrent illnesses, and possibly, virus strain. Three forms of the disease have been described: epidemic, endemic, and subclinical (Van Alstine et al., 1993b). In acute outbreaks of the disease in breeding herds, sows exhibited

anorexia, fever, reproductive failure, and occasionally death. Reproductive symptoms seen over a 7- to 8-week period included abortion, premature births, stillbirths, birth of small weak pigs, delayed estrus cycles, poor conception rates, and an increase in the rate of mummified fetuses (Keffaber, 1989; Hill, 1990). Pre-weaning mortality increased due to the weak condition of the pigs at birth, decreased sow lactation, and possibly direct effects of the virus (Keffaber, 1989; Hill, 1990). The clinical presentation in boars is less clear; possibly because fewer mature boars have been studied than sows and younger pigs. Boars infected with PRRSV showed variable signs of disease including listlessness, inappetence, fever, mild respiratory disease, and decreased libido (Hopper et al., 1992; White, 1992; Benfield et al., 1993; Swenson et al., 1994a).

Although clinical signs of respiratory disease was sometimes observed in all ages of swine during an acute outbreak of PRRSV, the severity of clinical respiratory disease decreased with the age of the pig. Pigs in the nursery or still on the sow exhibited more severe dyspnea than growing and finishing pigs (Keffaber, 1989). In comparison with related viruses, clinical signs associated with acute outbreaks of PRRSV were perhaps most similar to those seen with EAV. Both reproductive and respiratory components of disease have also been identified with EAV (Horzinek et al., 1992; Timoney and McCollum, 1987).

Endemic infection of a herd, especially nursery age pigs, may follow the epidemic form of PRRSV infection. Although reproductive performance and suckling pig mortality returned to normal levels after an acute outbreak of PRRSV, virus was maintained in nursery pigs for more than 2.5 years (Stevenson et al., 1993). If uncomplicated by secondary agents, nursery and grower pigs in herds endemically infected with PRRSV exhibited mild respiratory disease, occasional eyelid edema, and enlarged lymph nodes (Van Alstine et al., 1993b). Production losses due to an increased incidence of secondary infections with *Haemophilus parasuis*, *Streptococcus suis*, *Salmonella choleraesuis*, *Pasteurella multocida*, and *Actinobacillus pleuropneumoniae* were associated with herds endemically infected with PRRSV (Joo and Dee, 1993). These losses took the form of reduced average daily gain, reduced feed efficiency, and increased post weaning mortality. In addition to nursery age pigs, endemic PRRSV may also affect the breeding herd. A field study showed that cyclic reproductive failure sometimes occurred in infected herds (Dee and Joo, 1994b).

The third form of clinical presentation of PRRSV infection is asymptomatic or subclinical. Serological studies have demonstrated that herds may be infected with PRRSV without displaying clinical signs of the syndrome (Hill et al., 1993). In one study, sentinel pigs were used to demonstrate the circulation of infectious virus in clinically normal pigs

(Bilodeau et al., 1994). On the same farm, a retrospective serological study demonstrated that grower pigs were serologically positive to PRRSV 9 months prior to the development of clinical reproductive disease in the herd. The potentially long separation in time between virus entry and development of clinical signs in a herd may make determination of the source of virus into a herd difficult or impossible.

The ability to produce subclinical infections is a common attribute of the Arteriviridae. Asymptomatic infection of horses with EAV was shown to occur (Horzinek et al., 1992; Timoney and McCollum, 1985). In the case of SHFV, the species of the host determines how infection with the virus is expressed clinically. Patas monkeys (*Erythrocebus patas*), and other African monkeys, baboons (*Papio papio*), and African green monkeys (*Cercopithecus aethiops*), infected with SHFV virus were identified as clinically unaffected carriers (London, 1977). In contrast, macaques, including rhesus monkeys (*Macaca mulatta*), stump-tailed macaque monkeys (*Macaca speciosa*), and cynomolgus monkeys (*Macaca irus*), were very susceptible to severe and usually fatal infections (London, 1977; Palmer et al., 1968). Infection of mice with LDV has been characterized as a life long persistent infection which produces no clinical signs in its host except for elevation of plasma lactate dehydrogenase (Rowson and Mahy, 1975).

The clinical description of PRRS in Europe was nearly identical to that seen in the United States, with only slight differences. Hyperemia, cyanosis, or necrosis of extremities, especially ears, were seen in a small proportion of acutely infected animals (de Jong et al., 1991; Hopper et al., 1992).

Diarrhea was a consistent finding in young pigs in Great Britain (Hopper et al. 1992). Finisher pigs infected with PRRSV did not exhibit clinical signs of PRRSV-specific pneumonia but rather expressed the clinical pattern associated with the predominating pneumotropic bacteria present in the herd (Blaha, 1993).

Economic impact

A clinical outbreak of PRRS impacts the financial health of a swine herd as well as its physical health. Acute outbreaks can be financially devastating in a naive herd. The estimated loss of potential profits ranged from \$50.00 to \$314.00 per sow, in addition to estimated fixed costs of \$50.00 to \$250.00 per sow, in a study of clinical disease in 4 swine herds (Hoefling, 1990). In a financial analysis of a farm undergoing an acute outbreak of PRRS, decreased liveborn litter size, increased preweaning mortality, and decreased farrowing rates were shown to cause a net reduction of 3.8 pigs/sow/year resulting in a \$236.00 loss of profit per inventoried female (Polson et al., 1990).

Even after reproductive losses return to normal values, the financial impact of PRRS on an endemically infected herd may be substantial. Losses result from increased treatment costs, increased disease due to secondary bacterial pathogens, increased death loss, decreased average daily gain, and an increase in numbers of underweight pigs (Keffaber et al., 1992). Budget modeling techniques used to estimate the financial impact of PRRSV infection of nursery-aged pigs estimated a loss of up to \$18.21 per pig, assuming PRRS episodes of 6 weeks duration with 12% death loss and 50% decrease in average daily gain (Polson et al., 1994). In a study of 91 herds diagnosed as having PRRS by a statistical analysis of herd performance data, the economic loss attributed to PRRS was £65 per sow per year or about 55% of pig farm income (Brouwer et al., 1994).

Diagnosis

Clinical signs

Clinical signs of late term reproductive failure in breeding stock followed by, or concurrent with, respiratory disease in neonates, nursery, and grower pigs provide the basis for a presumptive diagnosis of PRRSV infection in a herd. When using clinical signs as a means of diagnosis, the case definition is extremely important. Many of the clinical signs of PRRS are shared by other reproductive and respiratory diseases of swine. In a survey of swine practitioners investigating the incidence and prevalence of PRRS, the

following case definition was used to determine a diagnosis of PRRS: anorexia, fever, stillbirths, abortions and/or early farrowings, mummified fetuses, respiratory signs in young pigs, increased scours and mortality of nursery pigs, decreased growth rate of nursery pigs, and decreased breeding performance, with the entire clinical episode continuing for 2 to 4 months (Zimmerman, 1991). In efforts to control the spread of PRRS in Europe, and prior to the development of diagnostic techniques, regulatory authorities adopted a case definition in which the presence of at least 2 of 3 parameters, present within a 14 day period, constituted a diagnosis of PRRS. The criteria were greater than 8% abortion or early farrowing rate, greater than 20% stillbirth rate, and first week mortality rate of pigs exceeding 25% (Cromwijk, 1991). Performance records were used in another study to identify herds which had experienced PRRS. The statistical analysis of 3 parameters (average number of stillborn piglets per litter; the average number of live piglets per litter; and the average piglet mortality before weaning) was used in this approach (Schukken et al., 1991). In endemically infected herds, gilts and sows may be free from clinical signs of reproductive disease, but clinical respiratory disease in nursery and grower pigs may still be apparent (Van Alstine et al., 1993b) making case definitions using reproductive criteria inappropriate.

Pathology

The demonstration of lesions typical of PRRS, especially in conjunction with clinical history, has also been used to form a presumptive diagnosis of PRRS. As with clinical signs, discerning between pathological lesions attributable to PRRSV infection and other pathogens is difficult. Gross or histopathological lesions are generally absent in mature females, stillborn fetuses, or aborted fetuses (Christianson et al., 1992; Collins et al., 1992; Zeman et al., 1993). Inflammatory processes in the endometrium, myometrium and placenta of the sow have been reported (Christianson et al., 1992; Stockhofe-Zurwieden et al., 1993). Interstitial pneumonia is the most consistent histopathological finding in neonate, nursery, and grower pigs (Pol et al., 1991; Collins et al., 1992; Done et al., 1992; Ramos et al., 1992; Zeman et al., 1993). Less frequently, PRRSV infection has been associated with lymphadenopathies (Bilodeau et al., 1991; Rossow et al., 1994a; Rossow et al., 1994b).

Virus isolation

Isolation of PRRSV provides confirmation of infection. Virus isolation in concert with typical clinical signs and pathology can provide a definitive diagnosis of disease. Initial identification of the etiologic agent of PRRS was delayed due to the narrow range of tissue culture cells in which the virus replicated. Primary cell cultures of PAM were first used to isolate the virus

in Europe, Spain, Canada, Taiwan, and Japan (Baron et al., 1992; Bøtner et al., 1994; Chang et al., 1993; Dea et al., 1992; Ohlinger et al., 1991; Paton et al., 1992a; Plana et al., 1992; Shimizu et al., 1994; Vynckier and Pensaert, 1993; Wensvoort et al., 1991). In contrast, PRRSV was first isolated in the United States on a commercial cell line CL2621 (Collins et al., 1992). Primary cell cultures of swine lung, tracheal epithelium, heart, kidney, and peripheral blood monocyte; primary cultures of chicken embryo fibroblasts; continuous cell lines of swine testis, porcine turbinate, porcine macrophages, bovine turbinate, African green monkey (Vero), baby hamster kidney (BHK-21), canine kidney (MDCK), and porcine kidney (PK-15); and embryonating hen eggs did not support replication of United States isolates of PRRSV (Christianson et al., 1992; Yoon et al., 1992a). Swine kidney (SK-2), porcine kidney (PK-15), and secondary porcine kidney cells and embryonated hen eggs were equally ineffective in propagating the Lelystad virus isolate (Wensvoort et al., 1991). Porcine peripheral monocyte cultures were shown to be susceptible to productive infections of a Canadian PRRSV isolate, providing a potential alternative to PAM for virus isolation assays (Voicu et al., 1994). In a comparison of PAM and CL2621 cultures in the isolation of PRRSV from tissue and serum samples submitted to the Minnesota Veterinary Diagnostic Laboratory, PAM were found to be more effective (Bautista et al., 1993b). Four of 33 isolates replicated only on

CL2621; 23 of 33 isolates replicated only on PAM. However, it is unclear how many different virus strains were actually represented by the various isolates.

The expense, inconvenience, storage difficulties, potential for contamination with other agents, and technical requirements associated with primary cell cultures make a PRRSV permissive continuous cell line preferable for virus isolation. Continuous cell lines that have been used are MA-104 cells derived from monkey kidney cells and a more permissive MA-104 clone (MARC-145) (Kim et al., 1993). Proprietary cell line CL2621 is almost certainly a MA-104 cell line, as well. However, even with the technical problems associated with their use, PAM remain the most sensitive cell type for isolation of PRRSV.

The restricted host cell permissiveness of PRRSV is similar to that seen in other arteriviruses. LDV has been effectively propagated in primary cultures of peritoneal macrophages, splenic macrophages, bone marrow macrophages, and mouse embryo cell, but not on established cell lines (Kowalchyk and Plagemann, 1985; Plagemann and Moennig, 1992; Yaffe, 1962). Embryonated chicken eggs; primary cell cultures of rhesus monkey kidney, African green monkey kidney, and rabbit kidney; and continuous cell lines of African green monkey kidney (Vero), baby hamster kidney (BHK-21), diploid human embryonic lung (WI-38), diploid rhesus monkey kidney

cells (BSTC 224), HeLa, Hep-2, rhesus monkey kidney (LMK) and human amnion (HA) were all ineffective in isolating or propagating SHFV (Tauraso et al., 1968). SHFV was successfully isolated and propagated *in vitro* in MA-104 and BS-C-1 (African green monkey kidney) cell lines and peritoneal macrophages from rhesus monkeys (Gravell et al., 1980b; Myers et al., 1972; Tauraso et al., 1968). The similarities between host cell specificity of SHFV and PRRSV are particularly striking. In contrast to the specificity of the other Arteriviridae, EAV will replicate on a variety of tissue culture cell types (Evans, 1964; McCollum et al., 1961; McCollum et al., 1971; Wilson et al., 1962).

Although *in vitro* propagation of PRRSV is restricted to relatively few cell types, it has been recovered from several different tissues. Virus has been recovered from tonsil, spleen, kidney, heart, trachea, liver, lymph nodes, lung, serum, pleural fluid, thymus, and bone marrow (Bøtner et al., 1994; Halbur et al., 1995b; Paton et al., 1992b; Rossow et al., 1994a).

Serum and lung tissue were suggested as generally the most rewarding samples for recovery of PRRSV (Joo, 1993). Isolation rates from serum (28%) were somewhat higher than from lung samples (20%) (Mendez-Trigo, 1993). Pigs farrowed by experimentally infected gilts were viremic up to 56 days (Mengeling et al., 1994), providing a large window of opportunity for virus isolation. Serum was considered the sample of choice because of the

extended viremia seen in nursery age pigs and the good stability of virus in serum during transport (Van Alstine et al., 1993b). Virus survived in serum samples at least 72 hours when held at room temperature (Van Alstine et al., 1993a). In contrast to young pigs, viremia may only last for 2 weeks in adult swine. As a consequence, sows and gilts infected during gestation may not be viremic at farrowing (Van Alstine et al., 1993b). Therefore, pigs of different ages, preferably after they have been commingled with older pigs for at least 3 weeks, should be sampled to ensure successful isolation of virus from a herd (Stevenson et al., 1994).

PRRSV was isolated from the tonsils, but not serum, of pigs 42 days after experimental challenge suggesting tonsil samples may also be used for diagnosis of PRRSV infection (Shimizu et al., 1994). Tissues from stillborn, aborted, or mummified fetuses are usually non productive for virus isolation attempts (Van Alstine et al., 1993b; Zeman et al., 1993). However, isolation of PRRSV from stillborn piglets has been reported (Bilodeau et al., 1994; Bøtner et al., 1994; Murakami et al., 1994; Plana et al., 1992). Virus survival times in tissues, in contrast to serum, greatly decreased at room temperature compared to storage at -20⁰ C or 4⁰ C (Van Alstine et al., 1993a). The authors concluded necropsy specimens should be kept frozen or chilled during transport to the diagnostic laboratory to ensure virus isolation.

Immunohistochemistry

Immunoperoxidase staining of frozen tissues of inoculated pigs demonstrated PRRSV infection of epithelial cells in bronchioles and alveolar ducts, pulmonary alveolar macrophages, cells lining alveolar septa, and cells in the red pulp of the spleen (Pol et al., 1991). A streptavidin-biotin based immunoperoxidase technique detected virus antigen in formalin fixed lungs of PRRSV inoculated pigs (Halbur et al., 1994). Using this technique, viral antigen was demonstrated in pulmonary alveolar macrophages, endothelial cells, macrophages in the heart, and macrophages and dendrite-like cells in tonsil, lymph nodes, thymus, and spleen (Halbur et al., 1994; Halbur et al., 1995a). Immunogold silver staining detected PRRSV infected cells in lungs, tonsils, lymph nodes, thymus, and kidneys (Magar et al., 1993).

Bioassay

Due to cytotoxic effects of semen on cell culture systems, a swine bioassay was developed to demonstrate the presence of PRRSV in the semen of infected boars (Swenson et al., 1994a). In this technique, 4- to 8-week old pigs were injected intraperitoneally with semen samples. Subsequent seroconversion for PRRSV antibodies, and/or virus isolation, indicated the semen samples contained infectious virus.

Serology

Serological tests used to detect anti-PRRSV antibodies include the indirect fluorescent antibody (IFA) test, serum virus neutralization (SVN) test, immunoperoxidase monolayer assay (IPMA), and enzyme-linked immunosorbent assay (ELISA).

The first serological test for detecting antibodies to PRRSV was the IPMA developed during the initial isolation of Lelystad virus (Wensvoort et al., 1991). The IPMA was able to detect antibodies to PRRSV 5 to 6 days after experimental inoculation (Ohlinger et al., 1992). Peak antibody titers were demonstrated within 7 days of first detection. Persistence of IPMA antibody titers varied from 4 to more than 12 months.

Protocols for IFA tests using continuous cell lines (Mengeling et al., 1993) and PAM (Yoon et al., 1992b) have been reported. Continuous cell line CL2621 cells and PAM were found to be equally effective in IFA tests (Bautista et al., 1993b). Antibodies to PRRSV were detected by the IFA test as early as 7 to 8 days post exposure, with peak antibody levels reached by 21 to 28 days (Frey et al., 1992; Hill et al., 1993; Yoon et al., 1992b).

The ELISA is amenable to mechanized reading and thus rapid analysis of a large number of samples. The development of indirect ELISAs for the detection of anti-PRRSV antibodies have been reported (Albina et al., 1992; Murtaugh et al., 1993). A blocking ELISA in which PRRSV specific

antibodies were used to coat microtiter plates, rather than virus antigen, was developed (Houben et al., 1995a). In a comparison with the IPMA, the blocking ELISA was found to be more sensitive without complications from background staining (Houben et al., 1995a). A commercial ELISA produced in kit form (HerdCheck®:PRRS, IDEXX Laboratories, Westbrook, ME) is also available.

Serum virus neutralization tests specific for PRRSV using CL2621 cells were described shortly after the virus was isolated in the United States (Benfield et al., 1992; Christianson et al., 1992). SVN antibodies were not detected in gnotobiotic pigs inoculated at 3 days of age, but were detected at 7 to 10 weeks post exposure in gnotobiotic pigs inoculated with PRRSV at 7 days of age and immunized with virus in Freund's incomplete adjuvant at 14 and 28 days (Nelson et al., 1994). Serum from convalescent sows had measurable SVN antibodies 3 weeks after experimental inoculation (Christianson et al., 1992). Later, a modified SVN test using MARC-145 cells was reported (Yoon et al., 1994). Antibody titers by this test were first detected 9 to 11 days following experimental inoculation.

A study was conducted comparing the performance of IFA, IPMA, ELISA, and SVN tests of sera from 8 experimentally infected pigs (Yoon et al., 1995b). Serum samples were collected prior to inoculation, daily for 15 days post inoculation (PI), and then weekly until 105 days PI. The IPMA test

first detected antibodies specific for PRRSV in 4 of 8 pigs on day 5 PI. The IFA, ELISA, and SVN first detected antibodies in one or more pigs on day 9 PI. All pigs had seroconverted by IFA, IPMA, and ELISA by day 11, 9, and 13 PI, respectively. Seroconversion by SVN occurred over a longer period of time. Four of eight pigs had seroconverted by day 15 PI and the remainder by day 28 PI. Using regression analysis, the antibody titers measured by ELISA, IFA, IPMA, and SVN were predicted to decline to undetectable levels by 137, 158, 324, and 356 days PI, respectively.

Selection of antigen has been shown to be an important consideration in serological diagnosis of PRRSV. Twenty four porcine sera collected from field cases in Europe and North America and sera collected from pigs experimentally infected with either a Dutch isolate (Lelystad virus) or a United States PRRSV isolate (ATCC VR-2332) were tested by IPMA for antibodies specific for one of 7 isolates originating from The Netherlands, Germany, and the United States (Wensvoort et al., 1992). In general, European sera reacted more strongly with European isolates and North American sera reacted more strongly with United States isolates; although cross reactions occurred. For the isolates and sera tested, it also appeared that European isolates are more antigenically homogeneous and United States isolates are more antigenically diverse. Serological differences between European and United States PRRSV isolates were found to be at

least partially due to polypeptide sequences encoded by the open reading frame 3 of the virus isolates (Katz et al., 1995).

Monoclonal antibodies have been utilized to provide insight into antigenic diversity of PRRSV isolates. Researchers at South Dakota State University developed monoclonal antibodies SDOW17, VO17, and EP147 which recognized epitopes on the 15 kd nucleocapsid (N) protein of PRRSV. These monoclonal antibodies were used to demonstrate that some epitopes were conserved in United States and European PRRSV isolates, but other epitopes were divergent between groups of isolates (Nelson et al., 1993). One of the monoclonal antibodies, SDOW17, was found to be specific for epitopes on both United States and European PRRSV isolates. Isolates of PRRSV from Quebec and Ontario, Canada were shown to be antigenically similar to United States isolates according to reactivity patterns with SDOW17, VO17, and EP147 monoclonal antibodies (Magar et al., 1995). In this same study, similar reactivity patterns with SDOW17 monoclonal antibody were obtained for North American isolates and a European Lelystad virus isolate, but the other 2 monoclonal antibodies did not react with the Lelystad isolate. However, in another study, a PRRSV isolate originating from Pennsylvania did not react with SDOW17 or EP147, but did react with VO17 monoclonal antibody, suggesting caution be used in relying

on a single monoclonal antibody for diagnostic purposes (Yoon et al., 1995a).

In addition to antigenic diversity, sampling strategies are also an issue in the diagnosis of PRRSV at the herd level. The variation in seroprevalence seen in herds and age groups within herds should be considered when determining the number of pigs to be sampled (Stevenson et al., 1994).

Assuming 10% prevalence in a 500 sow herd, 28 randomly selected sows would assure (95% confidence level) that at least one seropositive pig would be among the group. Stevenson et al. (1994) found that the proportion of PRRSV positive finisher pigs was usually much higher. Consequently, fewer finisher pigs samples were required to achieve an accurate diagnosis.

Assuming a prevalence of 50%, sampling of only 7 out of 10,000 pigs would ensure, with 95% confidence, detection of at least one seropositive animal. For these reasons, the investigators advised that adequate numbers of both breeding and finishing pigs be tested before assuming a herd is PRRSV free. Other investigators have suggested that testing 10 sows, 10 4-week old pigs, and 10 5- to 6-month old pigs is usually adequate to judge seroprevalence in a herd (Dee and Joo, 1995). Investigators advocated sampling 10- to 26-week old pigs if sample size was restricted (Freese and Joo, 1994).

Another important concern in evaluating serological profiles of herds is the presence of PRRSV antibodies in young pigs. Maternal antibodies in

pigs from naturally PRRSV infected sows were detected up to 4 weeks by IFA (Dee et al., 1993), 8 weeks by indirect ELISA (Albina et al., 1994), , and 10 weeks by blocking ELISA (Houben et al., 1995a; Houben et al., 1995b).

Polymerase chain reaction

A polymerase chain reaction (PCR) technique able to detect about 30 infectious PRRSV particles per ml of virus diluted in either tissue culture media or semen has been developed (Van Woensel et al., 1994). Detection of PRRSV by PCR from tissue homogenates of 4 experimentally infected pigs was reported (Suárez et al., 1994). The analytical sensitivity reported for this test was approximately 10^2 TCID₅₀ per gram of tissue, as measured by virus titration on porcine alveolar macrophages. At 5 days PI, viral RNA was detected in pulmonary macrophages, lungs, liver, kidney, spleen, submaxillar and inguinal lymph nodes, tonsil, plasma, buffy coat, and serum. In pigs sacrificed at 10 days PI, virus was only detected by PCR in lung macrophages, plasma, and serum. In a different study, PCR analysis detected the presence of PRRSV in lung samples from 3 of 4 pigs infected 4 to 10 days earlier; although, virus was demonstrated in all 4 pigs through virus isolation on PAM cultures (Mardassi et al., 1994). By using different primers, European and Canadian isolates could be differentiated. A PCR technique which consistently detected PRRSV in semen of experimentally infected boars has also been described (Christopher-Hennings et al., 1995).

Pathogenesis

As discussed earlier, immunohistochemistry techniques have demonstrated PRRSV infection of a variety of cell types in different organs. However, cells from the immune system, especially macrophages, appear to be the primary site of PRRSV replication (Molitor, 1993). Infection of macrophages is a consistent feature of arteriviruses.

Viral antigen was demonstrated, by a direct immunofluorescent test, in pulmonary macrophages 24 hours after horses were experimentally inoculated by aerosolized suspensions of EAV suggesting pulmonary macrophages were the site of initial replication of the virus (Crawford and Henson, 1973). Replication of LDV was shown to be restricted, apparently, to permissive macrophages (Onyekaba et al., 1989). Macrophages also appear to be the site of SHFV replication in acute cases of infection in patas monkeys (Gravell et al., 1986) and macaques (Gravell et al., 1980b).

Due to the predilection of PRRSV and the other Arteriviridae for macrophages, research on the pathogenesis of PRRSV has focused on the effects of infection on monocyte/macrophage function. Various immune parameters were followed in 1-, 4-, and 10-week-old pigs experimentally infected with PRRSV to determine if immunosuppression resulted from the infection (Molitor et al., 1992). The proportion of alveolar macrophages in alveolar washings decreased while the proportion of lymphocytes and

polymorphonuclear leukocytes increased in virus inoculated pigs in comparison to sham inoculated pigs. Alveolar macrophages collected from infected pigs 7 days post challenge produced lower levels of superoxide anions in response to phorbol-myristate acetate, although at 28 days PI alveolar macrophages from PRRSV infected pigs produced greater levels of superoxide than sham inoculated pigs. In addition, the PRRSV infected pigs produced a greater antibody response to pseudorabies vaccination than did the control pigs. Delayed type hypersensitivity responses were also increased in PRRS infected pigs. In another report, macrophages made up greater than 95% of the cells from lung lavages of non infected control pigs compared to 50% in infected pigs (Zhou et al., 1992). The proportion of alveolar macrophages increased to 80% by 27 days PI. Interleukin-1B levels in PRRSV inoculated pigs 7 days PI were increased while tumor necrosis factor and transforming growth factor B1 levels were the same as levels of control animals. Although NADPH-oxidase 90 kd-catalytic subunit mRNA levels were initially decreased in PRRS infected animals, these levels had returned to normal levels by 27 days PI. At day 14 PI, CD8⁺ and CD4⁻8⁻ T lymphocytes were decreased while CD4⁺ T cells increased. Helper T cells and CD4⁺8⁺ levels were increased, although within normal limits, on days 7 and 28 PI. The increased numbers of CD4⁺ T cells corresponded to high

PRRSV IFA antibody titers suggesting immune system enhancement. However, in sows experimentally inoculated with PRRSV during mid-gestation, CD4⁺ T cells, CD8⁺ T cells, and the CD4⁺/CD8⁺ ratio were decreased on days 3 to 11 PI (Christianson et al., 1993). Although differences in age of pigs and sampling dates precludes direct comparisons, the latter study suggested decreased T helper cell function occurred in response to PRRSV infection. Destruction of bronchiolar cilia (Done and Paton, 1995) and epithelial cells of nasal mucosa (Pol et al., 1991) have been reported. Taken collectively, these studies suggest PRRSV infection decreases the non specific immune response and thereby contributes to the pathogenesis of PRRSV-induced pneumonia, especially in conjunction with secondary infections. However, the effects of virus infection on antigen-specific humoral and cell mediated immune responses is less clear. The fluctuations in T cell subpopulations suggest PRRSV infection has a direct effect on immune system performance, but whether it is one of suppression or enhancement is unclear.

Exacerbation of other infectious diseases have been attributed to immunosuppression due to PRRSV infection. *Haemophilus parasuis*, *Streptococcus suis*, *Salmonella cholerasuis*, *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, swine influenza virus, encephalomyocarditis virus, pseudorabies virus, porcine cytomegalovirus,

porcine respiratory coronavirus, and porcine paramyxovirus are common secondary infections reportedly associated with herds chronically infected with PRRSV (Joo and Dee, 1993). Swine influenza virus, chlamydia, *H. parasuis*, *A. pleuropneumonia*, *P. multocida*, *P. haemolytica*, and *S. suis* respiratory infections in association with PRRSV infections have been recognized in Britain (Done and Paton, 1995). Increases in the incidence and severity of salmonellosis, *Escherichia coli* and *Clostridia* infections, polyserositis, greasy pig disease, atrophic rhinitis, swine dysentery, sarcoptic mange, and *S. suis* meningitis were also noted. Investigators demonstrated a positive association between seroprevalence of PRRSV and porcine respiratory corona virus, and between PRRSV and porcine influenza virus strain Arnsberg subtype H1N1 (Groschup et al., 1993). Investigators have suggested that concurrent infections with PRRSV were responsible for increased nursery mortality due to *S. choleraesuis* septicemia (Stevenson et al., 1993). Diagnostic laboratories in the upper Midwest reported increases in the diagnosis of *H. parasuis* in the years prior to 1992, the same time PRRSV became widespread (Molitor, 1994). The author cautioned that, although clinical and diagnostic laboratory data supported the contention that PRRSV increases the incidence of secondary bacterial infections, there is little experimental evidence.

Only two studies have shown an interaction between PRRSV and a secondary infection. Galina et al. (1994) inoculated 13-day-old pigs with *S. suis* 7 days after inoculation with either culture medium or PRRSV. The dually infected pigs developed suppurative meningitis (4/10), mononuclear perivascular cuffing in the brain (5/10), and yielded dense growth of *S. suis* (6/10), while non inoculated and *S. suis*-only inoculated pigs did not. Shimizu et al. reported increased severity in the pneumonia produced in pigs inoculated with *Mycoplasma hyorhinis* 5 days after PRRSV inoculation as compared to *M. hyorhinis*-only infected pigs. However, only 2 pigs were used in each group.

On the other hand, Cooper et al. (1995) was unable to demonstrate significant interactions between PRRSV and secondary bacterial infections under experimental conditions . In the first trial, 4- to 6-week-old pigs were inoculated with either *H. parasuis*, *S. suis*, *S. choleraesuis*, or *P. multocida* 7 days after half of the pigs were infected with PRRSV. In the second trial, pigs were inoculated with *S. suis* or *P. multocida* 2 days after half of the pigs were inoculated with PRRSV. Infection with PRRSV did not potentiate bacterial infection. In fact, mortality was greater in pigs infected only with *H. parasuis* or *S. suis*. The authors noted that there may be stressors and virulence factors present in field conditions that were not replicated under the experimental conditions.

Differences in degree of clinical respiratory scores, rectal temperature, percentage of lung with gross lesions of pneumonia, and microscopic lung lesion scores were demonstrated among 4-week-old cesarean-derived colostrum-deprived (CDCD) pigs inoculated with different PRRSV isolates including Lelystad virus (Halbur et al., 1995b; Halbur et al., 1996). The authors suggested that differences in virulence among field strains of virus were responsible for the variation in severity of clinical outbreaks of PRRS.

Differences in virulence of isolates of SHFV have been documented in both *in vivo* and *in vitro* systems. Isolates of SHFV, which caused asymptomatic infections in patas monkeys, and acute, fatal, hemorrhagic disease in macaques, replicated in patas peritoneal macrophages without producing cytopathic effects but caused cell lysis when propagated in rhesus peritoneal macrophages (Gravell et al., 1986). In contrast, isolates which caused acute disease in both patas monkeys and macaques demonstrated cytopathic effects in both patas and rhesus peritoneal macrophages.

Another potential factor in the pathogenesis of PRRSV infection is antibody dependent enhancement (ADE) of virus replication. Replication of infectious progeny virus was enhanced 10 to 100 times when diluted anti-PRRSV sera was mixed with PRRSV prior to inoculation of PAM (Choi et al., 1992). Further evidence of ADE of PRRSV replication was provided in a

study of fetuses inoculated *in utero* during mid-gestation (Christianson et al., 1993). Virus replication was enhanced in fetuses inoculated with PRRSV concurrently with anti-PRRSV antibody as compared to fetuses inoculated with PRRSV alone.

Results from a study by Yoon et al. (1996a) suggested that ADE virus yields *in vitro* were a consequence of both an increased infection rate of PAM and an increased yield of progeny virions from individual PAM. Further evidence of *in vivo* ADE of PRRSV was also presented. Pigs which were injected with subneutralizing amounts of PRRSV specific immunoglobulin prior to challenge had longer periods of viremia, as well as higher virus titers in serum, than did control pigs injected with non PRRS-specific serum globulin. The investigators suggested a scenario in which ADE might play a role in the pathogenesis of respiratory disease in young pigs. When maternal antibodies declined to a subneutralizing level, ADE of PRRSV was conjectured to cause increased replication of virus and severity of respiratory disease. Field isolates of PRRSV varied in their susceptibility to ADE induced by antibodies raised against a specific virus isolate (Yoon et al., 1996b). Concentrations of antibodies that were neutralizing to some isolates caused enhancement of virus yields of other isolates. These results have suggested the possibility of vaccine induced ADE of wild strain virus.

In addition to concurrent infections, strain differences of virulence, and ADE, other factors may be involved in the pathogenesis of PRRSV infection. Fumonisin, a mycotoxin, was found to be a significant risk factor associated with the occurrence of PRRSV in swine herds (Bane et al., 1992). Furthermore, the risk of clinical PRRSV infection was directly proportional to fumonisin concentrations in feed.

The pathogenesis of reproductive disease due to PRRSV has not been fully defined, but progress has been made. Transplacental infection of fetuses and typical late term reproductive disease were reported in sows experimentally inoculated 3 weeks prior to their expected farrowing dates (Christianson et al., 1992). PRRSV did not appear to cross the placentas of infected sows to fetuses, or from fetuses infected *in utero* to dams, when inoculation occurred in mid-gestation (Christianson et al., 1993). Transplacental infection of fetuses occurred more often in gilts inoculated after 90 days of gestation than in gilts inoculated between 31 and 72 days of gestation (Mengeling et al., 1994). The authors noted that actual crossing of the placenta may not occur until some time after 72 days of gestation; i.e., the virus may persist in the sow until transplacental infection is biologically possible. Therefore, if in an experimental study the sows were euthanized prior to this time, transplacental infection would not be evident. In a study in which fetuses were inoculated *in utero* with PRRSV, it appeared fetuses

infected during the first half of gestation were capable of replicating virus up to 31 days without severe pathological effects (Lager and Mengeling, 1995). However, fetuses infected in the second half of gestation seemed to succumb to the infection. Apparent intrauterine spread of PRRSV was also evident.

Characterization of the development of immunity to PRRSV infection is crucial to clinical management of PRRSV infected. It is known that infection produces protection against some aspects of clinical disease. Sows experimentally infected with PRRSV during gestation developed protective immunity against reproductive loss on subsequent challenge exposure (Gorcyca et al., 1993). Gorcyca et al. (1993) exposed six gilts to PRRSV at 86 to 93 days of gestation and allowed them to farrow. These sows were again challenged at approximately 93 days of their next gestation, as were 3 PRRSV-naïve control sows. Sows demonstrated improved live born, stillborn, and mummified fetus rates following challenge in their second gestation, as compared to their first litter and control sows. The onset of clinical signs following the decline of passive antibody levels in piglets born to sows infected at 90 days of gestation suggested that maternal antibodies were protective to offspring (Albina et al., 1994). Epidemiological studies of field cases in Germany also suggested that immunity developed in sows during the initial outbreak of PRRSV and prevented abortions in subsequent pregnancies (Busse et al., 1991). However, others have reported that, in

preliminary experiments, sows reinfected more than 5 months after initial infection went through clinical reproductive disease (Plana Durán et al., 1992).

Epidemiology

In 1990, a survey was conducted of the members of the American Association of Swine Practitioners (AASP). The practitioners' experience with PRRS was used to determine the distribution of affected herds. A defined set of clinical signs (case definition) was used as the criteria for recognizing a case. Of the 1305 AASP members from the United States and its territories, 677 responded to the survey. These respondents reported seeing clinical episodes matching the case definition in 1611 herds located in 19 states. Cases were widely distributed geographically, but followed areas of highest swine density. The earliest outbreak matching the case definition occurred in 1980. Six respondents recalled seeing their first case of PRRS in 1986. The number of practitioners who observed PRRS for the first time increased dramatically in the next 3 years. In 1989, 124 swine practitioners observed their first outbreaks of PRRS. Seemingly, PRRSV spread rapidly through the US swine population between 1986 and 1989. (Zimmerman, 1991)

Seroprevalence

Retrospective studies of the seroprevalence in the United States are available from several surveys. Yoon et al. (1992b) tested serum samples collected between 1981 to 1991 by IFA for the presence of anti-PRRSV antibodies. Samples collected in March of 1986 from a herd with a clinical history of PRRS were the first seropositives in the survey.

A retrospective study of PRRSV in the state of Iowa was conducted using serum samples collected from 89 randomly selected swine herds through the Iowa Pilot National Animal Health Monitoring System (NAHMS) (Owen et al., 1992). Two herds which were not NAHMS herds, but had comparable serologic and production records, were also included. The herds were distributed over 60 of Iowa's 99 counties. Participating herds were monitored for 12-18 month periods between 1984 and 1989, with serum samples collected on the farm or at slaughter. The study found that 9.6% of the swine and 3.8% of the 26 swine herds sampled were seropositive for PRRSV in 1985. This is the earliest date of PRRSV infection in the United States. By 1989, 51.7% of the swine and 83.3% of the 6 herds which were sampled were seropositive for PRRSV. From these results it was concluded that PRRSV entered the Iowa swine population prior to 1985 and spread rapidly thereafter.

The seroprevalence of the PRRSV in the United States was estimated in a study conducted with a national NAHMS database (Bautista et al., 1993c). These data were compiled in 1990 from 412 randomly selected swine herds from 17 states. One to 10 serum samples from mature female swine from each of the selected herds were tested for antibodies to PRRSV by the IFA test. At least one seropositive animal was detected in 36% of the sampled swine herds. The data indicated that PRRSV infected herds were present in all regions of the US. The seroprevalence of PRRSV followed areas of highest swine density with a moderately high prevalence in the Midwest. Eastern and Western states generally had a lower prevalence of PRRSV positive herds.

In a recent serological study, sera collected from swine in Ontario, Canada between 1978 and 1982 were assessed for anti-PRRSV antibodies (Carman et al., 1995). Single serum samples from 50 to 57 herds per year were analyzed. Two serum samples collected in 1979 were seropositive for PRRSV. These results indicate that PRRSV was present in Canadian swine as early as 1979 and represent the earliest serological evidence of PRRSV infection.

Another serological survey was performed on 837 serum samples to compare prevalence of antibodies as detected by IFA to the Lelystad virus and the United States isolate VR-2332 in the United States swine population

(Bautista et al., 1993a). The serum samples were collected in conjunction with the NAHMS survey of breeding animals or samples submitted to the Minnesota Veterinary Diagnostic Laboratories for detection of PRRSV antibody. Eighty seven farms from 18 states were represented. Of the 837 samples, 57.2% were seropositive for antibodies to one or both viruses. Of the positive samples, 43.8% were positive for VR-2332 only, 20.1% were positive for Lelystad only, and 36.1% were positive for both virus isolates. On a herd basis, 17.2% of the farms were negative for both strains. In regard to positive farms, 9.7% were positive for Lelystad only, 19.4% were positive for VR-2332 only, and 70.8% were positive for both strains. The study highlighted the problems present when only one virus strain is used as the indicator in the IFA test, i.e., false negatives occur which result in an incorrect diagnosis and an underestimation of seroprevalence.

Transmission

The methods and routes of transmission of PRRSV from pig-to-pig or herd-to-herd has not been clearly defined. In the initial outbreaks of the disease in Europe, airborne spread was assumed to be the way in which the disease moved from one herd to another when pig-to-pig contact, movement of semen, or other more direct means of transmission were not apparent. Airborne spread was suggested to be responsible for the conveyance of the disease a distance of 5 km from Germany to Denmark (Mortensen and

Madsen, 1992), and up to 3 km between field cases in Great Britain (Robertson, 1992; Edwards et al., 1992). More recent epidemiological evidence suggests that area spread is restricted to a distance of less than 2 km (Blaha and B  ker, 1995). Weather conditions of high humidity, low temperature, and favorable wind were offered as circumstantial evidence in support of airborne transmission (Robertson, 1992). The weather conditions during the first reported outbreak of PRRS in the Netherlands were studied to determine if an association between weather conditions and spread of the virus existed (Komijn et al., 1991). The initial case of PRRS in the Netherlands was reported on January 17, 1991, 20 km from the German border. Subsequent cases occurred to the west and southwest, and later, to the north of the initial case. Generally, the prevailing winds in the Netherlands were westerly, blowing from the Netherlands towards Germany. However, from January 14 to February 12, 1991 and February 26 to March 9, 1991 mild to moderate easterly winds prevailed. Weather conditions of moderate wind speeds and relative humidity greater than 60% were judged to be ideal for wind borne transmission of PRRSV based on experience with foot-and-mouth disease virus. However, the lack of knowledge of case dates, and thus the temporal spread of cases of PRRS, prevented the investigators from clearly establishing that airborne spread of the virus occurred. At the time the study was conducted the etiologic agent of PRRS

was unknown, so cases were determined on the basis of clinical signs. This undoubtedly led to misclassifications errors, i.e., false negative and false positive cases. The authors had correctly assumed that the etiologic agent of PRRS was a virus. However, now that PRRSV is more fully characterized, it seems inappropriate to use foot-and-mouth disease virus, an extremely stable, non enveloped picornavirus, as a model for airborne transmission of PRRSV.

Weather conditions, particularly relative humidity greater than 60%, were considered important evidence for airborne transmission of PRRSV. Information on persistence of viruses in aerosols does not entirely support this hypothesis. A study comparing the survivability of different aerosolized viruses determined that non enveloped viruses such as equine rhinovirus type 1, a picornavirus, survived better at relative humidity greater than 60% (Donaldson and Ferris, 1976). Conversely, enveloped viruses, including EAV, were more readily inactivated at higher relative humidity. This latter work suggests that PRRSV, an enveloped virus related to EAV, may not survive long enough in an aerosolized state during periods of high humidity to allow airborne transmission. No experimental evidence of airborne transmission of the PRRSV has been reported.

Movement of pigs was linked to the spread of PRRSV in field cases (Robertson, 1992). Research under experimental conditions has

documented transmission between pigs in direct contact and allowed investigation of how long pigs remain infectious. In one such study, 4 sows experimentally infected with PRRSV 99 days earlier were each commingled with 3 PRRS negative finisher pigs (Zimmerman et al., 1992). A non infected sow was also housed with 3 finishers to serve as a control group. Serum samples from the finishers were collected at weekly intervals for 84 days. Seroconversion by the IFA test and clinical observations showed that transmission had occurred. The use of convalescent, asymptomatic sows in the trial illustrated the potential of "recovered" animals to be the source of infection to naive herds.

Persistence of PRRSV infection was demonstrated in another study in which sentinel pigs seroconverted after they were placed in contact with pigs experimentally infected with PRRSV 56 days earlier (Terpstra et al., 1992). Transmission to sentinel pigs no longer occurred when they were placed in contact with pigs infected 140 days earlier or 182 days earlier and immunosuppressed with prednisolone-acetate.

In another study, pigs were placed in an isolation room and inoculated with PRRSV (Yoon et al., 1993). Three sentinel groups of pigs were sequentially placed in contact with the infected pigs and remained with them until the sentinel pigs were slaughtered. Virus isolation and IFA test results indicated that all of the sentinel pigs placed in contact with infected

pigs on day 3 or 10 PI contracted PRRSV. Two of the 4 sentinel pigs commingled with infected pigs on day 24 PI did not appear to become infected. The failure of these pigs to become infected suggested that the amount of virus in the room decreased with time. Alternatively, the pigs in the day 24 sentinel group may have been more resistant to infection because they were older when placed in contact with infected pigs.

How long pigs remained infectious was also investigated in a study in which the principal pigs became infected from their experimentally inoculated dams. The principal pigs transmitted PRRSV to susceptible pigs placed in direct contact with them at weaning at 4 weeks of age (Albina et al., 1994). In this same study, infected pigs were also placed in adjacent pens (fenceline contact) with susceptible pigs. The susceptible pigs remained seronegative for 9 weeks. At that time, 2 of the infected pigs were treated with prednisolone for 5 days and exchanged with 2 of the susceptible pigs. Over the next 7 weeks the susceptible pigs, both in direct and fenceline contact with the treated pigs, became infected with PRRSV. The study demonstrated that pigs were still harboring infective virus 15 weeks after initial seroconversion. It also demonstrated that relatively close contact was required for rapid transmission to occur.

Neither the location of PRRSV during chronic infection nor the mechanism allowing chronic infection have been established. A mechanism

presented to explain the ability of LDV to produce a persistent infection in the face of a strong antibody response (Onyekaba et al., 1989) may provide insights into the possibility of a comparable mechanism in swine. In LDV infection, a subpopulation of macrophages which are permissive to LDV are initially infected with LDV and produce a rapid increase in viral titers. Anti-LDV antibodies are not directly responsible for the decline of virus titers. Instead, virus is removed by the cytotoxic activity of the virus on permissive macrophages. A reduction in the population of permissive macrophages produces a resultant decrease in viral titers. Persistent infections are maintained at lower titers by virus replication in new permissive macrophages generated by the body.

Variation in the duration of persistent infections of the other Arteriviridae viruses suggest that host differences may be influential. Inapparent infection with SHFV in patas monkeys has been shown to persist for years in some patas monkeys (Gravell et al., 1980a). The blood of these persistent carriers contained infectious virus and was capable of inducing disease if inoculated into macaques. Other patas monkeys, however, became SHFV-free after 3 to 12 months of isolation. Stallions also showed variability in duration of persistent EAV infection with virus replication continuing in the reproductive tracts of some stallions for years (Timoney et al., 1986;

Timoney et al., 1987). The host attributes which are responsible for this variability in duration of infection are unknown.

Field case studies have also provided information about the transmission of PRRSV within herds. Studies of endemically infected herds suggested pigs usually became infected between 3 and 12 weeks of age with the nursery and grower age pigs serving as a reservoir of infection to the herd (Dee et al., 1993; Houben et al., 1995b; Paton and Drew, 1995; Stevenson et al., 1994). Some pigs remain free from infection for several weeks after weaning, but whether this is due to protective passive immunity or lack of exposure is unclear.

The shedding patterns of PRRSV in feces and nasal secretions have been investigated (Yoon et al., 1993). Virus isolation was performed on plasma, nasal swabs and feces collected from 4 pigs at 2 to 4 day intervals following inoculation with PRRSV. Virus was detected from plasma, feces, and nasal swabs up to 35, 35, and 38 days PI respectively. The authors suggested that isolation of PRRSV from feces more often than nasal swabs indicated that the fecal-oral route may be a more important mode of transmission than nose-to-nose contact. In another study, PRRSV was sporadically recovered from fecal swabs, nasal swabs, and postmortem urine samples from 1-, 4-, or 10-week old pigs (Rossow et al., 1994a). Virus was not recovered from 105 nasal swabs collected on days 1, 4, 7, 14, and 28 PI;

although virus was recovered from nasal swabs from 3 of 15 pigs on day 21 PI. In the case of fecal swabs, virus was not recovered from 105 swabs collected from day 1 through 21 PI; although virus was recovered from fecal swabs from 2 of 15 pigs on day 28 PI. Urine samples collected at postmortem 7 days PI were negative for virus isolation from 10 pigs, but positive from one of 9 pigs on day 28 PI. Similar results were seen in studies of EAV and LDV. EAV was recovered from nasopharynx samples up to 21 PI and from urine up to 22 PI in sequentially sacrificed horses (McCollum et al., 1971). Virus was recovered from urine, saliva, milk, and feces through days 9, 16, 16, and 135 PI respectively from mice inoculated with LDV (Notkins, 1965; Notkins and Scheele, 1963).

Transmission via boar semen was proposed as a mechanism of inter-herd spread of PRRS in field cases in Great Britain (Robertson, 1992). In an experiment conducted to determine if PRRSV was present in semen of infected boars, semen was collected twice weekly for 8 weeks from 4 boars that were experimentally infected with virus (Swenson et al., 1994a). A bioassay was used to detect the presence of virus. Semen was injected intraperitoneally into 4- to 6-week old seronegative pigs. Serum samples from these pigs were then tested by IFA. Seroconversion indicated the presence of PRRSV in the boars' semen. All 4 boars were found to shed virus in their semen from as early as 3 days post-challenge to as late as 43

days post-challenge. Virus was also recovered from the serum of seroconverted bioassay pigs, confirming that viable virus was excreted. EAV was isolated from stallion semen for even longer periods after inoculation, through day 135 PI (Neu et al., 1988). Stallions naturally infected with EAV have been shown to be capable of shedding virus in semen up to 5 years after initial infection (Timoney et al., 1986; Timoney et al., 1987).

Semen contaminated with PRRSV may produce infection in dams. Fresh undiluted semen from 2 PRRSV inoculated boars was combined and used to inseminate 2 seronegative gilts (Yaeger et al., 1993). Both gilts seroconverted, demonstrating transmission of PRRSV via semen occurred. LDV replication in persistently infected mice was localized in testis suggesting it, too, may be sexually transmitted (Anderson et al., 1995). In a 1984 outbreak of EAV in Thoroughbreds in Kentucky, epidemiological analysis indicated the virus was spread by infected stallions during breeding (Timoney and McCollum, 1985).

In a study in which gilts were inseminated with extended rather than fresh semen from boars infected with PRRSV, transmission did not occur (Swenson et al., 1994b). It was demonstrated through a bioassay that the semen contained infectious virus. *In vitro* studies indicated the extender did not inactivate the virus. The authors concluded the boar was excreting virus at levels insufficient to cause transmission via insemination. In a

second study, virus was recovered from the tonsils and ovaries of gilts inseminated with extended semen from PRRSV infected boars (Swenson et al., 1995b). The differences in results between these two studies may have been related to the titer of PRRSV in the semen. Thus, the minimum infectious dose (MID) of PRRSV may be route dependent, as has been shown with LDV. The MID of LDV when mice were inoculated by intraperitoneal or tail cartilage injection was 1 ID₅₀. In contrast, the MID for oral, vaginal, rectal, and ocular exposure was 10^{5.3}, 10^{5.3}, 10^{3.3}, and 10^{5.3} ID₅₀, respectively, suggesting mucosal barriers are protective. (Cafruny and Hovinen, 1988).

Host range

The PRRSV has a very limited host range. The only animals in which the virus has been shown to replicate are swine and some species of birds. Virus isolation from the feces of birds orally inoculated with PRRSV was attempted on days 0, 7, 10, 12, 14, 21, and 24 PI. PRRSV was recovered from the feces of guinea fowl on days 5 and 12 PI, from chickens on day 5 PI, and from Mallard ducks on nearly all sample days from Day 5 to 24 PI. No virus was isolated from Muscovy ducks. Although the birds did not demonstrate signs of clinical disease, the long period of fecal shedding indicated that the virus replicates in certain avian species (Zimmerman et al., 1996). Isolation attempts of PRRSV from rats and mice captured on a

swine farm endemically infected with PRRSV were unsuccessful. Attempts to experimentally infect laboratory mice and rats with PRRSV were also not fruitful. (Hooper et al., 1994)

Host specificity is a characteristic shared by members of the Arteriviridae family. Rabbits, hamsters, guinea pigs, mice, or chicken embryos did not demonstrate signs of infection when inoculated with EAV (Doll et al., 1957). Wild and laboratory mice *Mus musculus* and Asian mice *Mus caroli* are the only confirmed host species for LDV (Plagemann and Moennig, 1992; Rowson, 1980). Rats, Hooded rats, hamsters, guinea pigs, rabbits, deer mice, and dwarf hamsters did not become infected when inoculated with LDV (Notkins, 1965; Plagemann et al., 1963; Rowson and Mahy, 1985). Investigators were unable to isolate or demonstrate an immune response to SHFV in hamsters, guinea pigs, white mice, or white rats (Tauraso et al., 1968).

Environmental stability

Stability of PRRSV in the environment has direct implications for control of the virus in the field, but only one study has been carried out to date. The study was conducted to determine how long PRRSV persisted on various fomites stored at room temperature (Pirtle and Beran, 1996). Virus was not recovered from corn, swine starter, or denim cloth. Virus was recovered only on the same day of application from stainless steel, plastic,

boot rubber, alfalfa, wood shavings, straw, swine saliva, urine, and feces. Virus survived in city water, well water, saline G, and phosphate buffered saline for 11, 9, 6, and 4 days respectively. Further research on the persistence of the virus in the environment over a range of temperatures is needed.

Researchers have reported an effective protocol for the disinfection of nursery facilities. The nurseries were freed of PRRSV by pressure washing with hot water and disinfecting with formaldehyde three times, pumping pits between washings, and allowing the facilities to remain empty for 14 days (Dee et al., 1993).

Prevention and control

Understanding the means by which PRRS is perpetuated in herds and utilizing management strategies which reduce the likelihood of clinical outbreaks are the basis for preventing or controlling PRRSV infections in a herd. The purchase of PRRSV seronegative replacement gilts has been shown to contribute to recurrent reproductive failure in an endemically infected swine herd (Dee and Joo, 1994b). In contrast, a farm which used in-herd seropositive replacement gilts did not suffer recurrent reproductive failure. Eradication of PRRSV from endemically infected herds was accomplished by closing the herd to outside seedstock and utilizing farm raised replacement gilts (Dee and Pijoan, 1995). In another PRRSV positive

herd, PRRSV positive replacement gilts were raised at an offsite farm and only moved into the closed herd if IFA titers had decreased 4-fold. This strategy also controlled the spread of PRRSV in the breeding herd. The success of the control strategies was attributed to preventing introduction of replacement gilts harboring actively circulating virus. The ideal replacement gilt for introduction into a PRRS positive herd was described as one previously exposed, but no longer viremic and with a declining PRRSV antibody titer (Dee and Joo, 1995). The spontaneous eradication of PRRSV from an infected herd was attributed to the quarantine of incoming seedstock, all-in/all-out pig flow, and 2 week intervals between movement of pigs from the farrowing house to the nurseries. (Freese and Joo, 1994).

Providing replacement seedstock to negative herds requires special consideration if outbreaks of PRRSV are to be avoided. A protocol was developed for the sale and introduction of previously infected pigs into PRRSV negative herds without introducing PRRSV (Dee et al., 1994). Non carrier status of the pigs to be sold was established by the following criteria: titers to PRRSV decreased in previously infected pigs, sentinels placed in fenceline contact with previously infected pigs did not seroconvert, and all virus isolation attempts on sera from previously infected pigs and sentinel pigs were negative.

Onsite nursery depopulation and thorough disinfection were successfully used to eradicate PRRSV from nursery- and grower-age pigs (Dee and Joo, 1994a). Success depended on lack of active viral shedding or recent exposure to virus in the sow herd. In another approach, offspring from a PRRSV positive herd were raised free of PRRSV infection by utilizing off-site nursery facilities (Dee et al., 1993).

Preliminary results indicated an experimental inactivated vaccine protected sows from reproductive failure due to challenge with PRRSV (Plana Durán et al., 1992). The length of time PRRSV was shed in the semen of experimentally challenged boars was reduced by prior administration of an inactivated vaccine (Swenson et al., 1995a). A modified live PRRSV vaccine licensed for use in pigs 3- to 18-weeks in age was made commercially available in 1994. At the present time, the role of the modified live vaccine in the prevention and control of the disease is under consideration.

Statement of Problem

Porcine reproductive and respiratory syndrome can have a devastating impact on the health of a swine herd, as well as the financial success of the producer. Although much has been learned in regard to the epidemiology of PRRSV, many fundamental questions are currently unanswered. One of the most perplexing of these basic issues is the transmission of PRRSV.

The objectives of the work reported in this dissertation were to increase our understanding of the methods and routes of transmission, further delineate the portals of exit and duration of shedding of virus from the infected host, and determine the duration of infection of the host.

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TRANSMISSION OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS BY DIRECT, CLOSE, OR INDIRECT CONTACT

A paper submitted to Preventive Veterinary Medicine

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Abstract

The development and implementation of effective strategies for the prevention and control of porcine reproductive and respiratory syndrome virus (PRRSV) are dependent upon an accurate and complete understanding of the modes of transmission between infected and susceptible animals. The goal of this work was to further our understanding of PRRSV transmission in swine herds.

The transmission of PRRSV was followed between groups of pigs housed under conditions simulating current swine production systems. Five trials

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were conducted using 13 3- to 5-week old pigs in each trial. Pigs were housed in 1.2 m by 1.8 m nursery decks fitted with wire mesh flooring raised 40.6 cm above the sealed concrete floor. Three decks were placed parallel to one another in a 3.4 m by 7.5 m by 2.7 m high isolation room. The decks were placed 46 cm apart for the first 3 trials and 102 cm for trials 4 and 5. A single sheet of aluminum was suspended equidistantly in the space between one side deck and the center deck to inhibit the direct passage of biological materials from pigs in the center deck to the side deck. On the first day of each trial, 3 pigs (primary exposure) were placed in the center deck and intranasally inoculated with PRRSV (ATCC VR-2402). Two days later, 3 pigs (direct contact) were placed in the center deck with the inoculated pigs; 3 pigs (close contact) were placed in the side deck with no barrier; and 4 pigs (indirect contact) were placed in the side deck separated from the center deck by a sheet of aluminum.

Transmission was determined to have occurred when PRRSV was isolated from, or anti-PRRSV antibodies were detected in, serum collected on day 31. Transmission of PRRSV was demonstrated between the primary exposure and the direct contact groups in all 5 trials. In contrast, the close contact group became infected in 3 trials and the indirect contact groups became infected in 2 trials. This study demonstrated that transmission of PRRSV is most likely to occur when susceptible pigs are in direct contact

with infected pigs. Transmission occurred across short distances, but the failure of transmission to routinely occur between acutely infected and susceptible pigs separated by a distance of only 46 to 102 cm suggests that airborne transmission is much less likely to take place than previously believed.

Keywords

Porcine reproductive and respiratory syndrome, PRRS virus, epidemiology, aerosol, airborne, transmission.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV), a relatively recently identified virus of swine, was first isolated in 1991 (Wensvoort et al., 1991). Due to similarities in morphology, structural proteins, genome size and polyadenylation, and preference for replication in macrophages, it has been suggested that PRRSV be included in a recently proposed family, Arteriviridae, along with lactate dehydrogenase-elevating virus (LDV), equine arteritis virus (EAV), and simian hemorrhagic fever virus (SHFV) (Plagemann and Moennig, 1992; Meulenberg et al., 1993).

Clinical outbreaks of porcine reproductive and respiratory syndrome (PRRS) were first reported in North Carolina in 1987 (Dial et al., 1990; Hill, 1990). A survey of members of the American Association of Swine

Practitioners indicated that clinically affected herds were observed in all major U.S. swine producing regions by 1987 (Zimmerman, 1991).

One of the remarkable and consistent characteristics of PRRSV has been its rapid spread. Serologic testing of banked serum showed that PRRSV first infected swine in Iowa sometime between 1980 and 1985 and then spread rapidly throughout the state (Zimmerman et al., 1996). In a similar fashion, PRRSV spread through swine populations in Europe, North America, and Asia (Wensvoort et al., 1992; Bautista et al., 1993; Chang et al., 1993; Owen et al., 1992). So far, it has not been possible to explain the rapid worldwide spread of the virus.

Transmission by direct contact between inoculated and sentinel pigs has been demonstrated under experimental conditions (Terpstra et al., 1992; Zimmerman et al., 1992; Chang et al., 1993b; Yoon et al., 1993; Albina et al., 1994). Descriptive data collected in association with outbreaks in England suggested that airborne spread of the virus occurred up to 3 km, with spread over longer distances resulting from pig movement. (Edwards et al., 1992; Robertson, 1992). Airborne spread over a distance of at least 5 km was suspected to be responsible for the transmission of the virus from infected herds in Germany to the first documented case in Denmark (Mortensen and Madsen, 1992). More recent epidemiological evidence

suggests that area spread is restricted to a distance of less than 2 km (Blaha and B ker, 1995).

The development of effective strategies for the prevention and control of PRRS is entirely dependent upon the validity of our understanding of the transmission of PRRSV. The objective of this study was to increase our knowledge of PRRSV by observing the effect of different levels of exposure on the rate of transmission from experimentally infected to susceptible pigs.

Methods

Experimental design

Five trials were conducted using 3- to 5-week-old, conventionally raised pigs. Pigs were obtained from a herd periodically monitored for PRRSV and known to be free of the virus. Three 1.2 m by 1.8 m nursery decks fitted with nipple waters and gravity flow feeders were placed parallel to one another in a 3.4 m by 7.5 m by 2.7 m high isolation room (Figure 1). Legs fitted to the decks raised the wire mesh floors of the decks 40.6 cm off the sealed concrete floor. The walls of the nursery decks consisted of 73.7 cm high vertical bars (0.95 cm diameter) spaced 5.1 cm apart. In the first 3 trials, the nursery decks were placed 46 cm apart. In Trials 4 and 5, this distance was increased to 102 cm. A sheet of aluminum with the same dimensions as the sides of the nursery decks was suspended equidistantly between the center nursery deck and one of the side nursery decks. The

location of this barrier, i.e. to the north or south of the center deck, was determined by random selection prior to each trial.

At the beginning of each trial, 13 pigs were randomly assigned to 4 groups corresponding to 4 degrees of PRRSV exposure: primary exposure, direct contact, close contact, or indirect contact. The pigs in the primary exposure group (n=3) were placed in the center nursery deck and administered 0.5 ml of 2.0×10^5 TCID₅₀/ml of PRRSV (ATCC VR-2402) inoculum into each naris during inspiration. Two days post inoculation (PI), the pigs in the direct contact group (n=3) were placed in the center deck with the primary exposure group; the pigs in the close contact group (n=3) were placed in the side deck without the barrier; and the indirect contact group (n=4) were placed in the side deck separated from the center deck by the barrier. Each trial lasted 31 days from the time of primary exposure to termination.

Virus

The PRRSV isolate (ATCC VR 2402) was derived from clinically affected pigs from a herd experiencing an acute outbreak of PRRS. Homogenates of tissues from these pigs were inoculated into gnotobiotic pigs. In turn, PRRSV was isolated on porcine alveolar macrophages (PAM) from tissue homogenates from the inoculated gnotobiotic pigs. The isolate underwent

limiting-dilution cloning 3 times in PAM, then adaptation and plaque purification in a monkey kidney continuous cell line (MA104).

The titer of virus inoculum used in this study was determined by a direct fluorescent test. Serial 10-fold dilutions of virus were made in 96-well microtitration plates (Corning Glass Works, Corning, NY), using a high-glucose minimum essential medium (JRH Biosciences, Lenexa, KS) supplemented with 30 µg of neomycin sulfate (Sigma Chemical Company, St. Louis, MO) /ml and 1.2 mg of sodium bicarbonate/ml. Confluent MA104 cells were inoculated with virus dilutions in replicates of 8. Wells were observed for cytopathic effect (CPE) at 4 to 5 days after inoculation. The wells were fixed with 80% acetone/water solution and allowed to air dry. The cell monolayer was flooded with PRRSV specific fluorescent monoclonal antibody conjugate SDOW17 (Dr. David Benfield, South Dakota State University, Brookings, SD) and then placed in a humid 37 C incubator for 30 minutes. The plates were rinsed in a phosphate buffered saline bath for 5 minutes, then a distilled water bath for one minute. After air drying, the plates were observed under a fluorescent microscope. Tissue culture infective dose titers (TCID₅₀/ml) were calculated using the Kärber method (Schmidt and Emmons, 1989).

Sample collection

Blood samples were drawn at the start of the trial and on day 31 PI from the orbital sinus using modified capillary tubes (S/P® Brand Natelson Capillary Tubes, Baxter Healthcare Corporation, McGaw Park, IL) as previously described (Huhn et al., 1969) or the anterior vena cava using a single use system (Vacutainer®, Becton Dickinson Vacutainer Systems, Rutherford, NJ). Serum was harvested by allowing the blood to clot and centrifuging tubes at 1000 x g for 10 minutes. The serum samples were stored at -80 C until serological and virus isolation assays were conducted.

PRRSV serology

The presence of PRRSV antibodies was determined by an indirect fluorescent antibody (IFA) test. Eight-chamber slides (Nunc Inc, Naperville, IL) were inoculated with MA104 cells and incubated at 37 C for 24 hours. Wells were inoculated with PRRSV, then incubated at 37 C for an additional 36 to 48 hours. The slides were fixed in an 80% acetone/water solution, dried, and stored at -80 C until needed. Serum IFA titers were determined by making an initial 1:20 dilution of serum samples followed by twofold dilutions. The slides were incubated with serum dilutions for 30 minutes at 37 C, then rinsed. Goat anti-swine immunoglobulin fluorescent antibody conjugate (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD) was

added and the slides were incubated for an additional 30 minutes at 37 C, after which the slides were rinsed, dried, and read by ultraviolet microscopy.

Virus assay

Virus isolation was performed using PAM. The PAM were harvested from 4- to 6-week-old pigs by lung lavage, suspended in RPMI 1640 media (Sigma Chemical Company, St. Louis, MO) supplemented with 10% fetal calf serum (Sigma Chemical Company, St. Louis, MO), 10 mM HEPES (Sigma Chemical Company, St. Louis, MO), and antibiotics-antimycotics (Sigma Chemical Company, St. Louis, MO), then placed in 48-well plates (Corning Costar, Cambridge, MA) at a rate of 10^6 cells/well. The plates were incubated for 24 hours at 37 C in a 5% CO₂ environment. One-day-old cultures of PAM were inoculated in duplicate with each sample by replacing culture media with 0.25 ml of sample. Inoculated cells were incubated for 60 minutes at 37 C. One half ml of RPMI 1640 growth media was then added to all wells. The cells were incubated at 37 C in a 5% CO₂ humidified atmosphere and observed daily for up to 7 days for CPE. The presence of PRRSV in cultures exhibiting CPE was confirmed by a direct fluorescent test. For this, media from wells with CPE was subinoculated onto MA104 cell monolayers prepared on 96-well plates 24 hours prior to use. Inoculated cells were incubated for 48 hours at 37 C, then fixed with cold acetone:methanol (70:30). The presence of PRRSV antigen in cells was confirmed by staining

with monoclonal antibody SDOW17. Samples were considered negative after one blind passage.

Animal care and maintenance

Confounding the results through inadvertent transmission of PRRSV by persons carrying out routine daily tasks associated with animal care and maintenance was a matter of concern. To reduce this risk, 2 individuals trained in the control of infectious agents carried out all caretaking duties in all 5 trials. To avoid mechanical transmission by PRRSV-contaminated humans or fomites, pigs and decks were not handled or touched by caretakers once a trial began. Feeders were easily accessed and filling feeders did not require touching either pigs or pens. Feed for each deck of pigs was stored separately and individually in plastic barrels. The possibility of inadvertent transmission of infectious agents between decks via virus-contaminated urine and feces was a concern, as well. To facilitate waste removal, a "soaker" hose continuously dampened the floor with water. Water from the hose flowed the entire length of the decks to a gutter built along one wall. This facilitated the continuous removal of urine and prevented feces from drying or adhering to the floor surface. Once daily, a long handled squeegee was used to push waste materials from under the nursery decks to the gutter. Care was taken during cleaning procedures to avoid splashing water or generating aerosols. In the 2 trials in which pigs

died, the investigators wore freshly cleaned coveralls and reached over the sides of the decks to remove the dead pigs without contacting other pigs or the inside of the decks. In each case, the investigators promptly left the room with the carcass.

Room environment

The airflow patterns in the room were determined by the use of smoke sticks (Tel Tru Smoke Sticks, E. Vernon, Benicia, CA). Airflow velocity through the outlet vents was measured using an anemometer (Velocicalc Plus, Model 8360, TSI, St. Paul, MN). A chart recorder (TH8, Dickson, Addison, IL) was used to continuously monitor room temperature and relative humidity throughout the trials.

Results

Clinical observations

Two pigs died during the trials. One of the experimentally inoculated pigs in Trial 3 died acutely on Day 13 PI and one pig from the close contact group died on Day 19 PI of Trial 4. Both of these individuals were PRRSV infected. It was not possible to determine the exact cause of death or if PRRSV contributed to their deaths. Typical of isolate VR-2402, no overt clinical disease was seen in the remainder of the pigs, including the primary exposed pigs.

Airflow patterns

As shown in Figure 1, air entered the room from a ceiling vent located above the back of the center nursery deck. Air passed through the center nursery deck and moved toward the front of the room. Secondary airflow followed a circular movement in 3 dimensions causing a thorough dispersion of air throughout the room. The smoke flow also showed secondary airflow from the center nursery deck to the side decks. Smoke-laden air moved up and over the top of the aluminum barrier placed between the center deck and a side deck, indicating that aerosol-borne virus could potentially reach pigs behind the barrier. Air was found to flow through the outlet vents at a collective rate of 590 cubic feet per minute, providing 14.5 room air exchanges per hour.

The chart recorder provided continuous measurement of temperature and relative humidity. Room temperature and relative humidity readings taken from the recorder charts at 6 hour intervals (6:00 a.m., noon, 6:00 p.m., and midnight) for each day of the trials are presented in Figure 2.

IFA serology

Virus isolation and IFA results are presented in Tables 1 and 2. Serum samples with IFA antibody titers $\geq 1:20$ indicated infection had occurred, either through challenge inoculation or pig-to-pig transmission. All pigs were IFA seronegative ($< 1:20$) for PRRSV antibodies at the start of the trials.

All surviving inoculated pigs were seropositive on Day 31 PI. All of the direct contact pigs with the exception of one pig in Trial 5 were seropositive on Day 31 PI. One or more of the pigs in the close contact group from Trials 1, 2, and 4, including the close contact group pig which died on Day 19 PI, were seropositive. In Trials 3 and 5, the close contact pigs remained seronegative. The indirect contact pigs did not seroconvert in Trials 1, 2, and 5, but did in Trials 3 and 4.

Virus isolation

In several cases, pigs were no longer viremic at the time of sampling on day 31 PI but were serologically positive. In Trial 2, only one pig from the close contact group was seropositive but the other 2 pigs were viremic. Virus was not recovered in any of the cases in which all members of a group were seronegative.

Discussion

Even after several years of intense research effort, fundamental issues in the ecology of PRRSV remain unclear. This study was conducted to further our understanding of the transmission of PRRSV. Using infected animals as the source of virus, susceptible pigs were placed at different levels of controlled exposure to determine if transmission would occur. The selected experimental conditions were designed to represent specific types of

exposure likely to occur in the field: direct contact, close contact, and indirect contact.

Sentinel pigs placed in the deck with inoculated pigs were used to represent direct contact exposure. Equally, this group served to confirm that experimentally inoculated pigs shed infectious virus.

Close contact exposure was modeled by placing pigs in an adjoining nursery deck with no obstructions between the 2 decks. Decks were separated by a distance of 46 cm for Trials 1, 2, and 3, and 102 cm for the last 2 trials. At either distance, transfer of feces, urine, and other body secretions by splashing or splattering from the center deck to the adjacent deck occurred, but direct or nose-to-nose contact between pigs was not possible. The longer distance was used in the latter 2 trials to simulate contact between pens separated by aisles or walkways.

Indirect contact was simulated by placing an aluminum barrier equidistantly between the center pen containing the 3 inoculated and 3 direct contact pigs, and a side deck containing 4 susceptible pigs. Indirect contact represented exposure of adjacent pens with solid walls. By the exclusion of other routes, transmission to this group occurred by exposure to PRRSV-contaminated droplets from feces, urine, the respiratory tract, and/or other body fluids.

Virus isolation and IFA results were used to determine if transmission occurred. In some cases, pigs were no longer viremic at the end of the trial and infection was demonstrated by the presence of anti-PRRSV antibodies. If both virus isolation and serological results were negative, it was concluded that transmission had not occurred.

In a previous study, sentinel pigs placed in direct contact with inoculated pigs became viremic within 3 days of exposure and expressed IFA titers to PRRSV within 14 days of exposure (Yoon et al., 1993). In the current study, animals were given 29 days of exposure. All pigs placed in direct contact with inoculated pigs became infected, with a single exception. One direct contact pig in the fifth trial remained uninfected after 29 days of exposure to infected penmates.

Virus was transmitted to close contact pigs in 2 of the 3 trials in which the decks were separated by a space of 46 cm. The distance was increased to 102 cm in the last 2 trials to determine if transmission would still occur over distances representative of aisles between pens. The close contact pigs became infected in one of these latter 2 trials. More remarkable than the transmission of PRRSV across this relatively short space was the failure of transmission in 2 of the trials. Given the documented ability of PRRSV to move rapidly between herds and across entire continents, the absence of transmission under these circumstances was unexpected. The source of

virus for the close contact group was uncertain. Substantial amounts of feed, urine, feces, and possibly other body fluids, were splattered about the room by the infected pigs in the center deck. It is not known which of these materials served as a source of virus.

Similar results were seen in the indirect contact pigs. The placement of a barrier between decks was intended to prevent direct transfer of feed, feces, and body fluids while allowing transmission of virus via droplets and droplet nuclei. Airflow studies demonstrated that the primary airflow passed through the center nursery deck containing 5 to 6 infected pigs. Potentially, droplet nuclei containing virus particles could be moved via secondary airflow to pigs in the side decks. Transmission of PRRSV to the indirect contact group occurred in 2 of the trials. However, it became evident during the trials that the barrier did not prevent the transfer of feed, feces, and urine. Therefore, it was not possible to determine whether transmission occurred via aerosolized virus or by exposure to virus-contaminated materials from infected groups.

Airborne PRRSV has been suggested as a source of infection since the investigation of early outbreaks of the disease (Edwards et al., 1992; Mortensen and Madsen, 1992; Robertson, 1992). Therefore, the absence of transmission of PRRSV across short spaces in a single room containing 5 or more acutely infected pigs was unanticipated. In the field, the most widely

accepted explanation for the area spread of PRRSV has been transmission of aerosolized virus. Relative humidity and temperature during the trials were within ranges encountered under field conditions, and smoke studies indicated that air flow patterns were conducive to aerosol transmission, as well. For these reasons, we expected all susceptible pigs in the room to become infected within the 29 day exposure period.

To date, aerosol transmission of PRRSV from infected to susceptible pigs has not been demonstrated under experimental conditions. The relative difficulty observed in achieving transmission across a short space suggests that airborne transmission is less likely to occur than previously believed, at least under the conditions represented in these experiments. Estimates of the half-life of PRRSV in aerosols under a variety of temperature and relative humidity combinations are needed to define the prerequisites for aerosol transmission. On the basis of these results, it may also be important to seek alternative explanations for the area spread of PRRSV.

Information concerning the transmission of other arteriviruses may provide insight in the transmission of PRRSV. The minimum infectious dose (MID) of lactate dehydrogenase-elevating virus (LDV) when mice were inoculated by intraperitoneal or tail cartilage injection was one ID₅₀. In contrast, the MID for oral, vaginal, rectal, and ocular exposure was 10^{5.3}, 10^{5.3}, 10^{3.3}, and 10^{5.3} ID₅₀, respectively. These results emphasize the

importance of dose and route of exposure on transmission (Cafruny and Hovinen, 1988).

The ecological importance of differences in MID for different routes of exposure was exemplified in studies of LDV transmission. Susceptible 4- to 6-week-old CAF-1, C3H/Hen, BALB/c, and C57 Black male mice placed in the same cage as mice inoculated with LDV only rarely became infected (Notkins and Shochat, 1963; Notkins et al., 1964). In contrast, about 50% of the exposed mice contracted LDV when 4- to 6-week-old General Purpose (GP) Swiss male mice were used. It was noted that GP Swiss mice were more prone to fighting than the other strains of mice. If the incisors of both inoculated and exposed 6-month-old GP Swiss male mice were removed, transmission only rarely occurred. Transmission always occurred if the incisors of the inoculated mice were present, regardless of whether or not the incisors of the exposed mice were removed. If the incisors of only the principals were removed, transmission of virus still occurred a majority of the time. The results suggested that LDV could be transmitted by either injection of saliva or ingestion of blood and tissue. (Notkins et al., 1964).

These studies of LDV in mice may help explain why transmission consistently occurred between pigs in direct contact, but irregularly in pigs separated by even a short distance from PRRSV infected pigs. In the close contact and indirect contact groups, the quantity of PRRSV present in

materials transferred between decks of pigs was apparently not always sufficient to achieve the minimum infectious dose. If PRRSV were present, recent work suggests that the virus would have been quickly inactivated in urine, saliva, fecal slurry, and on non porous fomites (Pirtle and Beran, 1996).

In contrast to close contact and indirect contact exposures, direct contact nearly always resulted in transmission. As in the case of LDV-infected mice, direct contact provided an opportunity for repeated exposure of susceptible pigs to higher levels of virus. In all likelihood, the social dominance behavior and fighting common among pigs facilitated virus transmission.

These experiments effectively demonstrated that transmission of PRRSV does not require direct contact, although pig-to-pig contact is the most efficient mode of transmission. It was shown that transmission can occur between pigs separated up to 102 cm, although the means of transmission across this space were not determined. Since transmission between decks occurred, if less frequently than expected, the source of virus becomes a pertinent question. Shedding of PRRSV in urine has been demonstrated (Rossow et al., 1994), suggesting that urine splashed between decks may have provided the means for transmission. Virus-contaminated feces is another possibility, although the reports are not in agreement regarding the

presence of PRRSV in feces. Yoon et al. (1993) reported extensive fecal shedding by young pigs over a 35 day observation period, while Rossow et al. (1994) found only intermittent shedding in feces. Wills et al. (1996), using PRRSV isolate ATCC VR-2402, found no infectious virus in 56 fecal samples collected from 4 pigs over a period of 32 days, although viral RNA was demonstrated by PCR in 3 of 20 fecal samples.

This work evaluated the transmission of PRRSV under circumstances of exposure representative of field conditions. These results demonstrated that direct contact is the most efficient mode of PRRSV transmission; whereas, absence of physical contact sharply reduced transmission. The experimental design was not entirely successful in that aerosol exposure was confounded by contamination of the pen holding the indirect exposure pigs with urine, feces, and possibly other contaminated biomaterials from infected pigs in other pens. Consequently, aerosol transmission of PRRSV was not corroborated. At this point, we lack information regarding sources of the virus in the transmission cycle. This is imperative to our understanding of the mechanism(s) of pig-to-pig, as well as area spread. Therefore, our subsequent studies will focus on the characterization of routes and duration of virus shedding.

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Table 1. Summary of indirect immunofluorescent antibody (IFA*) test and virus isolation (VI†) results from serum.

Trial	Inoculated		Direct Contact		Close Contact		Indirect Contact	
	VI	IFA	VI	IFA	VI	IFA	VI	IFA
1	0/3	3/3	1/3	3/3	2/3	3/3	0/4	0/4
2	1/3	3/3	1/3	3/3	2/3	1/3	0/4	0/4
3	1/2	2/2	0/3	3/3	0/3	0/3	2/4	4/4
4	0/3	3/3	0/3	3/3	0/3	3/3‡	0/4	4/4
5	0/3	3/3	0/3	2/3	0/3	0/3	0/4	0/4

*Number of IFA seropositive pigs per total number of pigs in treatment group

†Number of PRRSV positive pigs per total number of pigs in treatment group

‡Serum from one pig taken at post mortem 19 days post inoculation

Table 2. Summary of PRRSV infection status of the groups during the trial periods.

Trial	Inoculated	Contact		
		Direct	Close	Indirect
1	+	+	+	-
2	+	+	+	-
3	+	+	-	+
4	+	+	+	+
5	+	+	-	-

- + Indicates recovery of PRRSV or seroconversion by indirect immunofluorescent antibody test in one or more members of a group
- Indicates neither recovery of virus nor seroconversion occurred in any members of a group.

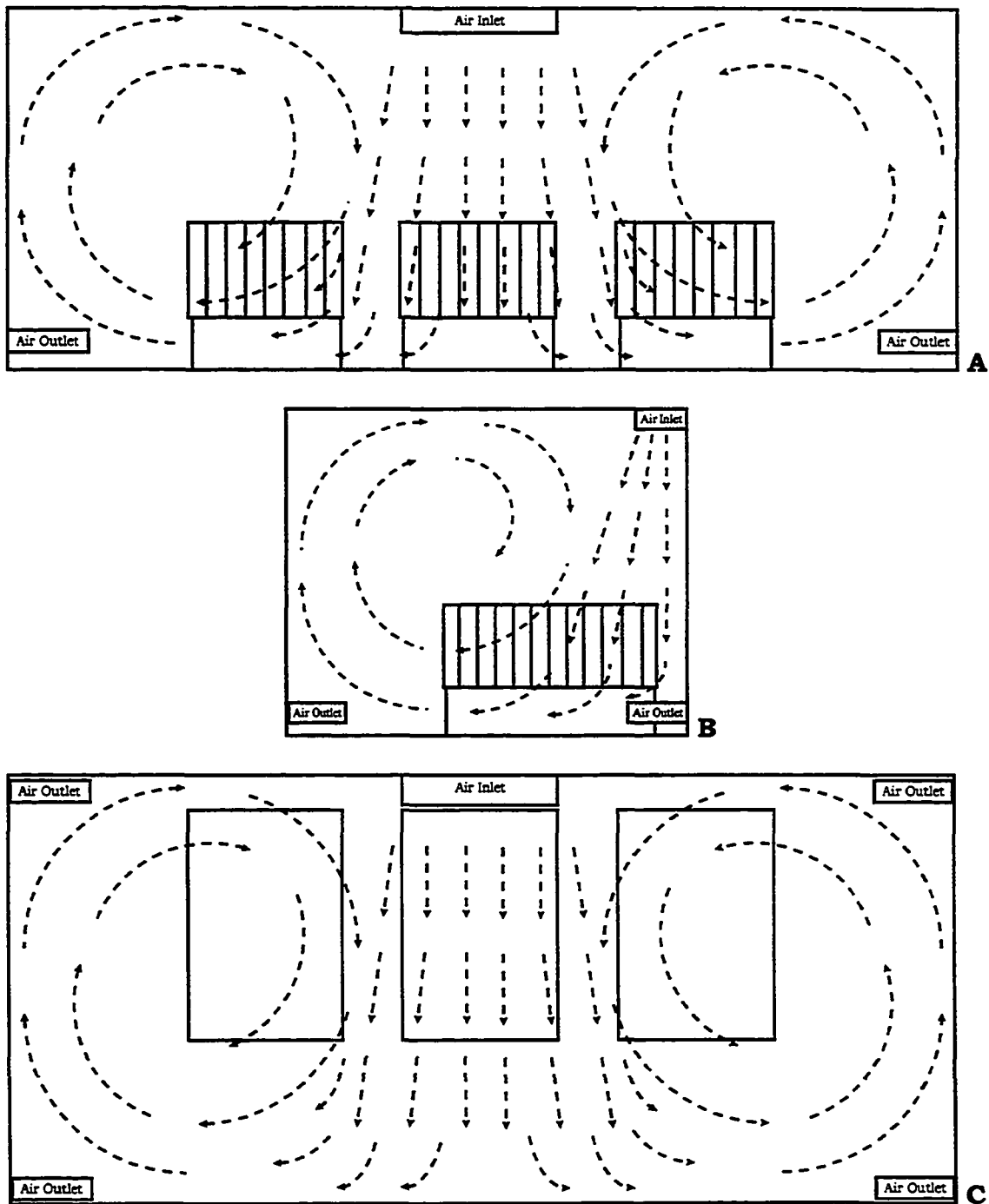


Figure 1. Arrangement of nursery decks in room and airflow patterns. Front to back view (A), side view (B), and top view (C).

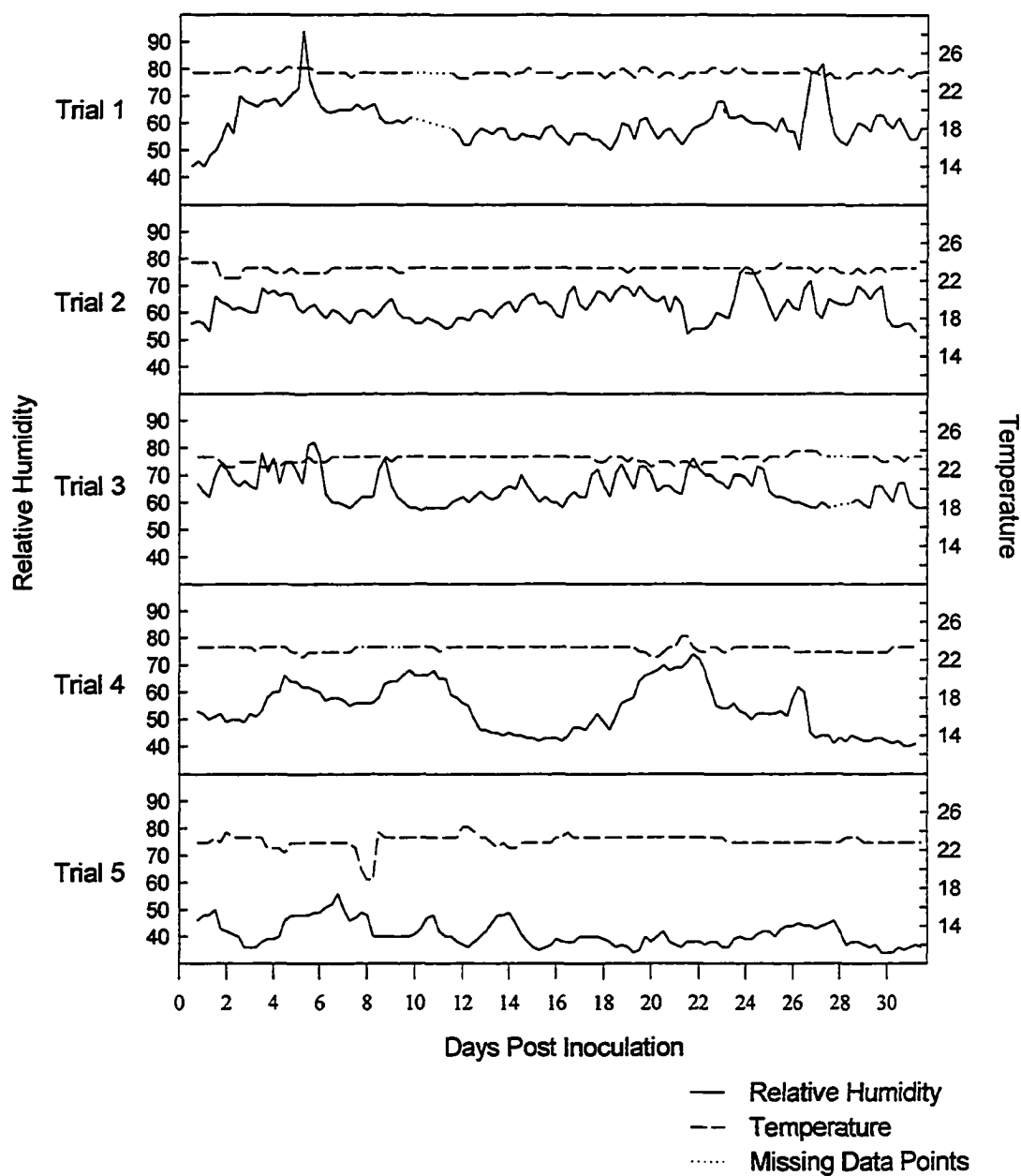


Figure 2. Room temperature (°C) and relative humidity (%) readings taken at 6 hour intervals during trial periods.

PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS: PORTALS OF EXIT

A paper submitted to Preventive Veterinary Medicine

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Abstract

This study was conducted to delineate potential sites of exit and duration of shedding of porcine reproductive and respiratory syndrome virus (PRRSV). Two replicates of 6 pigs each were conducted. Pigs were farrowed in isolation, weaned at 7 days of age, and housed in individual HEPA filtered isolation chambers. In each replicate, 3 pigs served as controls and 3 pigs were intranasally inoculated with PRRSV (ATCC VR-2402) at 3 weeks of age. On days 7, 14, 21, 28, 35, and 42 post inoculation (PI), pigs were anesthetized and intubated. While anesthetized, the following samples were

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collected: serum, saliva, conjunctival swabs, urine by cystocentesis, and feces. Upon recovery from anesthesia, the endotracheal tube was removed, rinsed, and the rinse retained. The sampling schedule was expanded in replicate 2 and serum, saliva, and oropharyngeal samples were collected from day 55 to day 124 PI at 14 day intervals. Virus isolation procedures were carried out on samples using porcine alveolar macrophages. Virus was isolated through day 14 from urine, day 21 from serum, day 35 from endotracheal tube rinse, day 42 from saliva, and day 84 from oropharyngeal samples. No virus was recovered from conjunctival swabs, fecal samples, or negative control samples. This is the first report of isolation of PRRSV from saliva. Virus-contaminated saliva, especially when considered in the context of social dominance behavior among pigs, may play an important role in PRRSV transmission. These results support previous reports of persistent infection with PRRSV with prolonged recovery of virus from tonsils of swine.

Keywords

Porcine reproductive and respiratory syndrome, PRRS virus, epidemiology, portals of exit, transmission, shedding

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV), an emerging virus, was first isolated in 1991 (Wensvoort et al., 1991). Based

on similarities in morphology, structural proteins, genome size and polyadenylation, and preference for replication in macrophages, it has been suggested that PRRSV be included in the proposed family Arteriviridae, along with lactate dehydrogenase-elevating virus (LDV), equine arteritis virus (EAV), and simian hemorrhagic fever virus (SHFV) (Plagemann and Moennig, 1992; Meulenberg et al., 1993).

A remarkable characteristic of PRRSV has been its rapid worldwide spread through domestic swine populations. The first outbreaks of the disease were reported in 1987 (Keffaber, 1989). The disease had been reported in 11 states in the United States and 2 provinces of Canada by 1990 (Hill, 1990). In Europe, the syndrome was first recognized in Germany in 1990 (Leyk, 1991). The disease spread rapidly through Europe appearing in Belgium (Varewyck, 1991), England (White, 1991), France (Baron et al., 1992), Holland (Wensvoort et al., 1991), Spain (Plana et al., 1992) and Taiwan (Chang et al., 1993) by 1991; Denmark by 1992 (Bøtner et al., 1994); and Japan (Murakami et al., 1994) by 1993. A study of the seroprevalence of PRRSV in swine in the state of Iowa revealed the number of infected herds increased from 3.8% (1/25) in 1985 to 63% (10/17) by 1988 (Owen et al., 1992). The means by which this high rate of transmission was achieved has not been clearly defined. In the initial outbreaks of the disease in Europe, transmission among herds was

frequently attributed to airborne spread (Edwards et al., 1992; Mortensen and Madsen, 1992; Robertson, 1992). Experimental trials, however, have not yet corroborated this hypothesis (Wills et al., 1994). Either the virus is not readily transmitted by the airborne route or it requires as-yet-undefined environmental conditions. More is known regarding other aspects of the transmission of PRRSV. Viable PRRSV has been shown to be present in semen collected from experimentally inoculated boars, although it was not initially suspected as a source of virus (Swenson et al., 1994). Gilts inseminated with fresh semen from experimentally infected boars developed antibodies against PRRSV, indicating transmission occurred via semen (Yaeger et al., 1993). Reports of the presence of PRRSV in feces, nasal secretions, and urine suggest other routes of transmission, as well (Yoon et al., 1993; Rossow et al., 1994).

In order to develop and implement successful prevention and control programs for PRRSV infection, a thorough understanding of its epidemiology is needed. However, many of the factors involved in the transmission of PRRSV have not been adequately characterized. The objective of this study was to further delineate potential portals of exit and duration of PRRSV shedding.

Materials and Methods

Experimental Animals and Design

Two replicates of 6 crossbred pigs each were conducted. Each replicate represented pigs from one litter. The sows originated from a herd periodically tested for PRRSV and known to be free of the virus. The dams and offspring were handled in such a fashion as to reduce the possibility of confounding the results with concurrent infections. Sows were placed in farrowing crates in isolation rooms and washed with chlorhexidine diacetate (Nolvasan Solution®, Fort Dodge Laboratories, Fort Dodge, IA) prior to farrowing, and given 4.0 mg/kg ceftiofur sodium (Naxcel®, UpJohn Company, Kalamazoo, MI) intramuscularly (IM) once daily for 7 days beginning 2-4 days prior to farrowing. Pigs were given 100 mg iron dextran (The Butler Company, Dublin, OH) IM at one and 10 days of age and 22.0 mg/kg ceftiofur sodium IM for 7 days beginning at 4 days of age. Pigs were weaned as a litter at 7 days of age and placed into individual HEPA-filtered, heated isolation chambers. At 3 weeks of age the pigs were randomly assigned to one of 2 treatments. Three pigs were inoculated with PRRSV (ATCC VR-2402) by instilling 0.5 ml of 2.7×10^5 TCID₅₀/ml (replicate 1) or 3.6×10^3 TCID₅₀/ml (replicate 2) inoculum into each naris during inspiration. Three pigs in each replicate were maintained as non inoculated controls. Housing and sampling of the pigs were identical in the 2 replicates

except that the inoculated pigs in replicate 2 were moved from the individual isolation units into a single isolation room 42 days after inoculation and monitored for an additional 124 days post inoculation (PI). One non inoculated control pig from replicate 2 was also placed in a separate isolation room and sampled over this time period.

Isolation Units

Specialized isolation units (Barrier Systems Inc., Tom River, NJ), each with 2 chambers, allowed total isolation of animals for an extended period. Air flowing into each unit was HEPA-filtered to remove environmental microorganisms. Out flowing air was also HEPA-filtered to prevent contamination of the room in which the units were located. The feeding system was equipped with an air lock system to prevent exposure of the enclosed animal to outside agents. Internal flushing mechanisms allowed for disposal of waste products while maintaining a sealed environment. All contact with external surroundings and other animals could be controlled, thus providing independent observations.

Virus

The PRRSV (ATCC VR 2402) isolate used in this study was originally isolated from a herd experiencing an acute outbreak of porcine reproductive and respiratory syndrome (PRRS). Homogenates of tissues collected from clinically affected young pigs were inoculated into gnotobiotic pigs. Virus

was recovered in porcine alveolar macrophages (PAM) inoculated with tissue homogenates from the inoculated gnotobiotic pigs. The isolate underwent limiting-dilution cloning 3 times in PAM, then adaptation and plaque purification in a monkey kidney continuous cell line (MA104).

The titer of virus inoculum used in this study was determined by making serial 10-fold dilutions of virus in 96-well microtitration plates (Corning Glass Works, Corning, NY), using a high-glucose minimum essential medium (JRH Biosciences, Lenexa, KS) supplemented with 30 µg of neomycin sulfate (Sigma Chemical Company, St. Louis, MO) /ml and 1.2 mg of sodium bicarbonate/ml. Virus dilutions were inoculated onto confluent MA104 cells in replicates of 8. Wells were observed for cytopathic effect (CPE) at 4 to 5 days after inoculation. The cell monolayer was fixed with 80% acetone/water and allowed to air dry, then flooded with PRRSV-specific fluorescent monoclonal antibody conjugate SDOW17 (Dr. David Benfield, South Dakota State University, Brookings, SD) and placed in a humid 37 C incubator for 30 minutes. The plates were rinsed in a PBS bath for 5 minutes and a distilled water bath for one minute. After air drying, the plates were observed under a fluorescent microscope. Tissue culture infective dose titers (TCID₅₀/ml) were calculated using the Kärber method (Schmidt and Emmons, 1989).

Radiographic protocol

Thoracic radiographs were taken on days -1, 13, 27, and 41 PI. The pigs were transported individually in clean, plastic containers from the isolation units to the radiology facilities. Only technicians who had no previous contact with swine that day were allowed to handle the pigs. The table surfaces used during the procedure were disinfected prior to contact with the pigs. Lateral recumbent and ventrodorsal recumbent views of the thorax were radiographed. Standard thoracic imaging techniques were used with a 101.6 cm tube film distance. Exposure parameters were adjusted based on pig size. Radiographs were evaluated by a board certified radiologist for the presence of respiratory disease.

Sampling Protocol

Biological samples for virus isolation were collected on days 7, 14, 21, 35, and 42 PI from all inoculated pigs and one randomly selected control animal. Body weights and serum samples were taken from all pigs on these days, as well as day 0 PI. To collect samples, each pig was weighed and premedicated with 0.06 mg/kg atropine sulfate (Phoenix Pharmaceuticals, St. Joseph, MO) IM and 1.0 mg/kg acepromazine maleate (PromAce®, Fort Dodge Laboratories, Fort Dodge, IA) IM. Fifteen minutes later, pigs were masked down with halothane (Halocarbon Laboratories, River Edge, NJ), intubated, and maintained on halothane until sampling was completed.

Following sample collection, the animals were allowed to recover and returned to their isolation chambers.

Serum, conjunctival swab, urine, saliva, feces, and tracheal rinse were collected for virus isolation. Blood samples were drawn from either the orbital sinus using modified capillary tubes (S/P® Brand Natelson Capillary Tubes, Baxter Healthcare Corporation, McGaw Park, IL) as previously described (Huhn et al., 1969) or the anterior vena cava using a single use system (Vacutainer®, Becton Dickinson Vacutainer Systems, Rutherford, NJ). Serum was harvested by allowing the blood to clot at room temperature for 30 minutes and centrifuging at 1000 x g for 10 minutes.

Conjunctival samples were taken using a dampened, sterile swab (Dacron®, E.I. du Pont de Nemours and Co., Inc., Baxter Healthcare Corporation, McGaw Park, IL). After swabbing, swabs were placed in a polystyrene tube (Falcon® 2054, Becton Dickinson Labware, Lincoln Park, NJ) containing 1.0 ml of normal saline solution (Baxter Healthcare Corporation, Deerfield, IL).

Urine was collected by cystocentesis using a 0.9 mm x 40 mm needle (Monoject®, Sherwood Medical, St. Louis, MO) and 3 ml syringe (Monoject®, Sherwood Medical, St. Louis, MO) following application of surgical scrub (Betadine®, Purdue Frederick Company, Norwalk, CT) and alcohol (Kendall

Curity®, Kendall Healthcare Products Company, Mansfield, MA) to the caudal ventral abdomen.

Saliva samples were collected by swabbing the sublingual oral cavity with a sterile swab and placing the swab in 1.0 ml of normal saline solution. Saliva samples were collected prior to the extraction of the endotracheal tube to avoid contamination of the oropharyngeal region and buccal cavity with PRRSV from the lower respiratory tract.

Fecal samples were collected using separate sterile fecal loops for each pig. Approximately 0.5 g of feces were collected from the rectum and suspended in 10.0 ml of normal saline solution.

When pigs were sufficiently recovered from anesthesia, the endotracheal tube was removed and rinsed in 2.5 ml of normal saline solution. The saline rinse was divided into 2 aliquots; one of which was submitted for bacteriological culture and the other for virus assay.

In replicate 2, in addition to the samples previously specified, serum, saliva, and oropharyngeal samples were collected approximately every 2 weeks from day 55 to 124 PI. Serum and saliva were collected as described above, but without anesthesia. To collect oropharyngeal samples, animals were restrained with a nose snare and the mouth held open with an oral speculum. A sterile stainless steel spoon with an elongated handle was used to scrape the oropharyngeal area, specifically targeting the palatine

tonsil. The material collected on the spoon was placed, with the aid of a sterile swab, into a capped polystyrene tube containing 1.0 ml of sterile saline.

Following collection, urine and endotracheal tube rinse samples were filtered through 0.22 μm nitrocellulose membrane filters (Corning Costar, Cambridge, MA) to remove bacterial contaminants. Saliva, conjunctival swab, and oropharyngeal samples were similarly filtered following vortexing and removal of the swab. Fecal samples were centrifuged at 2500 x g for 30 minutes, after which the supernatant was sequentially filtered through 0.45 μm and 0.22 μm nitrocellulose membrane filters. Samples were stored on wet ice until assayed for the presence of PRRSV later in the same day.

Virus Assay

Virus isolation procedures were carried out on serum, saliva, oropharyngeal, conjunctival swab, urine, endotracheal tube rinse, and fecal samples using PAM. Four- to 6-week-old pigs from a PRRSV-negative herd were used as PAM donors. The PAM were obtained by lung lavage, suspended in RPMI 1640 (Sigma Chemical Company, St. Louis, MO) media supplemented with 10% fetal calf serum (Sigma Chemical Company, St. Louis, MO), 10 mM HEPES (Sigma Chemical Company, St. Louis, MO), and antibiotics-antimycotics (Sigma Chemical Company, St. Louis, MO), and placed in 48-well plates (Corning Costar, Cambridge, MA) at a rate of 10^6

cells/well. The plates were incubated for 24 hours at 37° C in a 5% CO₂ environment. One-day-old PAM cultures were inoculated in duplicate with each sample by replacing culture media with 0.25 ml of sample. Inoculated cells were incubated for 60 minutes at 37° C. One half ml of RPMI 1640 growth media was then added to each well. The cells were then incubated at 37° C in a 5% CO₂ humidified atmosphere and observed daily for up to 7 days for CPE. The presence of PRRSV in cultures exhibiting CPE was confirmed by a direct fluorescent test. For this, the media from wells with CPE was subinoculated onto MA104 cell monolayers prepared on 96-well plates 24 hours prior to use. Inoculated cells were incubated for 48 hours at 37° C, then fixed with cold acetone:methanol (70:30) mixture. The presence of PRRSV antigen in cells was confirmed by staining with SDOW17. Samples were considered negative after one blind passage.

Bacteriology

Endotracheal tube rinses were plated on blood agar, Tergitol-7 (Difco Laboratories, Detroit, MI), MacConkey (Difco Laboratories, Detroit, MI) and PMD (Ackermann et al., 1994). Blood agar plates were prepared by mixing 5% citrated bovine blood with Tryptose Blood Agar Base (Difco Laboratories, Detroit, MI). Citrated bovine blood was prepared by collecting 2 liters of bovine blood in a sterile flask containing 40 g sodium citrate (Fischer Scientific) and 200 ml water. The PMD agar, which is selective for

Pasteurella multocida, was prepared by supplementing 5% blood agar with the following antibiotics to produce a plate concentration of 3.75 U/ml bacitracin, 5 µg/ml clindamycin, 0.75 µg/ml gentamicin, and 5 µg/ml amphotericin B (Sigma Chemical Company, St. Louis, MO).

Plates were divided and 2 samples were applied per plate. One blood agar plate and the selective and differential media plates were incubated aerobically. A second blood agar plate was also streaked and incubated anaerobically. Following incubation for 24 hours the plates were evaluated for significant bacteria. Organisms were identified by conventional methods of colony morphology, microscopic morphology, and biochemical techniques (Quinn et al., 1994).

Serology

Serum samples were submitted as a block to the Iowa State University Veterinary Diagnostic Laboratory for analysis. All serum samples were assayed by enzyme-linked immunofluorescent assay (ELISA) for anti-PRRSV antibodies. In addition, serum samples from days 0 and 42 PI from both replicates and day 124 serum samples from replicate 2 were submitted for serological assays for detection of antibodies against *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae*, swine influenza virus (SIV), transmissible gastroenteritis virus (TGEV), and pseudorabies virus (PRV).

For PRRSV serology, serum samples were completely randomized and then assayed by a commercially available ELISA (HerdChek: PRRS®, IDEXX Laboratories, Inc., Westbrook, ME) following the procedures described by the manufacturer. Samples were considered positive if the calculated sample to positive (S/P) ratio was 0.4 or greater. *M. hyopneumoniae*- and *A. pleuropneumoniae*-specific antibody titers were determined by microtitration complement-fixation tests based on a previously published protocol [Slavik et al., 1972]. A microtitration neutralization test (Snyder et al., 1981) was used to determine TGEV-specific antibody titers. Anti-SIV titers were assayed by a microtitration hemagglutination inhibition test [Snyder et al., 1981]. Sera were screened for the presence of PRV-specific antibodies by a commercial ELISA test kit (HerdChek:Anti-PRV(S)®, IDEXX, Westbrook, ME).

Results

Both groups of pigs in each replicate remained healthy with no observable clinical signs of disease. Radiographic evidence of thoracic disease was not observed in either group. Contaminants and normal flora including *Escherichia coli*, alpha *Streptococcus sp.*, *Staphylococcus sp.*, and *Pseudomonas sp.* were cultured from endotracheal tube rinses. Bacteriological cultures were negative for pathogenic organisms.

M. hyopneumoniae, *A. pleuropneumoniae*, and TGEV titers were negative on day 0, 42, and 124 PI serum samples. Pig 301 from replicate 2 was seropositive for PRV on day 124 PI. An additional serum sample from pig 301 collected on day 112 PI was tested to determine when this pig had seroconverted and the day 112 PI sample was negative.

Hemagglutination inhibition test results for SIV are presented in Table 1. All pigs were SIV seropositive on day 0 PI but titers had dropped at least 4 fold by day 42 PI with the exception of pig 305. All pigs from replicate 2 which were tested on day 124 were seronegative.

Serum samples collected on day 0 PI were negative for anti-PRRSV antibodies by ELISA for all pigs. All of the inoculated pigs seroconverted by ELISA test by day 14 PI and remained seropositive through the completion of the study. None of the negative control animals seroconverted during the study period.

Body weight gain over the 42 day period of the replicates was evaluated by analysis of variance (PROC GLM, SAS Institute Inc., Cary, NC). The mean weight gain for non inoculated and PRRSV inoculated pigs was 13.58 kg (SD=1.74) and 12.61 kg (SD=1.14) respectively. When replicate and treatment group were considered in the model, differences in weight gain between non inoculated and PRRSV inoculated pigs were not significantly different ($p=.2308$). If the replicate-treatment group interaction was added

to the model, the differences in weight gain between the two groups approached significant values ($p=0.1432$) suggesting a trend that PRRSV infection lowered weight gains. Mean weight gain of PRRSV infected and non inoculated pigs in the first replicate were very similar, 12.63 kg and 12.1 kg, respectively. In the second replicate, the non inoculated pigs (15.1 kg) considerably out performed the PRRSV infected pigs (12.6 kg).

In a separate analysis, repeated measurements of body weight over time were analyzed as a split plot design using conservative degrees of freedom (1, 8) in F tests. The analysis disclosed a significant interaction between replicate and day PI ($p=0.0188$) and a significant three way interaction among treatment group, replicate, and day PI ($p=0.0495$). The interaction between treatment group and day PI approached significance ($p=0.1525$). The mean body weights of the pigs from both groups were consistently similar in the first replicate. However, in the second replicate, the non infected pigs had steadily increasing mean body weights in comparison to the PRRSV infected pigs. These differences between the replicates were reflected in the replicate-day and replicate-day-treatment group interactions.

The presence and duration of shedding of virus was dependent on the sample assayed (Table 2). Serum samples from all inoculated pigs were positive for the presence of PRRSV on days 7 and 14 PI. One pig was

viremic on day 21 PI, as well. Virus was isolated from the saliva samples from 5 of 6 inoculated pigs on one or more days and recovered intermittently from the saliva of one pig through day 42 PI. Endotracheal tube rinse samples from 5 of 6 inoculated pigs were virus isolation positive on one or more days. Virus was isolated from endotracheal tube rinse samples through day 35 PI. Virus was isolated from the urine of 2 pigs, one on day 7 PI and the other on day 14 PI. Virus isolation results from oropharyngeal samples collected in replicate 2 are presented in Table 3. Virus was recovered from oropharyngeal samples up to day 84 PI. Oropharyngeal samples from one inoculated pig were virus isolation negative on all sampling dates. Virus was not recovered from conjunctival swab samples, fecal samples, negative control samples, or day 0 PI serum samples.

Discussion

Although PRRSV has been the focus of intensive research efforts for the last several years, many of the fundamental issues concerning its epidemiology have not been well characterized. Central to the issue of transmission are portals of exit and duration of shedding of the virus by swine. The purpose of this work was to more completely define these factors of transmission. Repeated sampling from individual pigs allowed the establishment of temporal patterns of virus distribution and shedding.

Recovery of PRRSV from serum, urine, endotracheal tube rinses, and saliva provided insight into potential routes of transmission.

Sows were medicated prior to and after farrowing with ceftiofur sodium to reduce the bacterial exposure of the pigs. Pigs were also medicated with ceftiofur sodium, weaned at 7 days of age, and placed into isolation chambers to prevent infection with potential pathogens. Bacteriological cultures and radiographic evaluations were used to assess the success of these measures. The lack of signs of respiratory disease by either radiographic or clinical evaluations, the absence of growth of pathogens from endotracheal tube rinses, and negative titers for *M. hyopneumoniae* and *A. pleuropneumoniae* suggest the pigs were free of bacterial respiratory diseases. Negative serological results for TGEV indicated that the pigs were not concurrently infected with porcine respiratory corona virus. The swine influenza titers profiles were consistent with declining maternal antibodies. Although the subclinical seroconversion of one pig to PRV between day 112 and 124 was of concern, it did not compromise the results because it occurred late in the experiment. Taken together these results indicated the pigs were free of common porcine respiratory viruses. The apparent absence of concurrent bacterial and viral respiratory disease agents allowed the characterization of PRRSV ecology without potential interactions with secondary agents.

The lack of clinical signs resulting from PRRSV infection seen in the current study has been a hallmark of PRRSV infection under experimental conditions, even though respiratory disease may be a major clinical component in field cases of the syndrome (Keffaber, 1990).

Histopathological lesions compatible with field cases of PRRS have been reproduced experimentally in gnotobiotic pigs (Collins et al., 1991; Collins et al., 1992) and cesarean derived, colostrum deprived pigs (Pol et al., 1991). Gnotobiotic pigs or cesarean derived, colostrum deprived pigs were not used in the current study because of concerns that they would not respond in a representative fashion to PRRSV infection. Thus, the pigs used in this study provided a basic model to study virus portals of exit and duration of shedding in conventionally raised pigs infected exclusively with PRRSV.

Although mean weight gain of non inoculated pigs was greater than that of inoculated pigs, the difference was not statistically significant. This was not surprising in light of the lack of other clinical signs of disease and the small sample size. The magnitude of differences in weight gain, if solely the result of PRRSV, were small enough to require more experimental units in order to adequately test the differences. Further work in quantifying production losses due to PRRSV is needed. It remains to be seen if measurable production losses occur in PRRSV infected pigs in the absence of secondary pathogens.

Negative virus isolation and ELISA results from day 0 PI serum samples confirmed that pigs were PRRSV-free prior to inoculation. Seroconversion measured by ELISA and demonstration of PRRSV in serum up to 21 days PI indicated that inoculated pigs were systemically infected. This is consistent with other reports (Terpstra et al., 1992; Stevenson et al., 1993; Yoon et al., 1993; Rossow et al., 1994). It should be noted that because of the prolonged period of viremia, the possibility of hematogenous spread via biting insects, contact with wounds, needles, and surgical instruments deserves serious consideration and further research as a mode of transmission. Hematogenous spread has been implicated in the transmission of related Arteriviridae viruses. For example, transmission of SHFV from patas to rhesus monkeys through the use of contaminated needles and a multiple dose vial has been reported (London, 1977). Studies of LDV in mice suggested non infected mice could acquire the virus by biting infected mice and ingesting virus laden tissues and blood (Notkins et al., 1964).

Virus was recovered from urine samples in 2 of 26 attempts. Urine samples were not obtained on several occasions due to the limitations imposed by the cystocentesis, as well as urination occurring shortly prior to sampling. The uncollected urine samples resulted in fewer opportunities to isolate PRRSV. This was especially evident in replicate 2 in which fewer

samples were collected and no virus was isolated from urine. Variation in the volume of urine produced prior to sampling may also have affected virus concentration in urine resulting in fewer successful isolation attempts. The rate of recovery in this study, however, is consistent with a previously published report in which virus isolation attempts from urine samples collected post mortem were negative in 10 pigs inoculated with PRRSV 7 days earlier and positive in 1/9 pigs inoculated 28 days earlier (Rossow et al., 1994). Virus isolation from urine has been reported in other arteriviruses. Equine arteritis virus (Neu et al., 1988) and LDV (Notkins and Scheele, 1963) have both been recovered from the urine of experimentally infected animals. The sporadic isolation of PRRSV from urine during the first 2 weeks of infection suggests that urine may be a source of virus to susceptible pigs early in infection.

There are no previous reports of isolation of PRRSV from saliva. In this study, saliva proved to be a rewarding sample for virus isolation with recovery of virus extending through day 42 PI. Although endotracheal tube rinse samples might be expected to have a higher PRRSV recovery rate through extensive contact of the tube with the respiratory tract, saliva samples proved to have roughly equivalent recovery rates. Prolonged isolation of PRRSV from saliva suggests that virus-contaminated saliva, especially when considered in the context of social dominance behavior

among pigs, may play an important role in PRRSV transmission. Studies in mice suggest that transmission of LDV occurs through the injection of saliva containing virus during biting and fighting among cage mates (Notkins et al., 1964).

Oropharyngeal samples were also fruitful samples for recovery of virus. Virus was recovered from oropharyngeal samples from 2 pigs until day 84 PI, i.e. 6 weeks after it was last isolated from any other sample. Virus was recovered from oropharyngeal samples up to day 157 PI in a previous study (Wills et al., 1996). Persistent asymptomatic infections are a consistent characteristic of the members of the proposed family Arteriviridae (Meulenberg et al., 1993; Plagemann and Moennig, 1992). Although the palatine tonsil was specifically targeted during collection, the oropharyngeal samples potentially contained blood traces, saliva, lacrimae, nasal secretions, and respiratory tract secretions. The absence of virus detected in conjunctival swabs, serum after day 21, saliva after day 42, and tracheal rinse samples after day 35 PI suggests that the source of virus in the oropharyngeal samples was not blood, saliva, or lacrimae. These findings provided more evidence that tonsil tissue harbors PRRSV during a persistent infection, but further research is needed to confirm the source of virus in the oropharyngeal samples. Such information may lead to insights

into transmission of virus as well as immune mechanisms involved in clearing PRRSV from the host.

In the current study, PRRSV was not isolated from either fecal or conjunctival samples. There are no previous reports of attempts to isolate PRRSV from conjunctiva. Recovery of PRRSV from fecal samples up to 35 days after experimental inoculation of pigs was reported (Yoon et al., 1993). However, PRRSV was not isolated from 105 fecal swabs collected over days 1, 4, 7, 14, and 21 PI; although, 2 of 15 fecal swabs taken 28 days post inoculation were positive (Rossow et al., 1994). The reason for discordance in results among these studies is not known, but could involve differences among PRRSV isolates, hosts, the enteric environment of pigs, methodology in sampling, or virus isolation protocols. Differences among these studies provide justification for further research in this area.

These results are important to understanding the epidemiology of PRRSV. Shedding of PRRSV occurs from a number of sites, including urine, saliva, and respiratory secretions. Whether PRRSV is commonly shed in feces and contributes to transmission is uncertain at this time. These routes of shedding provide a mechanism for pig-to-pig transmission seen in direct contact exposures. Once in the environment, the virus is relatively labile. At room temperature, virus was recovered only on the same day of application from stainless steel, plastic, boot rubber, alfalfa, wood shavings,

straw, swine saliva, urine, and feces. However, virus persisted in city water (11 days) and well water (9 days), suggesting virus-contaminated water may be a potential route of transmission (Pirtle and Beran, 1996). In some individual animals, PRRSV becomes a persistent infection. In this study, virus was recovered from oropharyngeal scrapings from 2 of 3 animals on day 84 PI. In the field, persistently infected individuals probably serve to perpetuate PRRSV infection in herds, as well as facilitate transmission between herds.

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Table 1. Swine influenza virus microtitration hemagglutination inhibition test results.

Replicate 1			Replicate 2			
Day PI			Day PI			
Pig	0	42	Pig	0	42	124
415	160	10	301	160	10	0
416	80	10	302	320	80	0
418	160	10	303	320	40	NT*
419	80	10	304	160	20	0
420	80	10	305	160	80	NT
421	160	10	306	320	40	0

* Not tested.

Table 2. Virus isolation results from replicates 1 and 2*.

Samples†	Pigs	Days after Inoculation					
		7	14	21	28	35	42
Serum‡	415	+	+	-	-	-	-
	419	+	+	-	-	-	-
	421	+	+	+	-	-	-
	301	+	+	-	-	-	-
	304	+	+	-	-	-	-
	306	+	+	-	-	-	-
Saliva	415	+	-	-	-	+	+
	419	-	+	+	-	-	-
	421	-	+	-	-	-	-
	301	-	-	+	-	+	-
	304	+	+	+	+	-	-
	306	-	-	-	-	-	-
Tracheal Rinse	415	-	+	+	-	-	-
	419	-	-	-	-	-	-
	421	+	-	-	-	-	-
	301	+	-	-	+	+	-
	304	+	+	+	-	-	-
	306	+	+	-	-	-	-
Urine	415	NC§	-	-	-	-	-
	419	NC	+	-	-	-	-
	421	+	-	-	-	-	-
	301	NC	NC	-	-	NC	NC
	304	-	-	-	-	NC	-
	306	-	NC	-	-	NC	NC

* Replicate 1 included pigs 415, 419, and 421. Replicate 2 included pigs 301, 304, and 306.

† All conjunctival, fecal, and negative control (n=6) samples were virus isolation negative

‡ Day 0 PI serum samples were virus isolation negative

§ Urine sample not collected

Table 3. Virus isolation from oropharyngeal samples from replicate 2.

Pigs	Days after Inoculation					
	55	69	84	97	112	124
301	+	-	+	-	-	-
304	+	+	+	-	-	-
306	-	-	-	-	-	-
302*	-	-	-	-	-	-

*Non inoculated control pig

PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS: A PERSISTENT INFECTION

A paper accepted by Veterinary Microbiology

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Abstract

Persistent infection with porcine reproductive and respiratory syndrome virus (PRRSV) was shown in experimentally infected pigs by isolation of virus from oropharyngeal samples for up to 157 days after challenge. Four 4-week-old, conventional, PRRSV antibody-negative pigs were intranasally inoculated with PRRSV (ATCC VR-2402). Serum samples were collected

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every 2 to 3 days until day 42 post inoculation (PI), then approximately every 14 days until day 213 PI. Fecal samples were collected at the time of serum collection through day 35 PI. Oropharyngeal samples were collected at the time of serum collection from 56 to 213 days PI by scraping the oropharyngeal area with a sterile spoon, especially targeting the palatine tonsil. Turbinate, tonsil, lung, parotid salivary gland, spleen, lymph nodes, and serum were collected postmortem on day 220 PI. Virus isolation (VI) on porcine alveolar macrophage cultures was attempted on all serum, fecal, and oropharyngeal samples, as well as tissues collected postmortem. Postmortem tonsil tissues and selected fecal samples were also assayed for the presence of PRRSV RNA by the polymerase chain reaction (PCR). Serum antibody titers were determined by IFA, ELISA, and SVN.

Virus was isolated from all serum samples collected on days 2 to 11 PI and intermittently for up to 23 days in 2 pigs. No PRRSV was isolated from fecal samples, but 3 of 24 samples were PCR positive, suggesting the presence of inactivated virus. Oropharyngeal samples from each pig were VI positive 1 or more times between 56 and 157 days PI. Oropharyngeal samples from 3 of 4 pigs were VI positive on days 56, 70, and 84 PI. Virus was isolated from 1 pig on day 157 PI, 134 days after the last isolation of virus from serum from this animal. Virus was isolated from oropharyngeal samples for several weeks after the maximum serum antibody response, as

measured by IFA, ELISA, and SVN tests. All tissues collected postmortem were VI negative and postmortem tonsil samples were also negative by PCR.

An important element in the transmission of PRRSV is the duration of virus shedding. The results of this study provided direct evidence of persistent PRRSV infection and explain field observations of long-term herd infection and transmission via purchase of clinically normal, but PRRSV infected, animals. Effective prevention and control strategies will need to be developed in the context of these results.

Keywords

Porcine reproductive and respiratory syndrome, PRRS virus, epidemiology, persistent infection, transmission

1. Introduction

As an emerging virus, porcine reproductive and respiratory syndrome virus (PRRSV), has been the focus of an intense research effort since the first report of its isolation in 1991 (Wensvoort et al., 1991). One of the outstanding features of PRRSV has been its high degree of transmissibility. First recognized clinically in 1987, PRRSV spread rapidly through domestic swine populations in Europe, North America, and Asia (Owen et al., 1992; Wensvoort et al., 1992; Bautista et al., 1993; Chang et al., 1993).

Although fundamental to the development of effective prevention and control strategies, the transmission of PRRSV among swine is not yet clearly

understood. Swine infected with PRRSV are known to shed infectious virus by several routes. Virus has been found in semen from experimentally inoculated boars (Swenson et al., 1994). Also, transmission occurred when gilts were inseminated with fresh semen from infected boars (Yaeger et. al., 1993). Virus has also been reported in feces, nasal secretions, and urine (Yoon et al., 1993; Rossow et al., 1994), suggesting other routes of transmission, as well. The purpose of this study was to expand our knowledge of PRRSV shedding patterns. We report the prolonged isolation of infectious PRRSV from oropharyngeal samples and new evidence that PRRSV produces a persistent infection in swine.

2. Materials and Methods

2.1 PRRSV

The PRRSV isolate (ATCC VR-2402) used in the experiment was originally derived from a pool of tissues from clinically affected young pigs from a herd undergoing clinical PRRSV infection. Inoculation of tissue homogenates into a gnotobiotic pig was followed by virus isolation (VI) in porcine alveolar macrophages (PAMs). The isolate was purified by 3 rounds of limiting dilution in PAMs, then plaque purified twice in an African monkey kidney continuous cell line (MA-104).

The titer of virus inoculum used in the study was determined by making serial 10-fold dilutions of virus in 96-well microtitration plates (Corning

Glass Works, Corning, NY, USA), using a high-glucose minimum essential medium (JRH Biosciences, Lenexa, KS, USA) supplemented with 30 µg of neomycin sulfate/ml (Sigma Chemical Company, St. Louis, MO, USA) and 1.2 mg of sodium bicarbonate/ml. Virus dilutions were inoculated onto confluent MA-104 cells in replicates of 8. Wells were observed for cytopathic effects (CPE) at 4 to 5 days after inoculation. The wells were fixed with 80% acetone/water and allowed to air dry. The cell monolayer was flooded with PRRSV fluorescent monoclonal antibody conjugate SDOW17 (David Benfield, South Dakota State University, Brookings, SD, USA) and placed in a humid 37 C incubator for 30 min. Plates were rinsed in a phosphate-buffered saline solution bath for 5 min and a distilled water bath for 1 min. After air drying, plates were observed under a fluorescent microscope. Tissue culture infective dose titers (TCID₅₀/ml) were calculated using the Kärber method (Schmidt and Emmons, 1989).

2.2 Experimental animals

Four 4-week-old pigs were obtained from a herd periodically tested for PRRSV and known to be free of the virus. Pigs were determined to be seronegative for PRRSV antibodies by indirect fluorescent antibody (IFA), serum virus neutralization (SVN), and enzyme linked immunosorbent assay (ELISA). Animals were housed in isolation facilities throughout the experiment. After a 4 day acclimatization period, pigs were intranasally

inoculated with 1 ml of PRRSV at a concentration of $10^{3.2}$ TCID₅₀/ml by instilling 0.5 ml of the inoculum into each nostril during inspiration.

2.3 Biological samples

Serum samples were collected for virus isolation (VI) every 2 to 3 days up to day 42 post-inoculation (PI) and then approximately every 14 days until day 213 PI. Serum samples were also collected for VI at necropsy on day 220 PI. Blood samples were drawn and the serum separated after 30 min at room temperature by centrifugation at 1000 x g for 10 min. Serum samples were refrigerated until VI procedures were started later in the same day. Samples for serological tests were stored at -80 C until the tests were performed.

Fecal samples for VI were collected at the time of serum collection through day 35 PI. Approximately 0.5 grams of feces were collected with a fecal loop and suspended in 10 ml of Hanks' balanced salt solution (HBSS; Sigma Chemical Company, St. Louis, MO, USA) containing 0.5% bovine serum albumin (BSA; Sigma Chemical Company, St. Louis, MO, USA) and antibiotic-antimycotics (500 IU/ml penicillin, 500 µg/ml streptomycin, 250 µg/ml gentamicin, 125 µg/ml amphotericin B). The suspension was clarified by centrifugation at 2500 x g for 30 min and supernatants were sequentially filtered through 0.45 µm and 0.22 µm nitrocellulose membrane filters (Costar Corporation, Cambridge, MA, USA). Virus isolation and

polymerase chain reaction (PCR) procedures were performed on the final filtrates.

Oropharyngeal samples for VI were collected at the time of serum collection on days 56 through 213 PI. Animals were restrained with a nose snare and the mouth held open with an oral speculum. A stainless steel spoon with an elongated handle was used to scrape the oropharyngeal area, specifically targeting the palatine tonsil. With the aid of a Dacron® (E.I. du Pont de Nemours and Co., Inc.) sterile swab (Baxter Healthcare Corporation, McGaw Park, IL, USA), the material collected on the spoon was placed into a capped tube containing 1 ml of sterile HBSS supplemented with 0.5% BSA and antibiotic-antimycotics. The swab was twirled in the medium, broken off, and left in the tube. Samples were vortexed thoroughly and the swabs removed aseptically with forceps. Afterwards, the suspension was clarified by centrifugation at 1500 x g for 10 min and filtered through a 0.22 µm nitrocellulose membrane filter. Virus isolation was carried out on the filtrates.

The pigs were euthanatized by electrocution and exsanguination under the supervision of United States Department of Agriculture, Food Safety and Inspection Service inspectors. Turbinate, tonsil, lung, parotid salivary gland, and spleen samples were collected, as well as tracheobronchial,

mediastinal, iliac, mesenteric, and parotid lymph nodes. Minced tissue specimens were suspended in 20 ml of cold HBSS supplemented with 50 µg/ml gentamicin and antibiotic-antimycotics (100 IU/ml penicillin, 100 µg/ml streptomycin, and 25 µg/ml fungizone). The tissue suspension was homogenized in a Stomacher 400 (Tekmar, Cincinnati, OH, USA) for 20 seconds, then centrifuged at 2000 x g for 15 min. The supernatants were aliquoted and frozen at -80 C until submitted for VI.

2.4 Virus assay

Virus isolation was conducted on PAMs collected by lung lavage from 4- to 6-week-old pigs obtained from a PRRSV-free herd. In preparation for VI, PAMs were placed in 48-well plates (Costar Corporation, Cambridge, MA, USA) at a rate of 10^6 cells/well with RPMI 1640 media (Sigma Chemical Company, St. Louis, MO, USA) supplemented with 10% fetal calf serum (Sigma Chemical Company, St. Louis, MO, USA), 10 mM HEPES (Sigma Chemical Company, St. Louis, MO, USA), and antibiotic-antimycotics (Sigma Chemical Company, St. Louis, MO, USA), then incubated for 24 hr at 37 C in a 5% CO₂ atmosphere.

All samples for VI were processed immediately following collection and each sample was run in duplicate. One-day-old PAM cultures in 48-well plates were inoculated with 0.25 ml samples of serum, fecal filtrates, oropharyngeal, or tissue filtrates and observed daily for CPE for up to 7 days

after inoculation. The presence of PRRSV in cultures showing CPE was confirmed by subinoculating onto MA-104 cell monolayers prepared on 8-chambered glass slides (Nunc, Inc., Naperville, IL, USA), incubating for 48 hours, and staining with PRRSV fluorescent monoclonal antibody conjugate SDOW17. Samples were considered negative after 1 blind passage.

2.5 Serology

Serum antibody titers were measured on samples collected on days 0, 7, 11, 14, 21, 28 and then approximately every 14 days until day 213 PI. Serum samples were randomized and assayed as a block by IFA, SVN, and ELISA. The IFA test was performed using the protocol described by Swenson et al. (1994). SVN test has been described by Yoon et al. (1995). A commercially available ELISA (HerdChek: PRRS, IDEXX Laboratories, Inc., Westbrook, Maine, USA) was performed following the procedures described by the manufacturer. The sample to positive (S/P) ratio was calculated for each sample, with a S/P ratio of 0.4 or greater considered positive.

2.6 Polymerase chain reaction

Tonsil samples collected postmortem and fecal filtrates collected on days 0, 7, 14, 21, 28, and 35 PI were assayed for the presence of PRRSV by PCR. To process tonsil samples for PCR, 1 gram of frozen tonsil tissue was minced slightly in a sterile petri dish, then 2 ml of HBSS was added to the tissue in a sterile plastic bag and homogenized in a Stomacher 80 (Tekmar,

Cincinnati, OH, USA) for 1 min. The supernatant was stored frozen at -80 C. Prior to RNA extraction, the supernatant was thawed and cell debris was removed by centrifugation at 14,000 x g for 15 s. Five hundred µl of the homogenized tonsil tissue supernatant or fecal filtrate was added to an equal volume of lysis buffer (4M guanidinium thiocyanate, 25 mM sodium citrate pH 7, 0.5% sarkosyl, 0.1 M 2-mercaptoethanol). Five hundred µl of the lysate was then added to an equal volume of phenol chloroform-isoamyl alcohol (24:1), vortexed, and centrifuged at 10,000 X g for 5 min. Further extractions, reverse transcription, and outer and nested PCR reactions are described elsewhere (Christopher-Hennings et al., 1995b).

3. Results

Virus isolation results varied among the tissues sampled. Virus was isolated from all serum samples collected on days 2 through 11 PI, then intermittently for up to 23 days in 2 of the animals (Table 1). Serum samples collected on days 25 through 220 were negative for VI. All VI attempts on fecal samples were negative, although viral RNA was detected by PCR analysis of fecal samples from pigs 141, 149, and 128 collected on days 7, 14, and 21, respectively. Oropharyngeal samples from all pigs were positive by VI 1 or more times between 56 and 157 days PI (Table 2). Virus was isolated from 3 of 4 pigs on 56, 70, and 84 days PI. Virus was isolated from 1 animal 157 days PI, which was 134 days after the last isolation of

virus from the serum of this pig. The VI results on oropharyngeal samples are presented in conjunction with serological test results in Figures 1, 2, and 3. Virus was isolated from oropharyngeal samples for several weeks after the maximum serum antibody response, as measured by IFA, ELISA, and SVN tests. Virus was not isolated from any of the tissue homogenates or serum collected following euthanasia on day 220 PI. Viral RNA was not detected by PCR analysis of tonsil tissue collected postmortem.

4. Discussion and conclusions

Research into the epidemiology of PRRSV is still in its infancy and many of the factors involved in transmission of PRRSV have not been defined. Portals of exit and entrance are important areas of continuing research. In part, the intent of this research was to study fecal shedding of PRRSV by swine. However, none of 64 samples collected from 4 pigs were positive by virus isolation. A PCR assay subsequently performed on a subsample of 24 samples detected viral RNA in 3 samples, 1 each from 3 different pigs sampled on days 7, 14, and 21 PI. The infrequency of PCR-positive samples suggested that PRRSV was shed intermittently and at low levels in feces. It has recently been reported that PRRSV is rapidly inactivated in fecal slurry (Pirtle and Beran, 1995). We suggest that the occasional presence of viral RNA and the absence of infectious virus in feces is compatible with intermittent shedding of virus at low levels with rapid inactivation of virus,

perhaps within the intestinal tract itself. There are 2 previously published reports of isolation of PRRSV from feces. Yoon et al. (1993) reported isolation of PRRSV from 55 of 154 fecal samples collected from principal and sentinel pigs. Fecal samples from 4 of 4 experimentally inoculated pigs were VI positive for up to 35 days. Similar to the results reported here, Rossow et al. (1994) isolated PRRSV from only 2 of 15 fecal swabs taken 28 days PI, while 105 fecal swabs collected over days 1, 4, 7, 14, and 21 PI were VI negative. The reason for differences among these studies is not known. Possibly, alterations in the intestinal tract due to physiologic or infectious causes may affect either the rate of shedding of PRRSV or the persistence of intact virus in feces. This is an area which requires further investigation.

A critical feature in the transmission of PRRSV is the duration of infection. Virus isolation from oropharyngeal samples for up to 157 days after experimental inoculation provided direct evidence for persistent infection with PRRSV. This evidence can be added to previous work providing indirect evidence of persistent PRRSV infection in swine. Zimmerman and others (1992) reported transmission by direct contact between susceptible animals and sows infected 99 days earlier. Albina and others (1994) demonstrated transmission of PRRSV by pigs infected more than 15 weeks earlier. Boars have been shown to shed infectious virus in

their semen for up to 43 days (Swenson et al., 1994). Viral RNA has been detected for up to 92 days in semen (Christopher-Hennings et al., 1995a).

The results of this study suggest that PRRSV resembles lactate dehydrogenase-elevating virus (LDV), equine arteritis virus (EAV), and simian hemorrhagic fever virus (SHFV) in its capacity to produce persistent infections (Plagemann and Moennig, 1992). In contrast to PRRSV, persistent infection in LDV and SHFV is characterized by a persistent viremia (Gravell et al., 1986; Plagemann and Moennig, 1992). Because of the sampling process utilized in this work, the oropharyngeal sample potentially consisted of blood traces, saliva, lacrimae, nasal secretions, and respiratory tract secretions. From the work done to date, the palatine tonsil can not be unequivocally stated to be the site of the persistent infection. Further research is needed to define the site(s) of infection.

Isolation of virus from oropharyngeal samples for several weeks after peak IFA, SVN, and ELISA antibody titers indicated that the immune responses measured by these tests were not central to the clearance of the virus from the host. However, negative VI attempts on tissues collected at necropsy, in conjunction with the negative PCR results on tonsil tissue collected postmortem, suggested that the immune system of the host was eventually able to clear PRRSV from the body. The results of this study hold profound implications for our understanding of the immunology and

epidemiology of PRRSV infections in swine. The development of effective PRRSV prevention and control strategies will need to be assessed in the context of this new information.

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Table 1. Virus isolation from serum.

	Days post inoculation																		
Pig No.	0	2	4	7	9	11	14	16	18	21	23	25	28	30	32	35	37	39	42
128	- ^a	+	+	+	+	+	-	+	-	+	+	-	-	-	-	-	-	-	-
135	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
141	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
149	-	+	+	+	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-

^aVirus isolation negative (-) or virus isolation positive (+).

Table 2. Virus isolation from oropharyngeal samples.

Pig No.	Days post inoculation											
	56	70	84	98	115	128	143	157	171	185	199	213
128	+ ^a	+	-	-	-	-	-	-	-	-	-	-
135	-	-	+	-	-	-	-	-	-	-	-	-
141	+	+	+	-	-	-	-	-	-	-	-	-
149	+	+	+	-	-	+	+	+	-	-	-	-

^aVirus isolation negative (-) or virus isolation positive (+).

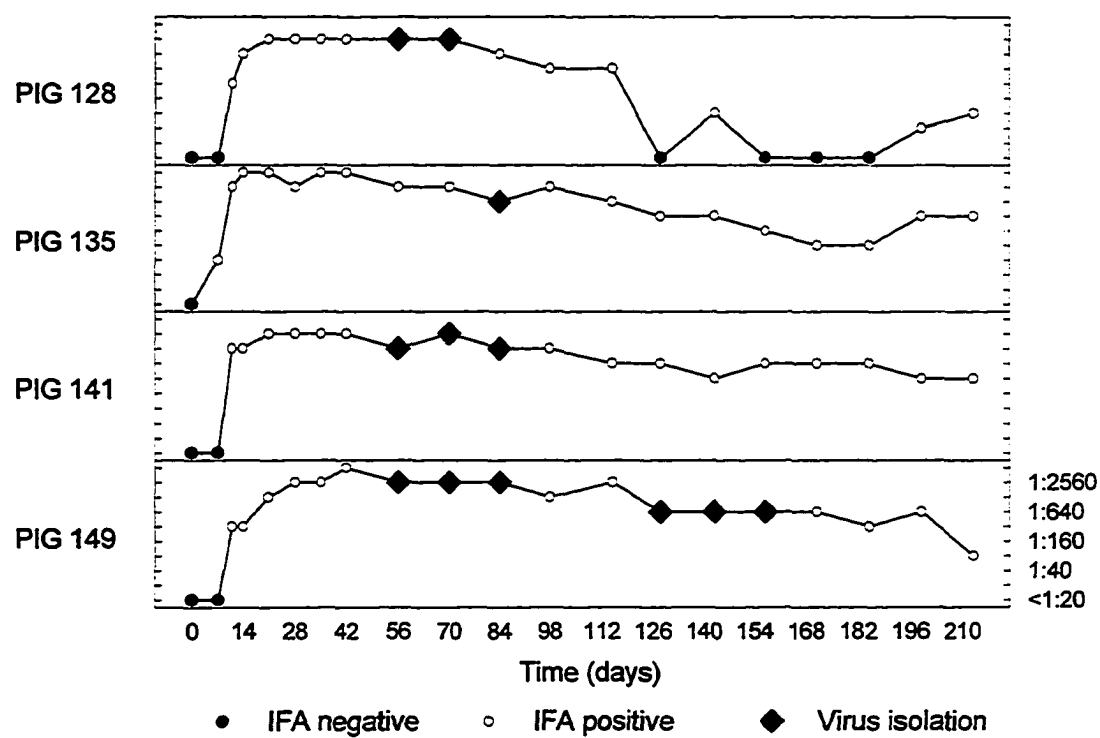


Figure 1. Results of IFA serological test and virus isolation from oropharyngeal samples.

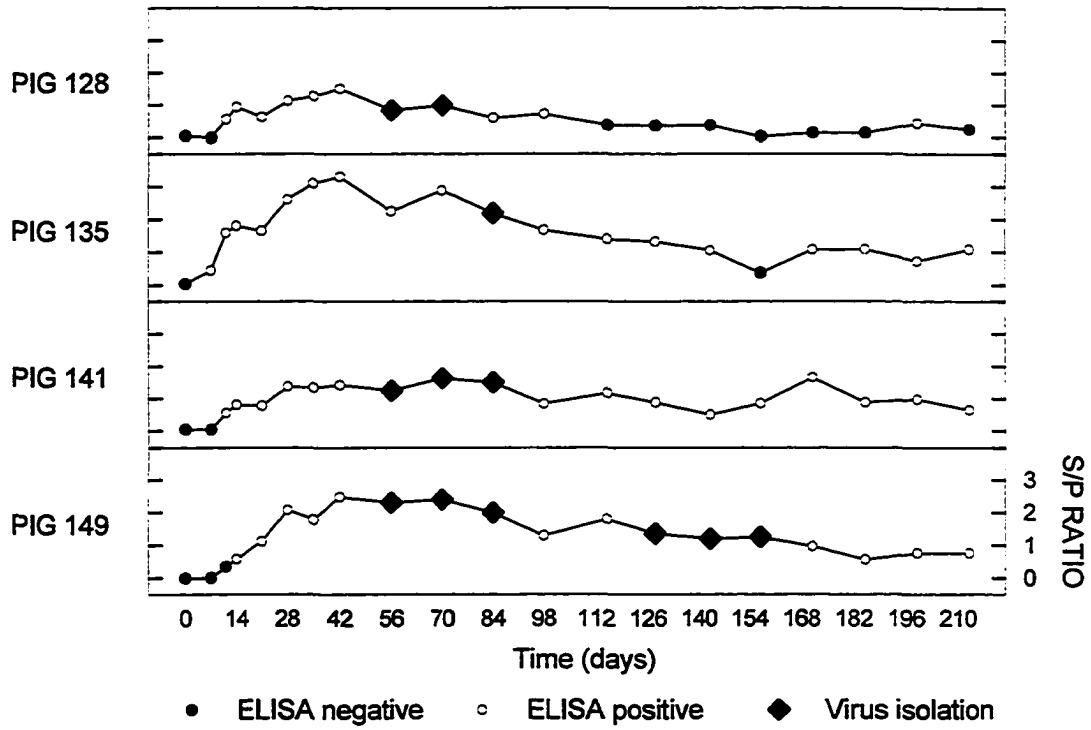


Figure 2. Results of ELISA serological test and virus isolation from oropharyngeal samples.

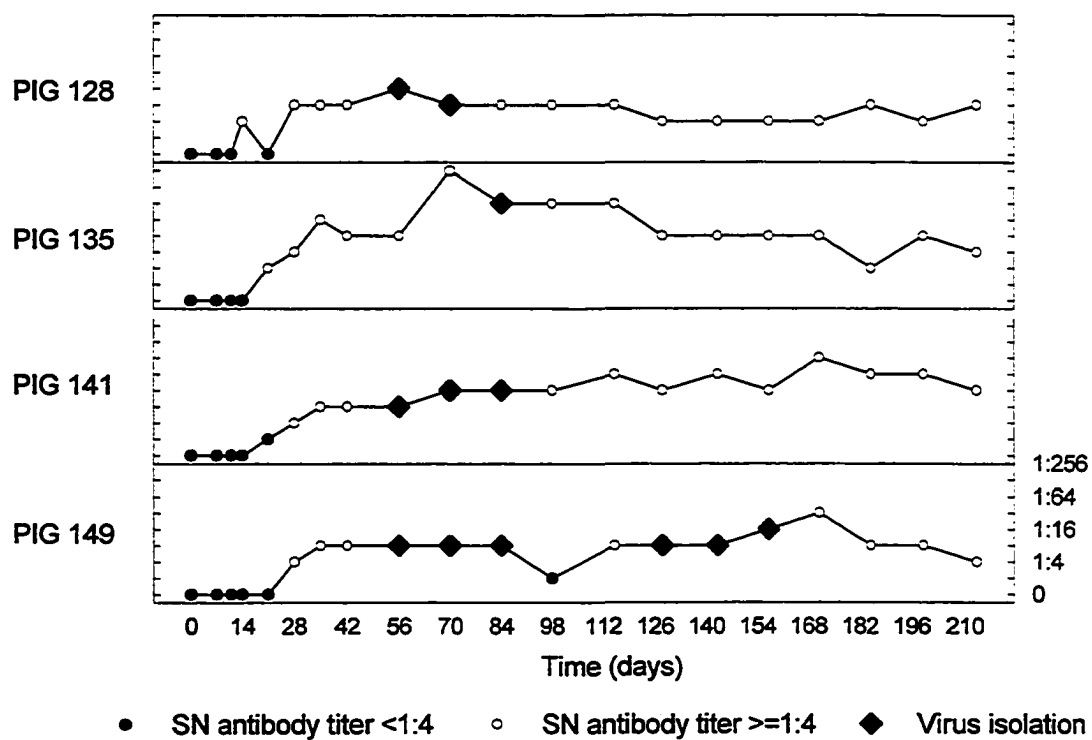


Figure 3. Results of SVN serological test and virus isolation from oropharyngeal samples.

GENERAL DISCUSSION

Porcine reproductive and respiratory syndrome virus (PRRSV) is a relatively recently identified viral disease of swine. It has gained the attention of swine producers, veterinary practitioners, and researchers alike because of its devastating impact on the health and financial well being of swine herds. PRRSV has presented a particular challenge to persons responsible for animal health because of its high degree of transmissibility. Previously absent from domestic swine, it has spread throughout the world in less than 10 years. Understandably, control of transmission of PRRSV within and between individual herds has posed a significant problem to swine producers and veterinarians. Thus, characterization of PRRSV transmission was an essential step to the development and implementation of prevention and control strategies. To that end, the studies presented in this dissertation have significantly advanced our goal of promoting and protecting animal health.

The studies described in this dissertation focused on 3 fundamental issues of PRRSV transmission: 1. methods and routes of transmission; 2. portals of exit and duration of shedding of virus from the infected host; and 3. duration of infection.

The first experiments established the relative rates of transmission of PRRSV from infected to susceptible pigs under circumstances of exposure

resembling field conditions. Nursery decks were arranged so that transmission of virus between pigs in direct, close, and indirect contact could be monitored. Transmission occurred most efficiently between pigs in direct contact. When direct physical contact was prevented, transmission was sharply reduced. The unexpectedly wide dispersion of urine, feces, and possibly other biological materials from pens of infected pigs to susceptible pigs prevented elucidation of the role of aerosols in the transmission of PRRSV. Further research is needed to substantiate postulated airborne spread of PRRSV and determine the environmental circumstances under which it occurs. In particular, estimates of the stability of PRRSV under different conditions of temperature and relative humidity is required. To determine how transmission occurred between pens, information was needed on the source of virus in the transmission cycle. This led to the next series of experiments.

The second study consisted of 2 parts. The objective of the initial phase was to characterize the routes and duration of shedding of PRRSV. Specific attention was focused on the possible presence of PRRSV in the biological materials that were potentially transferred between infected and susceptible pigs in the first study. Virus was isolated through day 14 post inoculation (PI) from urine, day 21 PI from serum, day 35 from endotracheal tube rinses, and day 42 from saliva. Virus was not recovered from fecal samples

or conjunctival swabs. Previously unreported, recovery of PRRSV from saliva was an important new finding. Virus-contaminated saliva, especially when considered in the context of social dominance behavior among pigs, probably plays an important role in transmission. Following the initial phase of the study, oropharyngeal, saliva, and serum samples were monitored for evidence of virus infection for 124 days. Virus was recovered from oropharyngeal samples through day 84 PI. These highly significant results provided direct evidence of persistent infection of swine with PRRSV. Because of its obvious significance to transmission, persistent infection was the focus of the third study.

The goal of the third investigation was to characterize persistent infections of PRRSV. Serum samples and oropharyngeal scrapings were collected periodically for over 7 months. Virus was recovered from oropharyngeal samples consistently through day 84 PI and from one pig up to day 157 PI, i.e., 135 days after virus was last recovered from serum. The duration of persistent infection was nearly twice the time documented in the second study and also exceeded estimates determined by contact transmission studies in previous reports. Serological test results provided insight into the relationship between persistent infection and the humoral immune response. Virus was recovered from oropharyngeal scrapings up to 150 days after seroconversion by IFA and ELISA. An important question is

whether levels of serum antibodies may decline to undetectable levels in persistently infected animals. All pigs from which virus was isolated were seropositive by IFA and ELISA in these studies. Additional work needs to be done to clarify this issue. The palatine tonsil was specifically targeted during collection of oropharyngeal scrapings. Recovery of virus from oropharyngeal scrapings suggested that the tonsil was probably the source, but future research is needed to confirm that the palatine tonsils are sites of persistent infection. It remains to be determined whether other sites in the body are reservoirs of PRRSV, as well.

Understanding the mechanism of persistency is hampered by the current deficit of information on the immunology of PRRSV. Identification of the cells and tissues which harbor virus during persistent infection would be the first step in understanding the interaction of PRRSV with the porcine immune system. The fact that PRRSV produces a persistent infection explains much of the descriptive epidemiology of the infection, but presents a significant challenge to the development of effective prevention and control programs. In the future, epidemiology and immunology will need to advance simultaneously, if we hope to provide viable solutions to PRRS.

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